The Immune-pathogenesis of Canine Atopic Dermatitis: Skin barrier, Microbiome and Inflammation

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The Immune-pathogenesis of Canine Atopic Dermatitis: Skin barrier, Microbiome and Inflammation

Immunopathogenese van Atopische Dermatitis in Honden: Huidbarrière, Microbioom en Inflammatie

(met een samenvatting in het Nederlands)

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Table of contents

List of abbreviations 6

Chapter 1: General introduction 7

Chapter 2: Scope of the thesis 25

Chapter 3: Altered lipid properties of the stratum corneum in Canine Atopic Dermatitis 31

Chapter 4: The bacterial and fungal microbiome of the skin of healthy dogs and dogs with atopic dermatitis and the impact of topical antimicrobial therapy, an exploratory study 51

Chapter 5: Antimicrobial peptides (AMP) and AD-related cytokines 83

Chapter 6: A canine keratinocyte cell line expresses antimicrobial peptide and cytokine genes upon stimulation with bacteria, microbial ligands and recombinant cytokines 99

Chapter 7: General discussion 115

Chapter 8: English Summary 127
Dutch Summary 131
Thai Summary 134

Chapter 9: Acknowledgements 137
Curriculum Vitae 142
List of publications 143

List of abbreviations

Abbreviation	Definition
16S	Ribosomal RNA gene of bacteria
AD	Atopic dermatitis
AMP	Antimicrobial peptide
APC	Antigen-presenting cell
CADESI	Canine Atopic Dermatitis Extent and Severity Index
cBD	Canine β-defensin
cDNA	Complementary deoxyribonucleic acid
CER	Ceramide
CFU	Colony-forming unit
CHOL	Cholesterol
CT, HD	Control/Healthy skin
DC	Dendritic cells
DLN	Draining lymph node
EGF	Epidermal growth factor
FFA	Free fatty acid
FTIR	Fourier transform infrared spectroscopy
HPTLC	High Performance Thin Layer Chromatography
K9CATH	Canine cathelicidin
KC	Keratinocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iTOL	Interactive tree of life
ITS	Internal transcribed spacer of the ribosomal gene in fungi
LC	Langerhans cell
LPP	Long periodicity phase
LS	Lesional atopic skin
MBC	Minimum bactericidal concentration
MHB	Miller Hilton broth
MHC	Major histocompatibility complex
MIC	Minimal inhibitory concentration
mRNA	Messenger ribonucleic acid
MS	Mass Spectrometry
MSCEK	A canine keratinocyte cell line
NLS	Non-lesional atopic skin
OTUs	Operational taxonomic units
PBS	Phosphate buffered saline
PCA	Principal component analysis
PDWT	Phylogenetic diversity whole tree
PRR	Pattern recognition receptor
RDA	Redundancy Analysis
RT-qPCR	Quantitative real-time polymerase chain reaction
SAXD	Small angle X-ray diffraction
SC	Stratum corneum
SD	Standard deviation
SEM	Standard error of mean
SPP	Short periodicity phase
TEWL	Transepidermal water loss
TGF-β	Transforming growth factor-β
Th1	T helper cell type I
Th2	T helper cell type II
Th17	T helper cell type 17
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin

Chapter 1

General introduction

Introduction

Atopic dermatitis (AD) is defined as a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features[1]. It is associated with immunoglobulin E (IgE) antibodies most commonly directed against environmental allergens[1]. Canine AD, sharing several characteristics with human AD[2-5], is one of the most common skin diseases occurring in dogs with a history of pruritus and recurrent skin or ear infections[6].

The prevalence of canine AD as established in canine populations varies between 3 and 15% depending, amongst others, on breed, geographic area and survey method[7]. The clinical signs are diverse and vary depending on age of onset, breed and breed-specific phenotypes, sex predisposition, seasonality, anatomical sites, and types of skin lesions (primary or secondary)[2,8]. Driven by allergen exposure, and depending on the types of immune cells responding the disease may present as acute or chronic[9-11]. Apart from prolonged impact on animal health and welfare, canine AD is a continuous (financial) burden to owners in view of treatment. Although current treatment options can alleviate clinical symptoms of canine AD in most patients, a cure that results in medication free disease remission is not available[12,13]. Moreover, the exact pathogenesis of canine AD, still to be unraveled and is subject of debate[8,13].

Several genes are likely to be related to abnormalities in immune response and barrier function in canine AD[14-16]. However, a direct relationship between genetic aspects and canine AD has not been established yet. In general, two hypotheses have been postulated for induction of AD. The first one, the “inside-out” hypothesis, considers AD primarily as an immunologic disease that is initiated by a disbalance in immune homeostasis resulting in over-activation of Th2-dominated immune responsiveness to allergens, that causes skin barrier dysfunction secondarily[17]. The second one, the “outside-in” hypothesis, postulates that AD is initiated by intrinsic defects of epithelial cells and the skin barrier, facilitating penetration of allergens resulting in activation of both innate and adaptive immunity[17,18]. Irrespective of the disease initiation, skin barrier disruption occurs in both disease models and affects skin microbe colonization[19,20], thereby disturbing the active functional balances between the naturally occurring (commensal) and opportunistic skin microbes, and the host’s immune response[21,22]. In this way, the host-microbe interaction (initially) taking place at the level of keratinocytes (KC) and other skin resident cells, may contribute to disease severity[18,21,23,24]. Besides, disease may in turn influence the microbiome[25,26]. Elucidation of the pathogenesis of canine AD may help diagnose the disease and also guide proper therapeutic strategies in this multifaceted disorder.

Clinical signs

Pruritus is the most common clinical signs of canine AD[2]. Both primary and secondary skin lesions, may be observed in canine AD[2,8]. The primary lesions are erythema, maculae and papulae. The secondary lesions, most likely resulting from self-trauma (scratching, biting, chewing, etc.), may be excoriation, self-induced alopecia, lichenification, hyperpigmentation, crusting, and seborrhea[27]. The predominant predilection sites of canine AD may vary between breeds, but the commonly affected areas on the body are face, ear pinnae, axilla, ventral abdomen, inguinal region and paws[28,29]. Most allergic dogs present atopy-related signs for a prolonged period of time, i.e. chronically and continuously[30]. The onset of the disease is typically observed between 6 months and 3 years of age[13]. The clinical signs also vary with respect to seasons, probably dependent on allergen exposure[8,13]. Certain breeds show predisposition to be affected, which can vary between geographical locations for different breeds, most likely depending on the variation in specific genetic make-up in local populations[8,31-34].

Etiology (pathogenesis)

Immune responses

In the process of activation of immune responses after encounter of allergens, as well as microbial antigens, KC produce cytokines (thymic stromal lymphopoietin or TSLP, IL-1, IL-6, transforming growth factor-β or TGF-β, etc.) and possibly antimicrobial peptides (AMP)[17,23,35]. The first contact between antigen (aeroallergen or microbial antigens) and antigen-presenting cells (APCs), which are the skin's Langerhans cells and dendritic cells (DCs), leads to their migration to the draining (regional) lymph nodes (DLN) where the antigens are presented to naive T cells[23]. In the presence of IL-4, for the increased production of which allergic individuals may be predisposed, naive T cells differentiate towards a Th2 phenotype and induce B lymphocytes to produce IgE that will bind to high-affinity IgE receptors on mast cells in tissues[36] (**Figure 1**). Upon secondary antigen exposure, T cells are reactivated, mast cells degranulate and a multitude of inflammatory cytokines such as histamine, IL-8, and IL-13 is released. This local microenvironment enhances migration of Th2 lymphocytes and eosinophils into the skin, resulting in increased local production of Th2 cytokines (e.g. IL-4, IL-5, and IL-13), which in turn stimulate IL-25 production by eosinophils. Next to enhancing a Th2 feedback loop eosinophils, producing IL-6, IL-1β, and TGF-β, may support Th17 differentiation during the acute phase of disease. Th17 cells in the skin can subsequently support IL-19 release by Th2 cells, which causes epidermal hyperplasia and release of AMP by keratinocytes and tissue remodeling activity of fibroblasts. The chronic phase of AD is characterized by a shift towards a stronger Th1 response, that is driven by IL-12 secreted by multiple cells such as

eosinophils, dermal dendritic cells (DDCs), and inflammatory epidermal dendritic cells (IDEC) (**Figure 1**)[36].

It is clear that the immune-pathogenesis of AD is complex. Next to the initial Th2 response and a more Th1 dominated chronic phase of disease regulatory T cells and Th17 cells are associated with the severity of AD[37,38]. There is evidence showing that expression of Th17 cytokines, IL-17 and IL-22, and the regulatory cytokine, IL-10, was significantly increased in lesional atopic skin[5,9,39-42].

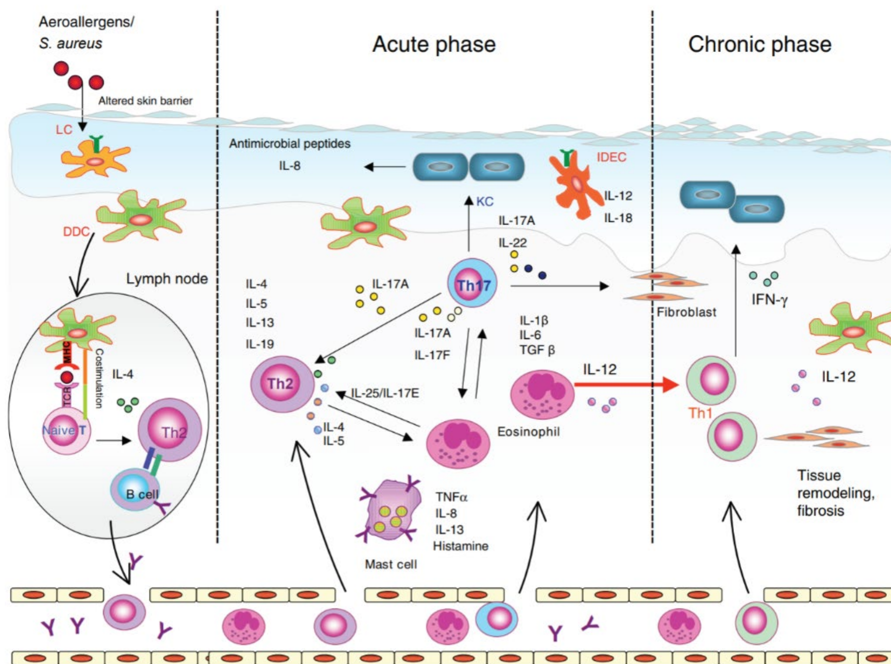

Figure 1. Activation of immune responses to allergens. *KC, keratinocyte; LC, Langerhans cell; MHC, major histocompatibility complex. This figure was originally published by Di Cesare et al., the Journal of investigative dermatology, A role for Th17 cells in the immune-pathogenesis of atopic dermatitis?, 2008; Vol. 128 issue 11, pages 2569-2571. DOI: https://doi.org/10.1038/jid.2008.283. Copyright © 2008 The Society for Investigative Dermatology, Inc. All rights reserved. Elsevier User license: https://www.elsevier.com/about/policies/open-access-licenses/elsevier-user-license.*

Barrier function

The epidermis of the skin consists of four layers; stratum basale, stratum granulosum, stratum spinosum and the outermost part of the skin called stratum corneum (SC) (**Figure 2**). The stratum corneum (SC), a “brick and mortar” structure consisting of corneocytes (terminally differentiated keratinocytes) embedded in a lipid matrix[43], acts as the primary barrier (of the skin) against physical, chemical and microbial insults[44]. Lamellar

bodies, organelles extruded from the corneocytes of the epidermis provide the major lipid classes of the SC[45,46], ceramides (CERs), free fatty acids (FFAs) and cholesterol (CHOL)[47-49].

In humans it has been shown that the SC lipid matrix between the corneocytes consists of two types of crystalline lamellar phases[50,51]. Those with repeat distances of approximately 6 nm, namely the short periodicity phase (SPP) lamellae and those with repeat distances of approximately 13 nm[52-54], called the long periodicity phase (LPP) lamellar organization, the latter of which is essential for the decreased permeability of the skin as compared to the skin organized by the shorter lamellar phase[55-57] (**Figure 2**).

The lateral packing of these lamellae, may be classified as dense, the orthorhombic pattern, less densely organized, the hexagonal pattern or liquid in fact a disordered pattern (**Figure 2**). The orthorhombic pattern is most abundant in control human skin, whereas the hexagonal organization is less present. In AD patients the latter it is more abundant, and its lower density may impair the skin barrier.

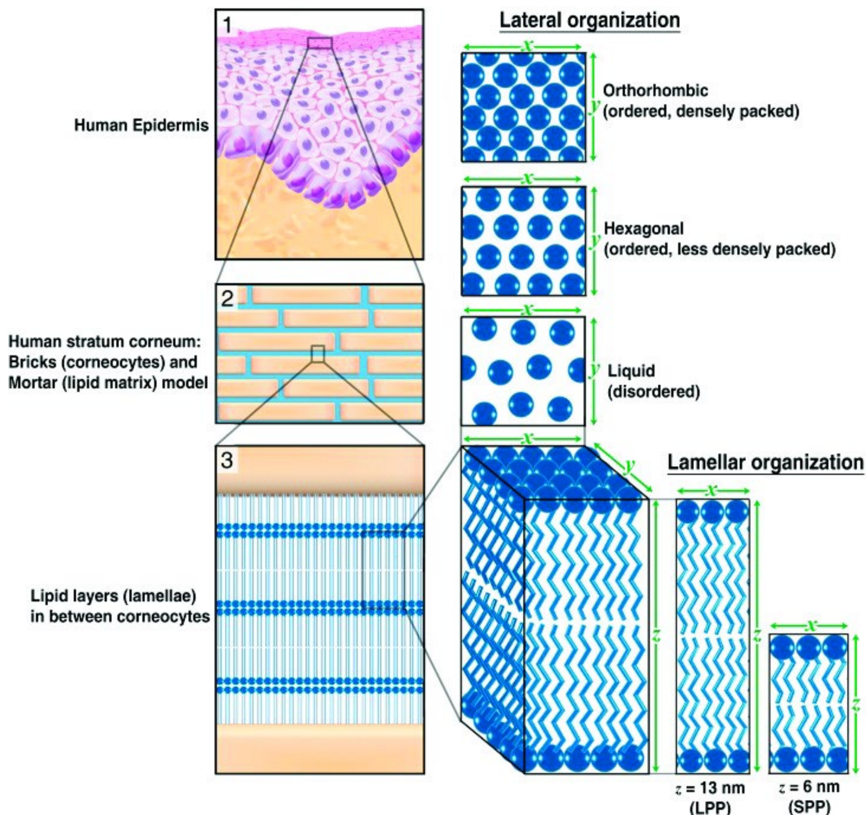

Figure 2. Lamellar and lateral organization in human stratum corneum.

This figure was originally published in the Journal of Lipid Research. Janssens et al. Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. J. Lipid Res. 2012; 53(12), 2755-2766. © the American Society for Biochemistry and Molecular Biology.

As described above, major lipid classes involved in structural and functional aspects of the lamellae are CERs, FFAs and CHOL (**Figure 3**)[58]. CERs is a class of lipids essential for the barrier structure and function that has been found to be markedly decreased in AD patients due to the fact that they are retained in lamellar bodies in KC[59,60]. CERs consist of a sphingoid base and an acyl chain, nomenclature according to Motta et al[61]. The base can be either sphingosine (denoted by S), phytosphingosine (P), 6-hydroxyspingosine (H) or dihydroxysphingosine (dS), the acyl chain is either non-hydroxy (N), α-hydroxy (A) or ω-hydroxy acyl chain ester linked to a linoleate (EO). In each of the CER subclasses there is a variation in carbon chain lengths[62]. In human SC, 12 ceramide subclasses are identified and denoted by CER[NS],CER[NH], CER[NP], CER[NdS], CER[AS], CER[AH], CER[AP], CER[AdS], CER[EOS], CER[EOH], CER[EOP] and CER[EOdS][61,63,64]. In canine SC, the same ceramide subclasses were identified except CER[EOdS][65,66].

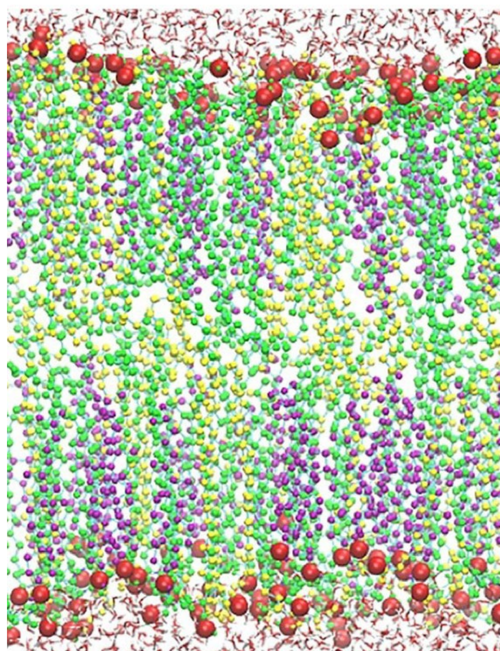

Figure 3. **Schematic representation of the skin lipid bilayer indicating positioning of the major lipid classes CER (Green), CHOL (Purple), FFA (Yellow) molecules, and water molecules (Top and bottom area).** *Polar heads (Oxygen) are shown in red sphere.*

This figure was partly adapted from the figures originally published in Frontiers in Pharmacology, Insight into the molecular dynamic simulation studies of reactive oxygen species in native skin membrane, Front. Pharmacol., 2018, Vol. 9, Article 644. DOI: 10.3389/fphar.2018.00644. *Copyright © 2018 Yadav, Kumar, Choi, Sharma, Misra and Kim. This open-access article is distributed under the terms of the Creative Commons Attribution License (CC BY) (https:// creativecommons.org/ licenses/by/4.0/).*

In previous studies it was observed that an increase in the level of CERs with a short chain length, C34 in particular, resulted in a reduction in the spacing in lamellar organization hence possibly decreased formation of LPP indicating changes in the lamellar phases[62,67], leading to an increased permeability of the skin barrier[57].

Next to CERs, there are FFA and CHOL as important lipids in the "mortar" constitution. Studies performed in lipid model systems showed that a reduction in FFA level and a reduction in chain length of CER increase the conformational disordering of lateral lipid packing, resulting in an impaired skin barrier properties[68,69]. Whereas CHOL resides in the mortar it has a crucial role for the "bricks", particularly in the KC differentiation and desquamation[70].

It was shown that changes in lipids e.g. an increase in the fraction of C34 CER levels forming a hexagonal packing[71], a reduction in the chain length of the FFAs (≤ 20 carbon atoms) and the CERs (≤42 carbon atoms)[71,72] and a decrease in total lipid content (CHOL+FFA+CERs) in SC[73-75]; cause a reduction in the skin barrier function measured by increased permeability and transepidermal water loss (TEWL) in AD patients.

Skin barrier disruption in general, as part of the proposed "outside-in" hypothesis of AD pathogenesis, is associated with altered lipid composition, and organization, has been shown to play a role in human AD[71,72,75,76]. Only little is known about skin barrier (dys-) function in canine atopic dermatitis[65,77,78]. Comparison of the lipid composition, and the lamellar and lateral organization of lipids in SC of AD and healthy dogs may shed light on the consequences of the differences in the skin lipid properties for the skin barrier function in AD.

Disrupted skin barrier may be caused by genetic mutations[79], for example the alterations in lipid metabolism or in structural proteins due to mutation of candidate genes located on chromosome 17 (LOC483120) or on chromosome 27 (PKP2), that are associated with defective skin barrier function in canine AD[80-82]. Apart from genetic factors, the abnormalities of the skin barrier can be the results of diet and/or microbiome changes[25], that may also play a role in the "inside-out" hypothesis.

Skin microbiome

The skin surface is colonized by a diversity of microorganisms including bacteria, fungi, viruses and mites; and colonization differs depending on anatomical sites, host factors and environmental factors[83]. The interaction between host and microbes can be commensal, symbiotic or pathogenic[84]. In healthy skin, the commensal bacteria such as *S. epidermidis* help maintaining skin homeostasis by regulating local inflammatory responsiveness involving components like KC and other skin resident cells, cytokines and AMP[21,22,85]. Disturbance of homeostasis by changes in skin barrier and local immune responsiveness increases the risk of pathogenic activity of skin microbes[21,22,86]. Moreover, it was shown that breaching of skin homeostasis due to a disturbed or diseased skin

tended to alter the composition of the microbiome[19,20]. For example, bacterial diversity was reduced and the microbial community was predominantly localized by the overgrowth of *Staphylococci*, *S. aureus* in particular, during exacerbation or flare of AD disease in humans[20].

The (secondary) infection of the skin is the most common complication in canine AD which is correlated with the clinical severity, occasionally resulting in unresponsiveness to the antimicrobial treatment[87]. Seen the high incidence of infection in AD, the pathogens may play a major role in both inside-out or outside-in hypotheses[21,88-90], and this has not been clearly elucidated in either human or canine AD. To study the interaction between host and predominant microorganisms may help clarify the link between the AD disease symptoms, the barrier disruption and the frequent skin infection.

Based on conventional culture of skin swabs *Staphylococcus pseudintermedius* is most commonly shown in case of bacterial overgrowth found on AD dog skin, whereas *Malassezia pachydermatis* is the main yeast strain found by culture[91,92]. Identification of the skin microbiome has developed, and high throughput sequencing and analysis methods are able to provide more details about the diversity of the microbiome than that found by culture-based methods[93,94]. Only limited data of the canine skin microbiome analyzed by this molecular approach have been reported[95-97].

Antimicrobial peptides (AMP)

Keratinocytes (KC) and their products are the main components of the skin barrier[17,23]. An important role of KC as part of the innate immune system is the production of AMP when activated through a spectrum of pattern recognition receptors (PRRs) and cytokine receptors that are expressed both constitutively (in homeostasis) as well as upregulated upon infection and through inflammatory stimuli[23,98]. AMP have many beneficial properties to the host such as broad-spectrum antimicrobial activity (Gram-negative and Gram-positive bacteria, fungi and viruses), immunomodulatory functions, stimulation of wound vascularization, re-epithelialization of healing skin and antitumor activity[98].

In dog skin, AMP expressed by epithelial tissue are defensins and cathelicidins[98-100]. Defensins are categorized into three subfamilies α, β, and θ based on disulfide bonds formed by amino acid residues[101]. The β-defensins (BD) may be expressed both in skin and the respiratory, gastrointestinal and reproductive tracts[98], whereas the α- and θ-defensins have not been found in dogs[102-104]. The amino acid sequences of canine BD (cBD) showed homology with those of other species varying from 51% to 80%[99,100,105]. Five β-defensin genes, cBD1, cBD102, cBD103 (most prominent), cBD122 and cBD127 have been recognized in the skin of control and atopic dogs[105]. Cathelicidin molecules consist of three domains: a signal sequence, a (conserved) propeptide domain and a mature peptide[98]. Humans (LL-37), mice (CRAMP), dogs (K9CATH) and cats (feCath) express a single

cathelicidin whereas horses, pigs, cows, goats and chickens express multiple cathelicidins[98]. The N-terminus (a conserved region) of K9CATH shares more than 80% similarity to that of other species whereas the C-terminus mature peptide (a highly variable region) displayed most similarity to that of human LL-37 at 46%[100].

Several conditions met in AD contribute to changes in AMP expression. AD is typically associated with skin inflammation and infection, that have shown to induce AMP expression in human skin[106,107] as well as dog skin[108,109]. Variable expression of AMP was observed at different body sites of healthy dog skin[110]. However, currently little information is available on the differential expression of skin AMP in dogs with AD. The fact that some areas of the skin are prone to be affected by AD (predilection sites i.e. axilla and inguinal region) while others (non-predilection site i.e. lateral thorax) are less involved[28], may be due to differential expression of AMP and as a consequence the severity of the lesions in those areas may vary from mild to severe[111].

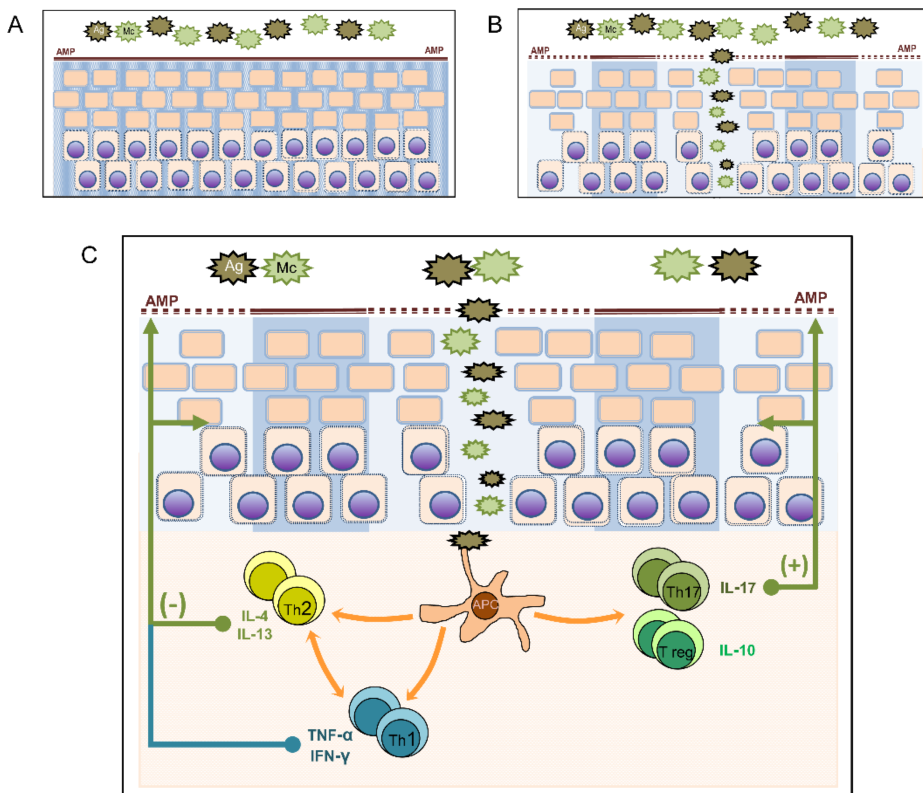

Figure 4. Loss of homeostasis. *A) An intact skin covered with AMP B) A disrupted skin barrier in AD diminishes local AMP production and renders the skin more susceptible to infection (microbes, Mc) or allows allergen (Ag) to easily penetrate the skin. C) The immune response in the tissue triggered by allergen penetration or microbial infection may either aggravate (-) the AMP production and contribute to disruption of skin barrier or stimulate (+) the AMP production. (Abbreviations; AMP: antimicrobial peptide; APC: antigen presenting cells)*

Skin barrier disruption reflected by skin disease may cause differential local expression of AMP that influences the constitution of the microbiome e.g. lower AMP levels may result in a diminished antimicrobial barrier increasing susceptibility to infection in human AD[112,113], possibly resulting in exacerbation of disease (**Figures 4A-B**).

In addition, several studies in humans have shown that imbalance of immune responsiveness in skin like the presence of a Th2 dominated response (IL-4 and IL-13) decreases the expression level of AMP (**Figure 4C**)[114-119]. On the other hand, the presence of IL-1, IL-17 and IL-22 in the skin tends to have a stimulatory effect on AMP production (**Figure 4C**)[117,118]. Thus, the clinical disease states at predilection sites of AD may alter AMP expression in dog skin as well.

Various components derived from the skin microbiome interact with different types of pattern recognition receptors (PRRs) expressed by skin cells[120,121]. The interactions between microbial ligands and specific receptors result in induction of (pro-inflammatory cytokines (e.g. TNF-α and IL-17) that contribute to alterations of innate (AMP) and adaptive immune responsiveness[23,35] (**Figure 5**). This may further aggravate skin barrier dysfunction.

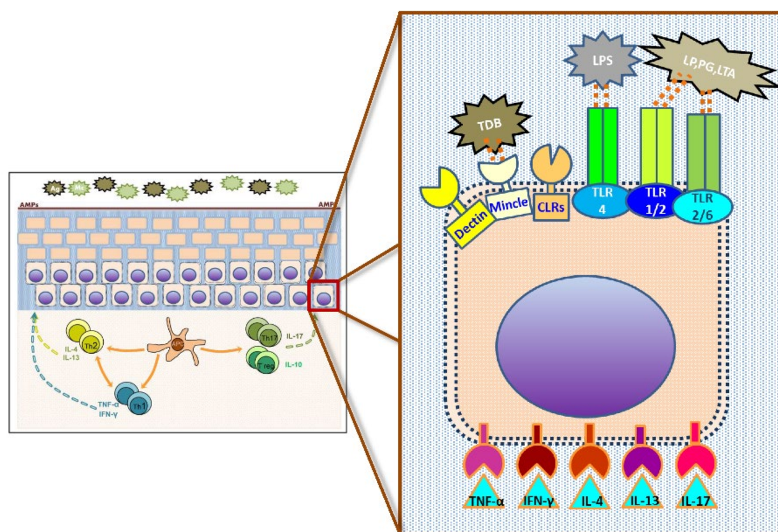

Figure 5. The interaction of microbial components and keratinocytes. *Microbial components interact with different types of PPR expressed by keratinocytes e.g. lipopolysaccharide (LPS) interacts with TLR4 ligand; lipoprotein (LP), peptidoglycan (PG), lipoteichoic acid (LTA) interact with TLR1, 2 or 6 ligands; trehalose-6,6-dibehenate (TDB) interacts with the Mincle ligand. Loss of balance in response to microbial and inflammatory (cytokine) stimuli may alter innate (AMP) and adaptive immunity and disturb skin homeostasis resulting in development of clinical symptoms.*

Diagnosis

Up to date, a sub-group of the International Committee for Allergic Diseases in Animals (ICADA) developed a set of practical guidelines to diagnose canine AD that consisting of following three crucial steps[27]:

1. *Ruling out of other skin conditions with clinical signs that can resemble, or overlap with canine AD.*
2. *Detailed interpretation of the historical and clinical features of the condition. A new tool to assist with interpretation of these findings is the application of clinical criteria known as "Favrot's criteria".*
3. *Assessment of skin reactivity by IntraDermal Testing (IDT) or detection of IgE by Allergen-Specific IgE Serology (ASIS) testing.*

Pruritus can be caused by several factors such as skin infection (bacteria, yeast), skin infestation by ectoparasites (e.g. flea, demodicosis, cheyletiellosis, pediculosis) and other types of skin allergy (food adverse reaction, flea allergic dermatitis, contact allergic dermatitis). Hence, to diagnose canine AD, other possibilities that cause pruritus should be primarily excluded[6,27].

Next, the new tool known as "Favrot's criteria" should be used to diagnose canine AD, for which 5 or 6 criteria should be fulfilled (**Table 1**)[27].

Identification of causal allergens may be performed by IDT or ASIS. IDT is a *in vivo* diagnostic tool that gives an immediate response, wheal formation within 10-30 minutes, to a specific allergen injected intradermally in small amounts[122]. ASIS testing is a serological approach to determine allergen-specificity of IgE[123]. The most common allergens in canine AD are house dust mites (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), storage mites, pollens, molds, and epidermal allergens (e.g. from human and cat)[29]. The interpretation of allergy testing should be done with caution. The skin test results can be confounded by the use of some drugs i.e. anti-histamine, anti-inflammatory agents, and antibiotics[123]. The total serum IgE measurement may not be related with the clinical signs or the results of IDT[124]. Also, IgE levels in atopic and normal dogs might not be significantly different[125]. Variations among laboratories in term of standardization, test procedures and quality control may cause difficulties in outcome, interpretation and comparison of tests[123,126]. Finally, diagnostic testing should be considered based on individual circumstances[123].

Table 1. Favrot's criteria.

	Use	Reliability
Set 1:	• Use for clinical studies and adapt required criteria based on the goal of the study.	• 5 criteria:
1. Age at onset <3 years	• If higher specificity is required, 6 criteria should be fulfilled (e.g., drug trials with potential side effects)	Sens. 85.4 %
2. Mostly indoor	• If higher sensitivity is required, 5 criteria should be fulfilled (e.g., epidemiological studies)	Spec. 79.1 %
3. Corticosteroid-responsive pruritus		
4. Chronic or recurrent yeast infections		• 6 criteria:
5. Affected front feet		Sens. 58.2 %
6. Affected ear pinnae		Spec. 88.5 %
7. Non-affected ear margins		
8. Non-affected dorso-lumbar area		
Set 2:	• Use to evaluate the probability of the diagnosis of canine AD	• 5 criteria:
1. Age at onset < 3 years	• 5 criteria should be fulfilled	Sens. 77.2 %
2. Mostly indoor	• Do not use alone for diagnosis of canine AD, and rule out resembling diseases	Spec. 83 %
3. "Alesional" pruritus at onset		• 6 criteria:
4. Affected front feet		Sens. 42 %
5. Affected ear pinnae		Spec. 93.7 %
6. Non-affected ear margins		
7. Non-affected dorso-lumber area		

This table was originally published in BMC Veterinary Research. Canine atopic dermatitis: detailed guidelines for diagnosis and allergen identification. BMC Vet. Res., 2015, Vol. 11, 196-015-0515-5. Copyright © Hensel et al. 2015. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0).

Treatments

There is no specific treatment to cure AD. The best strategy of disease control is combined intervention, multimodal treatments, depending upon the individual, the stage of disease, disease severity and the distribution of lesions[6,127]. There are three principal points of consideration which are i) Identification and avoidance of flare factors ii) Improvement of skin and coat hygiene and care iii) Reduction of pruritus and skin lesions with pharmacological agents, depending on the stages of disease, i.e. acute, flare or chronic[127]. Recently, a new biological agent, a monoclonal antibody specific for IL-31 was approved, it reduces pruritus and skin lesions in canine AD[128].

Bacterial and yeast infection of the skin and in the ears are common causes of disease flare, systemic and/or topical antimicrobial therapy are recommended to alleviate the symptoms[127,129], based on clinical presentation and culture or cytology results[130]. Since AD is a chronic disease and prolonged use of certain drugs may cause side effects, medication that produces the least (unwanted) side effects is preferential for the individuals health[6,127]. Topical treatment (bathing, spraying) that can reduce the severity of clinical signs and relieve pruritus is strongly recommended[131,132]. The medicated shampoo (Malaseb®) contains chlorhexidine 2% and miconazole 2% shown to be the most

effective ingredients against bacteria and yeast activity as compared to other products described previously[133-135].

Lipid (essential fatty acids, EFA) supplementation in diet is being recommended to help normalize the constitution of intercellular lipids in the SC of AD dog skin, to improve the quality of the skin barrier and possibly to help diminish skin inflammation[127]. The administration of oral medications such as anti-histamine, glucocorticoids, oclacitinib, immunomodulatory and essential fatty acids are widely used to reduce pruritus and/or skin lesions, thus to improve the skin barrier. However, the outcome of these therapies may vary depending on individual, disease stages of acute or chronic[6,127]. Oral glucocorticoids e.g. prednisone or prednisolone and oclacitinib showed rapid action in the treatment of acute flare of canine AD. Calcineurin inhibitor i.e. ciclosporin was more effective in chronic canine AD[127]. Currently, allergen-specific immunotherapy which is considered as an immune-modulator, that gives rise to success rates of up to 60%, most likely prevents disease recurrence and prolongs disease remission[127]. The variation in success rate can be due to the variety in type and duration of medication combinations, bathing, diet management, the approach to rule out other pruritic diseases, the history taken based on client perception and client emotion, difficulty in administering the medications and financial constraints[136].

References

1. Halliwell, R. Revised nomenclature for veterinary allergy. *Vet. Immunol. Immunopathol.* **114**, 207-208 (2006).
2. Griffin, C. E. & DeBoer, D. J. The ACVD task force on canine atopic dermatitis (XIV): clinical manifestations of canine atopic dermatitis. *Vet. Immunol. Immunopathol.* **81**, 255-269 (2001).
3. Marsella, R. Fixing the skin barrier: past, present and future--man and dog compared. *Vet. Dermatol.* **24**, 73-6.e17-8 (2013).
4. Santoro, D. & Rodrigues Hoffmann, A. Canine and Human Atopic Dermatitis: Two Faces of the Same Host-Microbe Interaction. *J. Invest. Dermatol.* **136**, 1087-1089 (2016).
5. Pucheu-Haston, C. M. *et al*. Review: Lymphocytes, cytokines, chemokines and the T-helper 1-T-helper 2 balance in canine atopic dermatitis. *Vet. Dermatol.* **26**, 124-e32 (2015).
6. Olivry, T. *et al*. Treatment of canine atopic dermatitis: 2010 clinical practice guidelines from the International Task Force on Canine Atopic Dermatitis. *Vet. Dermatol.* **21**, 233-248 (2010).
7. Hillier, A. & Griffin, C. E. The ACVD task force on canine atopic dermatitis (I): incidence and prevalence. *Vet. Immunol. Immunopathol.* **81**, 147-151 (2001).
8. Bizikova, P. *et al*. Review: Clinical and histological manifestations of canine atopic dermatitis. *Vet. Dermatol.* **26**, 79-e24 (2015).
9. Nuttall, T. J., Knight, P. A., McAleese, S. M., Lamb, J. R. & Hill, P. B. Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis. *Clin. Exp. Allergy* **32**, 789-795 (2002).
10. Hill, P. B. & Olivry, T. The ACVD task force on canine atopic dermatitis (V): biology and role of inflammatory cells in cutaneous allergic reactions. *Vet. Immunol. Immunopathol.* **81**, 187-198 (2001).
11. Marsella, R. & Olivry, T. The ACVD task force on canine atopic dermatitis (VII): mediators of cutaneous inflammation. *Vet. Immunol. Immunopathol.* **81**, 205-213 (2001).
12. Olivry, T. & Sousa, C. A. The ACVD task force on canine atopic dermatitis (XIX): general principles of therapy. *Vet. Immunol. Immunopathol.* **81**, 311-316 (2001).
13. Saridomichelakis, M. N. & Olivry, T. An update on the treatment of canine atopic dermatitis. *Vet. J.* **207**, 29-37 (2016).
14. Plager, D. A., Torres, S. M., Koch, S. N. & Kita, H. Gene transcription abnormalities in canine atopic dermatitis and related human eosinophilic allergic diseases. *Vet. Immunol. Immunopathol.* **149**, 136-142 (2012).
15. Schamber, P., Schwab-Richards, R., Bauersachs, S. & Mueller, R. S. Gene expression in the skin of dogs sensitized to the house dust mite Dermatophagoides farinae. *G3 (Bethesda)* **4**, 1787-1795 (2014).
16. Merryman-Simpson, A. E. *et al*. Gene (mRNA) expression in canine atopic dermatitis: microarray analysis. *Vet. Dermatol.* **19**, 59-66 (2008).
17. Novak, N. & Leung, D. Y. M. in *Pediatric Allergy: Principles and Practice* (eds Leung, D. Y. M. & Sampson, H.) 552-63 (W.B.Saunders, Edinburgh, 2010).
18. Elias, P. M., Hatano, Y. & Williams, M. L. Basis for the barrier abnormality in atopic dermatitis: outside-inside-outside pathogenic mechanisms. *J. Allergy Clin. Immunol.* **121**, 1337-1343 (2008).
19. Zeeuwen, P. L. *et al*. Microbiome dynamics of human epidermis following skin barrier disruption. *Genome Biol.* **13**, R101 (2012).
20. Kong, H. H. *et al*. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res.* **22**, 850-859 (2012).
21. Miller, L. S. & Cho, J. S. Immunity against Staphylococcus aureus cutaneous infections. *Nat. Rev. Immunol.* **11**, 505-518 (2011).
22. Laborel-Preneron, E. *et al*. Effects of the Staphylococcus aureus and Staphylococcus epidermidis Secretomes Isolated from the Skin Microbiota of Atopic Children on CD4+ T Cell Activation. *PLoS One* **10**, e0141067 (2015).
23. Oyoshi, M. K., He, R., Kumar, L., Yoon, J. & Geha, R. S. Cellular and molecular mechanisms in atopic dermatitis. *Adv. Immunol.* **102**, 135-226 (2009).
24. Elias, P. M. & Schmuth, M. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. *Curr. Opin. Allergy Clin. Immunol.* **9**, 437-446 (2009).
25. Santoro, D. *et al*. Review: Pathogenesis of canine atopic dermatitis: skin barrier and host-micro-organism interaction. *Vet. Dermatol.* **26**, 84-e25 (2015).
26. Bjerre, R. D., Bandier, J., Skov, L., Engstrand, L. & Johansen, J. D. The role of the skin microbiome in atopic dermatitis: a systematic review. *Br. J. Dermatol.* **177**, 1272-1278 (2017).
27. Hensel, P., Santoro, D., Favrot, C., Hill, P. & Griffin, C. Canine atopic dermatitis: detailed guidelines for diagnosis and allergen identification. *BMC Vet. Res.* **11**, 196-015-0515-5 (2015).

28. Favrot, C., Steffan, J., Seewald, W. & Picco, F. A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. *Vet. Dermatol.* **21**, 23-31 (2010).
29. Bizikova, P. *et al*. Review: Role of genetics and the environment in the pathogenesis of canine atopic dermatitis. *Vet. Dermatol.* **26**, 95-e26 (2015).
30. Olivry, T. & International Task Force of Canine Atopic Dermatitis. New diagnostic criteria for canine atopic dermatitis. *Vet. Dermatol.* **21**, 123-126 (2010).
31. Jaeger, K. *et al*. Breed and site predispositions of dogs with atopic dermatitis: a comparison of five locations in three continents. *Vet. Dermatol.* **21**, 118-122 (2010).
32. Picco, F. *et al*. A prospective study on canine atopic dermatitis and food-induced allergic dermatitis in Switzerland. *Vet. Dermatol.* **19**, 150-155 (2008).
33. Tarpataki, N., Papa, K., Reiczigel, J., Vajdovich, P. & Vorosi, K. Prevalence and features of canine atopic dermatitis in Hungary. *Acta Vet. Hung.* **54**, 353-366 (2006).
34. Zur, G., Ihrke, P. J., White, S. D. & Kass, P. H. Canine atopic dermatitis: a retrospective study of 266 cases examined at the University of California, Davis, 1992-1998. Part I. Clinical features and allergy testing results. *Vet. Dermatol.* **13**, 89-102 (2002).
35. Bitschar, K., Wolz, C., Krismer, B., Peschel, A. & Schittek, B. Keratinocytes as sensors and central players in the immune defense against Staphylococcus aureus in the skin. *J. Dermatol. Sci.* **87**, 215-220 (2017).
36. Di Cesare, A., Di Meglio, P. & Nestle, F. O. A role for Th17 cells in the immunopathogenesis of atopic dermatitis? *J. Invest. Dermatol.* **128**, 2569-2571 (2008).
37. Koga, C., Kabashima, K., Shiraishi, N., Kobayashi, M. & Tokura, Y. Possible pathogenic role of Th17 cells for atopic dermatitis. *J. Invest. Dermatol.* **128**, 2625-2630 (2008).
38. Berker, M. *et al*. Allergies – A T cells perspective in the era beyond the TH1/TH2 paradigm. *Clinical Immunology* **174**, 73-83 (2017).
39. Pucheu-Haston, C. M. *et al*. A canine model of cutaneous late-phase reactions: prednisolone inhibition of cellular and cytokine responses. *Immunology* **117**, 177-187 (2006).
40. Olivry, T., Dean, G. A., Tompkins, M. B., Dow, J. L. & Moore, P. F. Toward a canine model of atopic dermatitis: amplification of cytokine-gene transcripts in the skin of atopic dogs. *Exp. Dermatol.* **8**, 204-211 (1999).
41. Marsella, R., Olivry, T. & Maeda, S. Cellular and cytokine kinetics after epicutaneous allergen challenge (atopy patch testing) with house dust mites in high-IgE beagles. *Vet. Dermatol.* **17**, 111-120 (2006).
42. Nograles, K. E. *et al*. IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. *J. Allergy Clin. Immunol.* **123**, 1244-52.e2 (2009).
43. Chandrasekaran, S. K. & Shaw, J. E. Factors influencing the percutaneous absorption of drugs. *Curr. Probl. Dermatol.* **7**, 142-155 (1978).
44. Borkowski, A. W. & Gallo, R. L. The coordinated response of the physical and antimicrobial peptide barriers of the skin. *J. Invest. Dermatol.* **131**, 285-287 (2011).
45. Feingold, K. R. & Elias, P. M. Role of lipids in the formation and maintenance of the cutaneous permeability barrier. *Biochim. Biophys. Acta* **1841**, 280-294 (2014).
46. Elias, P. M. *et al*. Formation and functions of the corneocyte lipid envelope (CLE). *Biochim. Biophys. Acta* **1841**, 314-318 (2014).
47. Bouwstra, J. A. *et al*. Phase behavior of isolated skin lipids. *J. Lipid Res.* **37**, 999-1011 (1996).
48. Holleran, W. M., Takagi, Y. & Uchida, Y. Epidermal sphingolipids: metabolism, function, and roles in skin disorders. *FEBS Lett.* **580**, 5456-5466 (2006).
49. Man MQ, M., Feingold, K. R., Thornfeldt, C. R. & Elias, P. M. Optimization of physiological lipid mixtures for barrier repair. *J. Invest. Dermatol.* **106**, 1096-1101 (1996).
50. Madison, K. C., Swartzendruber, D. C., Wertz, P. W. & Downing, D. T. Presence of intact intercellular lipid lamellae in the upper layers of the stratum corneum. *J. Invest. Dermatol.* **88**, 714-718 (1987).
51. Bouwstra, J. A., Gooris, G. S., van der Spek, J. A. & Bras, W. Structural investigations of human stratum corneum by small-angle X-ray scattering. *J. Invest. Dermatol.* **97**, 1005-1012 (1991).
52. Groen, D., Gooris, G. S. & Bouwstra, J. A. New insights into the stratum corneum lipid organization by X-ray diffraction analysis. *Biophys. J.* **97**, 2242-2249 (2009).
53. McIntosh, T. J., Stewart, M. E. & Downing, D. T. X-ray diffraction analysis of isolated skin lipids: reconstitution of intercellular lipid domains. *Biochemistry* **35**, 3649-3653 (1996).
54. Bouwstra, J., Pilgram, G., Gooris, G., Koerten, H. & Ponec, M. New aspects of the skin barrier organization. *Skin Pharmacol. Appl. Skin Physiol.* **14 Suppl 1**, 52-62 (2001).
55. Groen, D., Poole, D. S., Gooris, G. S. & Bouwstra, J. A. Is an orthorhombic lateral packing and a proper lamellar organization important for the skin barrier function? *Biochim. Biophys. Acta* **1808**, 1529-1537 (2011).
56. Grubauer, G., Feingold, K. R., Harris, R. M. & Elias, P. M. Lipid content and lipid type as determinants of the epidermal permeability barrier. *J. Lipid Res.* **30**, 89-96 (1989).

57. de Jager, M. *et al*. A novel in vitro percutaneous penetration model: evaluation of barrier properties with p-aminobenzoic acid and two of its derivatives. *Pharm. Res.* **23**, 951-960 (2006).
58. Yadav, D. K. *et al*. Insight Into the Molecular Dynamic Simulation Studies of Reactive Oxygen Species in Native Skin Membrane. *Frontiers in pharmacology* **9**, 644; 644-644 (2018).
59. Marsella, R. & Samuelson, D. Unravelling the skin barrier: a new paradigm for atopic dermatitis and house dust mites. *Vet. Dermatol.* **20**, 533-540 (2009).
60. Fartasch, M. The epidermal lamellar body: a fascinating secretory organelle. *J. Invest. Dermatol.* **122**, XI-XII (2004).
61. Motta, S. *et al*. Ceramide composition of the psoriatic scale. *Biochim. Biophys. Acta* **1182**, 147-151 (1993).
62. Mojumdar, E. H., Kariman, Z., van Kerckhove, L., Gooris, G. S. & Bouwstra, J. A. The role of ceramide chain length distribution on the barrier properties of the skin lipid membranes. *Biochim. Biophys. Acta* **1838**, 2473-2483 (2014).
63. van Smeden, J. *et al*. LC/MS analysis of stratum corneum lipids: ceramide profiling and discovery. *J. Lipid Res.* **52**, 1211-1221 (2011).
64. Masukawa, Y. *et al*. Characterization of overall ceramide species in human stratum corneum. *J. Lipid Res.* **49**, 1466-1476 (2008).
65. Yoon, J. S. *et al*. Alteration of stratum corneum ceramide profiles in spontaneous canine model of atopic dermatitis. *Exp. Dermatol.* **20**, 732-736 (2011).
66. Chermprapai, S. *et al*. Altered lipid properties of the stratum corneum in Canine Atopic Dermatitis. *Biochim. Biophys. Acta* **1860**, 526-533 (2018).
67. Thakoersing, V. S. *et al*. Nature versus nurture: does human skin maintain its stratum corneum lipid properties in vitro? *Exp. Dermatol.* **21**, 865-870 (2012).
68. Uchiyama, M., Oguri, M., Mojumdar, E. H., Gooris, G. S. & Bouwstra, J. A. Free fatty acids chain length distribution affects the permeability of skin lipid model membranes. *Biochim. Biophys. Acta* **1858**, 2050-2059 (2016).
69. Mojumdar, E. H., Gooris, G. S. & Bouwstra, J. A. Phase behavior of skin lipid mixtures: the effect of cholesterol on lipid organization. *Soft Matter* **11**, 4326-4336 (2015).
70. Feingold, K. R. & Jiang, Y. J. The mechanisms by which lipids coordinately regulate the formation of the protein and lipid domains of the stratum corneum: Role of fatty acids, oxysterols, cholesterol sulfate and ceramides as signaling molecules. *Dermatoendocrinol* **3**, 113-118 (2011).
71. Janssens, M. *et al*. Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. *J. Lipid Res.* **53**, 2755-2766 (2012).
72. van Smeden, J. *et al*. The importance of free fatty acid chain length for the skin barrier function in atopic eczema patients. *Exp. Dermatol.* **23**, 45-52 (2014).
73. van Smeden, J., Janssens, M., Gooris, G. S. & Bouwstra, J. A. The important role of stratum corneum lipids for the cutaneous barrier function. *Biochim. Biophys. Acta* **1841**, 295-313 (2014).
74. Janssens, M. *et al*. Lipid to protein ratio plays an important role in the skin barrier function in patients with atopic eczema. *Br. J. Dermatol.* **170**, 1248-1255 (2014).
75. Imokawa, G. *et al*. Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *J. Invest. Dermatol.* **96**, 523-526 (1991).
76. Janssens, M. *et al*. Lamellar lipid organization and ceramide composition in the stratum corneum of patients with atopic eczema. *J. Invest. Dermatol.* **131**, 2136-2138 (2011).
77. Reiter, L. V., Torres, S. M. & Wertz, P. W. Characterization and quantification of ceramides in the nonlesional skin of canine patients with atopic dermatitis compared with controls. *Vet. Dermatol.* **20**, 260-266 (2009).
78. Marsella, R., Olivry, T., Carlotti, D. N. & International Task Force on Canine Atopic Dermatitis. Current evidence of skin barrier dysfunction in human and canine atopic dermatitis. *Vet. Dermatol.* **22**, 239-248 (2011).
79. Nuttall, T. The genomics revolution: will canine atopic dermatitis be predictable and preventable? *Vet. Dermatol.* **24**, 10-8.e3-4 (2013).
80. Roque, J. B. *et al*. Atopic dermatitis in West Highland white terriers is associated with a 1.3-Mb region on CFA 17. *Immunogenetics* **64**, 209-217 (2012).
81. Tengvall, K. *et al*. Genome-wide analysis in German shepherd dogs reveals association of a locus on CFA 27 with atopic dermatitis. *PLoS Genet.* **9**, e1003475 (2013).
82. Wood, S. H. *et al*. Gene expression in canine atopic dermatitis and correlation with clinical severity scores. *J. Dermatol. Sci.* **55**, 27-33 (2009).
83. Grice, E. A. & Segre, J. A. The skin microbiome. *Nat. Rev. Microbiol.* **9**, 244-253 (2011).
84. NIH HMP Working Group *et al*. The NIH Human Microbiome Project. *Genome Res.* **19**, 2317-2323 (2009).

85. Nakatsuji, T. *et al*. Antimicrobials from human skin commensal bacteria protect against Staphylococcus aureus and are deficient in atopic dermatitis. *Sci. Transl. Med.* **9**, 10.1126/scitranslmed.aah4680 (2017).
86. Cho, I. & Blaser, M. J. The human microbiome: at the interface of health and disease. *Nat. Rev. Genet.* **13**, 260-270 (2012).
87. Tarpataki, N. Recent developments in canine atopic dermatitis: a review. *Acta Vet. Hung.* **54**, 473-484 (2006).
88. Nakamura, Y. *et al*. Staphylococcus delta-toxin induces allergic skin disease by activating mast cells. *Nature* **503**, 397-401 (2013).
89. Kita, K. *et al*. Activation of bacterial ceramidase by anionic glycerophospholipids: possible involvement in ceramide hydrolysis on atopic skin by Pseudomonas ceramidase. *Biochem. J.* **362**, 619-626 (2002).
90. Oizumi, A. *et al*. Pseudomonas-derived ceramidase induces production of inflammatory mediators from human keratinocytes via sphingosine-1-phosphate. *PLoS One* **9**, e89402 (2014).
91. Miller, W. H., Griffin, C. E., Campbell, K. L. & Muller, G. H. in *Muller and Kirk's Small Animal Dermatology7: Muller and Kirk's Small Animal Dermatology* (Elsevier/Mosby, 2013).
92. DeBoer, D. J. & Marsella, R. The ACVD task force on canine atopic dermatitis (XII): the relationship of cutaneous infections to the pathogenesis and clinical course of canine atopic dermatitis. *Vet. Immunol. Immunopathol.* **81**, 239-249 (2001).
93. Dekio, I. *et al*. Characterization of skin microbiota in patients with atopic dermatitis and in normal subjects using 16S rRNA gene-based comprehensive analysis. *J. Med. Microbiol.* **56**, 1675-1683 (2007).
94. Petrosino, J. F., Highlander, S., Luna, R. A., Gibbs, R. A. & Versalovic, J. Metagenomic pyrosequencing and microbial identification. *Clin. Chem.* **55**, 856-866 (2009).
95. Rodrigues Hoffmann, A. *et al*. The skin microbiome in healthy and allergic dogs. *PLoS One* **9**, e83197 (2014).
96. Meason-Smith, C. *et al*. What is living on your dog's skin? Characterization of the canine cutaneous mycobiota and fungal dysbiosis in canine allergic dermatitis. *FEMS Microbiol. Ecol.* **91**, 10.1093/femsec/fiv139. Epub 2015 Nov 4 (2015).
97. Bradley, C. W. *et al*. Longitudinal Evaluation of the Skin Microbiome and Association with Microenvironment and Treatment in Canine Atopic Dermatitis. *J. Invest. Dermatol.* **136**, 1182-1190 (2016).
98. Leonard, B. C., Affolter, V. K. & Bevins, C. L. Antimicrobial peptides: agents of border protection for companion animals. *Vet. Dermatol.* **23**, 177-e36 (2012).
99. Wingate, K. V., Torres, S. M., Silverstein, K. A., Hendrickson, J. A. & Rutherford, M. S. Expression of endogenous antimicrobial peptides in normal canine skin. *Vet. Dermatol.* **20**, 19-26 (2009).
100. Sang, Y. *et al*. Canine cathelicidin (K9CATH): gene cloning, expression, and biochemical activity of a novel pro-myeloid antimicrobial peptide. *Dev. Comp. Immunol.* **31**, 1278-1296 (2007).
101. Selsted, M. E. & Ouellette, A. J. Mammalian defensins in the antimicrobial immune response. *Nat. Immunol.* **6**, 551-557 (2005).
102. Patil, A. A., Cai, Y., Sang, Y., Blecha, F. & Zhang, G. Cross-species analysis of the mammalian beta-defensin gene family: presence of syntenic gene clusters and preferential expression in the male reproductive tract. *Physiol. Genomics* **23**, 5-17 (2005).
103. Patil, A., Hughes, A. L. & Zhang, G. Rapid evolution and diversification of mammalian alpha-defensins as revealed by comparative analysis of rodent and primate genes. *Physiol. Genomics* **20**, 1-11 (2004).
104. Tang, Y. Q. *et al*. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science* **286**, 498-502 (1999).
105. van Damme, C. M., Willemse, T., van Dijk, A., Haagsman, H. P. & Veldhuizen, E. J. Altered cutaneous expression of beta-defensins in dogs with atopic dermatitis. *Mol. Immunol.* **46**, 2449-2455 (2009).
106. Harder, J. *et al*. Enhanced expression and secretion of antimicrobial peptides in atopic dermatitis and after superficial skin injury. *J. Invest. Dermatol.* **130**, 1355-1364 (2010).
107. Gambichler, T. *et al*. Differential mRNA expression of antimicrobial peptides and proteins in atopic dermatitis as compared to psoriasis vulgaris and healthy skin. *Int. Arch. Allergy Immunol.* **147**, 17-24 (2008).
108. Santoro, D., Marsella, R., Bunick, D., Graves, T. K. & Campbell, K. L. Expression and distribution of canine antimicrobial peptides in the skin of healthy and atopic beagles. *Vet. Immunol. Immunopathol.* **144**, 382-388 (2011).
109. Santoro, D., Bunick, D., Graves, T. K. & Segre, M. Evaluation of canine antimicrobial peptides in infected and noninfected chronic atopic skin. *Vet. Dermatol.* **24**, 39-e10 (2013).
110. Lancto, C. A., Torres, S. M., Hendrickson, J. A., Martins, K. V. & Rutherford, M. S. Altered expression of antimicrobial peptide genes in the skin of dogs with atopic dermatitis and other inflammatory skin conditions. *Vet. Dermatol.* **24**, 414-21, e90 (2013).
111. Olivry, T., Marsella, R., Iwasaki, T., Mueller, R. & International Task Force On Canine Atopic Dermatitis. Validation of CADESI-03, a severity scale for clinical trials enrolling dogs with atopic dermatitis. *Vet. Dermatol.* **18**, 78-86 (2007).

112. Howell, M. D. The role of human beta defensins and cathelicidins in atopic dermatitis. *Curr. Opin. Allergy Clin. Immunol.* **7**, 413-417 (2007).
113. Ong, P. Y. *et al*. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* **347**, 1151-1160 (2002).
114. Nomura, I. *et al*. Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J. Immunol.* **171**, 3262-3269 (2003).
115. Howell, M. D. *et al*. Interleukin-10 downregulates anti-microbial peptide expression in atopic dermatitis. *J. Invest. Dermatol.* **125**, 738-745 (2005).
116. Kisich, K. O., Carspecken, C. W., Fieve, S., Boguniewicz, M. & Leung, D. Y. Defective killing of Staphylococcus aureus in atopic dermatitis is associated with reduced mobilization of human beta-defensin-3. *J. Allergy Clin. Immunol.* **122**, 62-68 (2008).
117. Eyerich, K. *et al*. IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response. *J. Allergy Clin. Immunol.* **123**, 59-66.e4 (2009).
118. Wolk, K. *et al*. IL-22 increases the innate immunity of tissues. *Immunity* **21**, 241-254 (2004).
119. Gittler, J. K. *et al*. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *J. Allergy Clin. Immunol.* **130**, 1344-1354 (2012).
120. Afshar, M. & Gallo, R. L. Innate immune defense system of the skin. *Vet. Dermatol.* **24**, 32-8.e8-9 (2013).
121. de Koning, H. D., Simon, A., Zeeuwen, P. L. & Schalkwijk, J. Pattern recognition receptors in infectious skin diseases. *Microbes Infect.* **14**, 881-893 (2012).
122. Hill, P. B., Hillier, A. & Olivry, T. The ACVD task force on canine atopic dermatitis (VI): IgE-induced immediate and late-phase reactions, two inflammatory sequences at sites of intradermal allergen injections. *Vet. Immunol. Immunopathol.* **81**, 199-204 (2001).
123. Olivry, T., Saridomichelakis, M. & International Committee on Atopic Diseases of Animals (ICADA). Evidence-based guidelines for anti-allergic drug withdrawal times before allergen-specific intradermal and IgE serological tests in dogs. *Vet. Dermatol.* **24**, 225-e49 (2013).
124. Marsella, R. & Girolomoni, G. Canine models of atopic dermatitis: a useful tool with untapped potential. *J. Invest. Dermatol.* **129**, 2351-2357 (2009).
125. Hill, P. B., Moriello, K. A. & DeBoer, D. J. Concentrations of total serum IgE, IgA, and IgG in atopic and parasitized dogs. *Vet. Immunol. Immunopathol.* **44**, 105-113 (1995).
126. Plant, J. D., Neradelik, M. B., Polissar, N. L., Fadok, V. A. & Scott, B. A. Agreement between allergen-specific IgE assays and ensuing immunotherapy recommendations from four commercial laboratories in the USA. *Vet. Dermatol.* **25**, 15-e6 (2014).
127. Olivry, T. *et al*. Treatment of canine atopic dermatitis: 2015 updated guidelines from the International Committee on Allergic Diseases of Animals (ICADA). *BMC Vet. Res.* **11**, 210-015-0514-6 (2015).
128. Michels, G. M. *et al*. A blinded, randomized, placebo-controlled, dose determination trial of lokivetmab (ZTS-00103289), a caninized, anti-canine IL-31 monoclonal antibody in client owned dogs with atopic dermatitis. *Vet. Dermatol.* **27**, 478-e129 (2016).
129. Hillier, A. *et al*. Guidelines for the diagnosis and antimicrobial therapy of canine superficial bacterial folliculitis (Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases). *Vet. Dermatol.* **25**, 163-75, e42-3 (2014).
130. Beco, L. *et al*. Suggested guidelines for using systemic antimicrobials in bacterial skin infections: part 1—diagnosis based on clinical presentation, cytology and culture. *Vet. Rec.* **172**, 72-72 (2013).
131. Nam, E. H. *et al*. Evaluation of the effect of a 0.0584% hydrocortisone aceponate spray on clinical signs and skin barrier function in dogs with atopic dermatitis. *J. Vet. Sci.* **13**, 187-191 (2012).
132. Olivry, T., Mueller, R. S. & THE INTERNATIONAL TASK FORCE ON CANINE ATOPIC DERMATITIS. Evidence-based veterinary dermatology: a systematic review of the pharmacotherapy of canine atopic dermatitis. *Vet. Dermatol.* **14**, 121-146 (2003).
133. Young, R., Buckley, L., McEwan, N. & Nuttall, T. Comparative in vitro efficacy of antimicrobial shampoos: a pilot study. *Vet. Dermatol.* **23**, 36-40, e8 (2012).
134. Bond, R., Rose, J. F., Ellis, J. W. & Lloyd, D. H. Comparison of two shampoos for treatment of Malassezia pachydermatis-associated seborrhoeic dermatitis in basset hounds. *J. Small Anim. Pract.* **36**, 99-104 (1995).
135. Mueller, R. S., Bergvall, K., Bensignor, E. & Bond, R. A review of topical therapy for skin infections with bacteria and yeast. *Vet. Dermatol.* **23**, 330-41, e62 (2012).
136. Dell, D. L., Griffin, C. E., Thompson, L. A. & Griffies, J. D. Owner assessment of therapeutic interventions for canine atopic dermatitis: a long-term retrospective analysis. *Vet. Dermatol.* **23**, 228-e47 (2012).

Chapter 2

Scope of the thesis

Scope of the thesis

Atopic dermatitis (AD) is a pruritic chronic inflammatory skin disease with a complex pathogenesis[1], that occurs in multiple species[2]. Canine AD shares several characteristics with human AD[3-7]. The specific immune-pathogenesis of AD in dogs is not well-established yet, but in general, both a defective epidermal barrier and a propensity to develop secondary infections are well recognized features of atopic dermatitis, similar to AD in humans[7]. It has been widely assumed that these abnormalities reflect consequences of immunologic abnormalities, the historical "inside-out" view of atopic dermatitis pathogenesis[8]. Some research groups, however, have proposed the "outside-in" view of disease pathogenesis. This hypothesis entails an intrinsic abnormality of the skin barrier with consequent increased permeability, not merely as an epiphenomenon, but rather as the 'driver' of disease activity, whereas the extent of this defect parallels severity of AD[8,9] (**Figure 1A**). During progress of the disease, skin barrier pathophysiology may further aggravate[10,11] (**Figure 1B**). Invasion of skin microbes, a common complication in canine AD, may affect proper functioning of skin resident cells including keratinocytes, resulting in altered production of AMP and (pro-)inflammatory cytokines. This leads to exacerbation of clinical symptoms as observed both in humans[8,12] and dogs[9,13] (**Figure 1B**).

The overall aim of the research described in this thesis is to contribute to further elucidation of the immune-pathogenesis of canine atopic dermatitis (canine AD) (**Figure 2**). Skin barrier changes, more precisely alterations in stratum corneum lipid composition and physical constitution in AD versus healthy dogs are investigated and addressed in **Chapter 3.** The differences in microbiome (bacteria and fungi) of the skin between AD (lesional and non-lesional) and healthy skin and at different AD predilection sites are investigated and addressed in **Chapter 4**. In addition, the consequences of topical antimicrobial treatment for the microbiome constitution are also investigated and described. Differential expressions *in vivo* of AMP and AD-related cytokine genes in healthy and AD (lesional and non-lesional) skin at different AD predilection sites, and before, during and after topical antimicrobial treatment are investigated and addressed in **Chapter 5**. The interactions between skin cells (keratinocytes) and bacteria, microbial ligands, AMP and cytokines *in vitro* are investigated and addressed in **Chapter 6**. Study of these different subjects not only contributes to a more comprehensive understanding of canine AD, but may in future result in design of diagnostic and therapeutic strategies for canine AD.

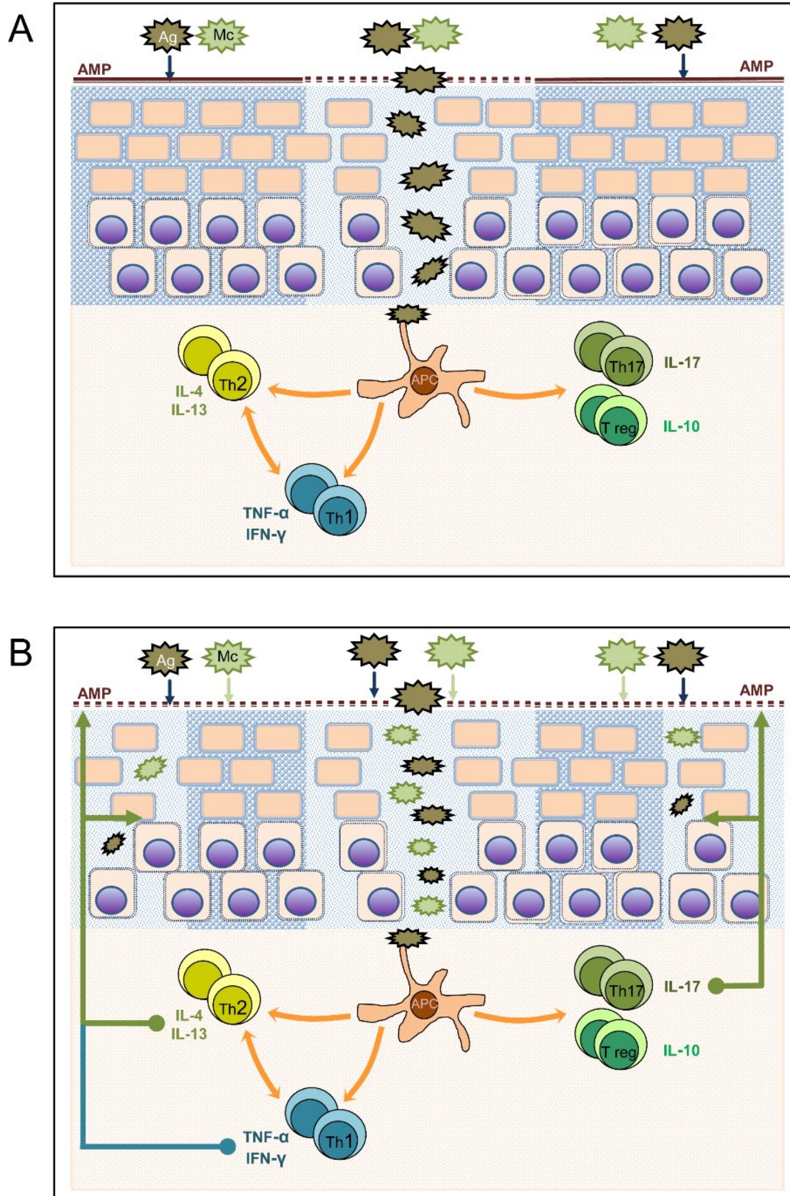

Figure 1. Factors related to the two hypotheses for pathogenesis of canine atopic dermatitis. *A) The "Outside-in" hypothesis; Barrier abnormality allows allergens (Ag) to easily penetrate the skin and interact with antigen-presenting cells (APC), and subsequently to induce both innate (antimicrobial peptides, AMP) and adaptive (T reg, Th1, Th2, Th17) immunity. B) The "Inside-out" hypothesis; Hyperimmune responsiveness to allergens cause skin barrier disruption, production of AMP decreases and that of (Pro-) inflammatory cytokines increase. Both in A) and B) skin microbial (Mc) colonization may result in disease exacerbation.*

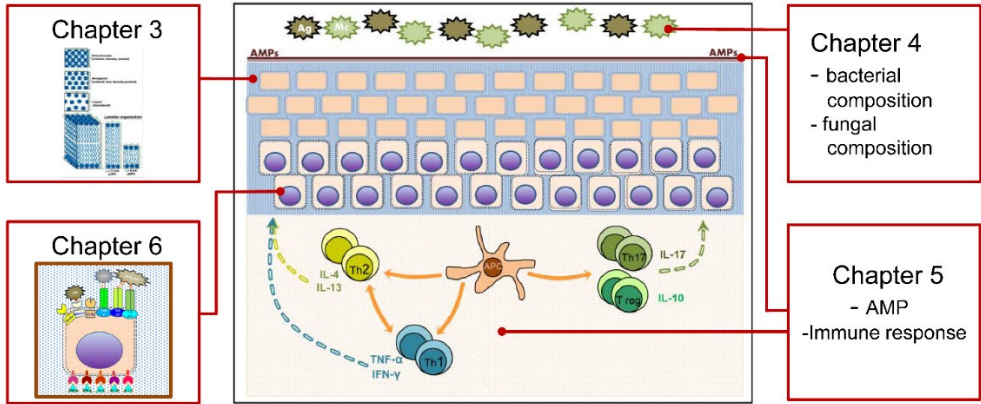

Figure 2. Overview of AD-related subjects described in this thesis as related to chapters of the PhD Thesis.

References

1. Hensel, P., Santoro, D., Favrot, C., Hill, P. & Griffin, C. Canine atopic dermatitis: detailed guidelines for diagnosis and allergen identification. *BMC Vet. Res.* **11**, 196-015-0515-5 (2015).
2. Freudenberg, J. M., Olivry, T., Mayhew, D. N., Rubenstein, D. S. & Rajpal, D. K. The comparison of skin transcriptomes confirms canine atopic dermatitis is a natural homologue to the human disease. *J. Invest. Dermatol.* (2018).
3. Griffin, C. E. & DeBoer, D. J. The ACVD task force on canine atopic dermatitis (XIV): clinical manifestations of canine atopic dermatitis. *Vet. Immunol. Immunopathol.* **81**, 255-269 (2001).
4. Marsella, R. Fixing the skin barrier: past, present and future--man and dog compared. *Vet. Dermatol.* **24**, 73-6.e17-8 (2013).
5. Santoro, D. & Rodrigues Hoffmann, A. Canine and Human Atopic Dermatitis: Two Faces of the Same Host-Microbe Interaction. *J. Invest. Dermatol.* **136**, 1087-1089 (2016).
6. Pucheu-Haston, C. M. *et al*. Review: Lymphocytes, cytokines, chemokines and the T-helper 1-T-helper 2 balance in canine atopic dermatitis. *Vet. Dermatol.* **26**, 124-e32 (2015).
7. Gedon, N. K. Y. & Mueller, R. S. Atopic dermatitis in cats and dogs: a difficult disease for animals and owners. *Clinical and translational allergy* **8**, 41; 41-41 (2018).
8. Novak, N. & Leung, D. Y. M. in *Pediatric Allergy: Principles and Practice* (eds Leung, D. Y. M. & Sampson, H.) 552-63 (W.B.Saunders, Edinburgh, 2010).
9. Santoro, D. *et al*. Review: Pathogenesis of canine atopic dermatitis: skin barrier and host-micro-organism interaction. *Vet. Dermatol.* **26**, 84-e25 (2015).
10. Howell, M. D. The role of human beta defensins and cathelicidins in atopic dermatitis. *Curr. Opin. Allergy Clin. Immunol.* **7**, 413-417 (2007).
11. Ong, P. Y. *et al*. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* **347**, 1151-1160 (2002).
12. Elias, P. M. & Steinhoff, M. "Outside-to-inside" (and now back to "outside") pathogenic mechanisms in atopic dermatitis. *J. Invest. Dermatol.* **128**, 1067-1070 (2008).
13. Fazakerley, J. *et al*. Staphylococcal colonization of mucosal and lesional skin sites in atopic and healthy dogs. *Vet. Dermatol.* **20**, 179-184 (2009).

Chapter 3

Altered lipid properties of the stratum corneum in Canine Atopic Dermatitis

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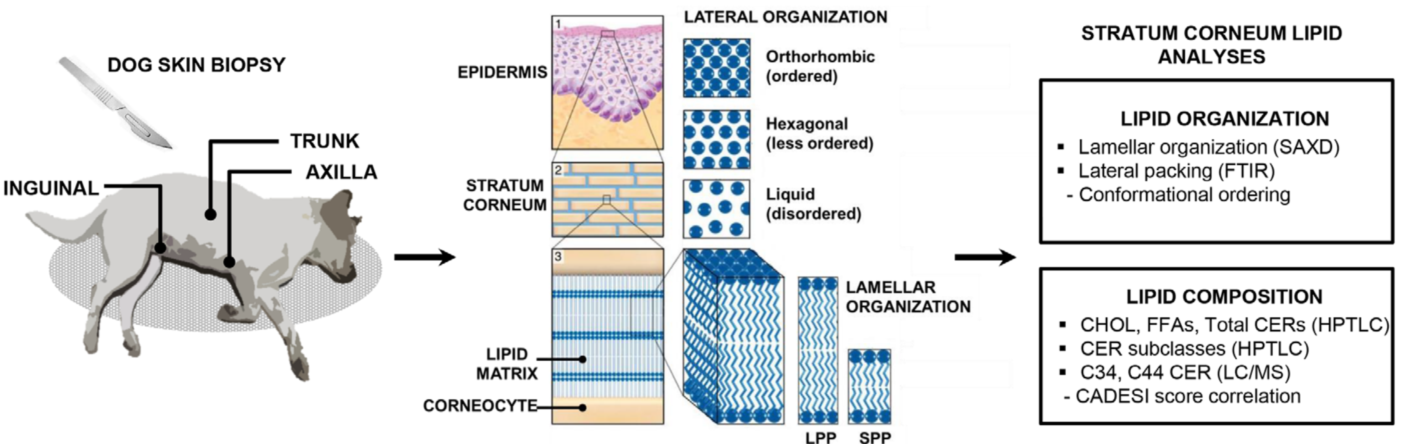
Graphical abstract



Partly adapted from the figure originally published in Journal of Lipid Research. Janssens et al. Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. *Journal of Lipid Research*. 2012; *53*(12), 2755-2766. © the American Society for Biochemistry and Molecular Biology.

ABSTRACT

Skin barrier disruption plays a role in the pathogenesis of atopic dermatitis (AD) in humans. However, little is known about skin barrier (dys-) function in canine atopic dermatitis. The properties of lipids located in the outermost layer of the skin, the stratum corneum (SC) are considered to be important for the barrier. In the present study the lipid composition and lipid organization of the SC of AD dogs and control dogs were examined. The lipid composition of lesional AD skin as compared to control skin, showed a reduced free fatty acid level and a decreased ratio of ceramide[NS] C44/C34, in which C44 and C34 are the total numbers of carbon atoms of the sphingosine (S) and non-hydroxy (N) acyl chains. As a consequence of the observed changes in lipid composition in AD lesional skin the lamellar organization of lipids altered and a shift from orthorhombic to hexagonal lipid packing was monitored. Simultaneously an increased conformational disordering occurred. These changes are expected to compromise the integrity of the skin barrier. The C44/C34 chain length ratio of ceramide[NS] also showed a decreasing nonlinear relationship with the AD severity score (CADESI). Taken together, canine atopic skin showed alterations in SC lipid properties, similar to the changes observed in atopic dermatitis in humans, that correlated with a disruption of the skin barrier. Hence lipids play an important role in the pathogenesis of canine atopic dermatitis.

Keywords: stratum corneum; lamellar organization; lipid composition; atopic dermatitis; canine.

1. Introduction

Atopic dermatitis (AD) in dogs, like in humans is a genetically predisposed chronic inflammatory and pruritic skin disease[1,2]. The pathogenesis of canine AD is not well understood and one of the paradigms is that skin barrier dysfunction may facilitate allergen penetration into the epidermal layers and subsequently induction of both innate and adaptive immune responsiveness causing clinical symptoms in sensitive individuals[1,3]. This may further deteriorate the barrier function, influence the microbiome of the skin and may lead to exacerbation of clinical symptoms as observed in AD in humans[4-6].

The stratum corneum (SC), the outermost layer of the epidermis, acts as the primary physical barrier of the skin. The “brick and mortar” structure of the SC consists of corneocytes (the bricks) embedded in a lipid matrix (the mortar)[7]. Integrity of the SC, particularly the lipid matrix, is important in maintaining the skin barrier function[8-11]. Previous studies have shown changes in lipid properties in non-lesional and lesional skin of human AD[9,12-16]. The main lipid classes are ceramides (CERs), free fatty acids (FFAs) and cholesterol (CHOL)[17-19]. Studies of human SC revealed that CERs, FFAs and CHOL assemble in two crystalline lamellar phases with repeat distances of approximately 6 and 13 nm, referred to as the short (SPP) and long periodicity phases (LPP), respectively[20,21]. The lipids within the lamellae may be organized in an orthorhombic lateral packing (very dense), a hexagonal lateral packing (less dense) or a liquid packing (high conformational disordering). Whereas the orthorhombic pattern is most abundantly present in SC of healthy human skin, it was shown that the fraction of lipids forming a hexagonal lateral packing is increased in SC of AD skin compared to that in control skin[9,14,22]. The altered lipid organization in AD skin can be correlated with the changes in lipid composition in the SC[23]. In more detail, a reduction in the skin barrier function in AD patients correlated with i) a decrease in total lipid content in SC[13,24], ii) a reduced chain length of the FFAs and the CERs[9,14], iii) an increase in the fraction of lipids forming a hexagonal packing[9].

Currently only limited information is available concerning the lipid composition[6,25] and lipid organization[26-28] in SC of dog skin. Since in humans the impaired skin barrier plays an important role in the pathogenesis of AD, in the present study we examined the lipid composition, the lamellar and lateral organization in SC of lesional and non-lesional skin of AD dogs as well as control animals. Lesional atopic skin showed changes in the lipid composition and organization similar to those observed in atopic dermatitis in humans.

2. Material and Methods

2.1 Animals

Three control dogs and five AD dogs were included in this study. The control Beagle dogs, owned by the Utrecht University Animal facility unit, aged between 1 and 3 years. The AD dogs (Bedlington beagle crossbreeds), owned by the Department of Clinical

Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, aged between 2 and 9 years. The atopic dogs met the diagnostic criteria for AD and other causes of pruritus were ruled out[29]. The severity of AD lesions was evaluated by the third version of the CADESI (Canine Atopic Dermatitis Extent and Severity Index) at each site (local score) and 62 body sites (total score) as described previously[30,31].

2.2 Skin biopsies and SC isolation

Prior to taking biopsies of the skin, hair was shaved at two sites that are commonly lesional in AD dogs (axilla and inguinal) and one site commonly non-lesional (trunk). Skin biopsies (10x10 cm^2) of both control and AD dogs were taken, by surgical blade excision immediately after euthanasia for purposes unrelated to this study. The SC was isolated from skin biopsies with small modifications of the method described by Tanojo et al[32]. Briefly, subcutaneous fat was removed and the remaining part of the skin was stretched on a polystyrene foam block. A dermatome was used to cut the skin at the proper thickness of 0.3-0.6 mm. The dermatomized skin was collected on filter paper soaked with 0.1% trypsin solution (in PBS) in a petri dish at 4 ^{o}C. After 24 hours, dishes were placed at 37 ^{o}C for 3 hours and the SC was peeled off from the epidermis at room temperature. Subsequently the SC was washed with 0.1% trypsin inhibitor solution (in PBS) and stored at room temperature in a plastic bag containing silica gel and argon gas until further analyses.

2.3 Small angle X-ray diffraction

To examine the lamellar lipid organization in SC, small angle X-ray diffraction (SAXD) was used. Measurements were performed at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) at station BM26B. Prior to the measurements, the SC was hydrated for 24 hours over a solution of 27% NaBr. The SAXD patterns were detected with a Pilatus 1M detector (1043 x 981 pixels) and a sample to detector distance of 2 meter, for a period of 2 x 150 seconds. The scattering profile of the X-rays of the SC samples was recorded as a function of its scattering vector (q) defined by $q= 2\pi \sin \theta/\lambda$ (λ is the wavelength of the X-rays, either 0.1033 or 0.124 nm, and θ is the angle of the scattered X-rays)[33]. The diffraction pattern of a lamellar phase is characterized by a series of equidistant peaks. The position of each peak can be denoted by its q-value or by its corresponding spacing, which is equal to $2\pi/q$. When dealing with a lamellar phase, the diffraction peaks attributed to such a phase are located at an equidistant position in the diffraction curve. This means that the nth order peak is located at a q-value being $n.q_1$ (the position of the 1st order diffraction peak of that lamellar phase). For calibration silver behenate/Cholesterol was used.

2.4 Fourier transform infrared spectroscopy (FTIR)

To analyze the lateral lipid organization, a Varian 670-IR spectrometer equipped with a broad band mercury-cadmium-telluride detector was used and the spectral resolution was 1 cm^{-1}. Absorption of infrared light of wavelengths ranging between 400 cm^{-1} and 4000 cm^{-1} was recorded[34]. Each spectrum was an average of 2560 scans and was collected during a temperature increase of 1°C between 0 °C and 90 °C. Using the software Varian Resolution Pro 4.1.0.101[35-37], all spectra were baseline-corrected and deconvoluted before analysis. After correction we focused on two regions of the spectra. To obtain information about conformational ordering of the chains, the positions of the CH_2 symmetric stretching within the wavelength range 2840-2860 cm^{-1} were determined. The changes in the position of the CH_2 symmetric stretching vibrations as function of temperature were determined as described previously[36]. The positions of the stretching vibration provide information on the conformational ordering. When the lipids are highly ordered, the CH_2 symmetric stretching frequencies are less than 2850 cm^{-1}. When the lipids exhibit a high conformational disordering, the liquid phase, the peak positions of the CH_2 symmetric stretching vibrations are higher than 2853 cm^{-1}. The temperatures of the transitions from the orthorhombic to the hexagonal phase and the hexagonal to the liquid phase were determined from the plots. The midpoint temperature was taken as the transitional temperature by curve fitting with five linear functions to use a six-pair-parameter of the temperature and frequency[35].

To obtain information about the lateral packing, also the scissoring vibration in the spectrum was monitored. For CH_2 scissoring vibration, appearance of vibrations at both the frequencies 1463 and 1473 cm^{-1} (**Table 1**) indicate the presence of an orthorhombic phase in the sample, whereas the presence of vibration at approximately 1467 cm^{-1} only, represents a hexagonal or liquid phase.

Table 1. The most prominent infrared absorption frequency regions in FTIR analyses[10,38,39].

Frequency/cm^{-1}	Assignment	Remarks
2846-2855	CH_2 symmetric stretching	Frequency increases when chain becomes disordered
1463 and 1473	CH_2 scissoring	Orthorhombic phase
1467	CH_2 scissoring	Hexagonal phase
1466	CH_2 scissoring	Disordered phase

2.5 Lipid extraction

To determine the lipid composition, SC lipids were extracted by the method described by Thakoersing et al.[40] using a modified Bligh and Dyer extraction procedure. The organic phases collected were dried under a stream of nitrogen gas at 40 °C dissolved in chloroform: methanol (2:1) and stored at -20 °C.

2.6 High Performance Thin Layer Chromatography (HPTLC) and Mass Spectrometry (MS)

The lipid composition focusing on CERs, FFAs and CHOL present in the SC were analyzed by HPTLC. Using this approach 8 different CER subclasses or combinations of subclasses can be separated. CERs consist of a sphingoid base and an acyl chain, nomenclature according to Motta et al[41]. The base can be either sphingosine (denoted by S), phytosphingosine (P), 6-hydroxyspingosine (H) or dihydroxysphingosine (dS), the acyl chain is either non-hydroxy (N), α–hydroxy (A) or ω-hydroxy acyl chain ester linked to a linoleate (EO). Hence CER subclasses to be distinguished are CER[EOS], CER[NS/NdS], CER[EOP], CER[NP], CER[EOH], CER[AS/NH], CER[AP], CER[AH][42,43], in which CER[NS] and CER[NdS] together with CER[AS] and CER[NH] cannot be separated with HPTLC. The extracted lipids were assessed according to the method of Thakoersing et al.[40] and Ponec et al[44]. Briefly, samples that were dissolved in chloroform: methanol (2:1) were reconstituted individually at a concentration of 1 mg/ml of lipids and 40, 80 and 120 µg quantities were sprayed on the HPTLC plate (silica gel 60, Merck, NJ, USA) using a CAMAG Linomat IV device (Muttenz, Switzerland). A synthetic lipid mixture at a concentration of 1 mg/ml was used as a standard for the lipid identification and quantification. These calibration samples (standard) were applied in different quantities (2, 4, 6, 8, 10, 15 and 20 µg) next to the experimental samples. The standard mixture consisted of 0.318 µg/ml of CHOL, 0.318 of µg/ml FFAs (C16, C18, C20, C22, C23, C24 and C26), 0.064 µg/ml of CER[EOS], 0.042 µg/ml of CER[EOP], 0.093 µg/ml each of CER[NS], CER[NP] and CER[AS], 0.038 µg/ml of CER[AP]; and 0.053 µg/ml of cholesterol sulphate. Separation of lipid classes and subclasses was established using four different solvent mixtures according to the procedure of Ponec et al[44]. Subsequently the plate was dried and stained with $CuSO_4$/Cu $(AcO)_2$ mixture and charred at 80 °C (CHOL and its sterol derivatives are visible) and 170 °C (all the saturated compounds are visible). Finally, the HPTLC plate was scanned and data analyzed. The calibration curves for all the compounds of the synthetic standard mixture were calculated using GraphPad Prism 6.05. Using the calibration curves of the individual lipid classes and CER subclasses, the quantities of CHOL, FFAs and CER subclasses in the experimental samples were calculated.

Samples were also analyzed by chromatography combined with mass spectrometry (LC/MS) using an Acquity UPLC H-class (Waters, Milford, MA, USA) connected to an XEVO TQ-S mass spectrometer (Waters, Milford, MA, USA). Samples were run using full scan analysis in positive ion mode (350-1200 m/z) under atmospheric pressure chemical ionization (APCI) as described elsewhere[45]. Separation was performed on a pva-silica column (5 µm particles, 100 × 2.1 mm i.d.; YMC, Kyoto, Japan). As an internal standard CER[NS] with a protiated sphingoid base and a deuterated acyl chain with 24 carbon atoms was used. From the ion maps the peak areas corresponding to CER[NS] and CER[NdS] with a total hydrocarbon chain length of 34 carbon atoms were

integrated and compared to the CER[NS] and CER[NdS] with a total chain length of 44 carbon atoms respectively.

2.7 Statistical analyses

Mean, standard deviation (SD) and standard error of the mean (SEM) were calculated with GraphPad Prism 6.05. Non-parametric tests: independent samples Kruskal-Wallis corrected by Dunn's multiple comparisons testing were used for comparison between any contrasts of non-lesional AD, lesional AD and control sample groups, and analyzed by GraphPad Prism 6.05 and Stata. The correlation between the carbon chain length of CER[NS] and AD severity score (CADESI) was tested by Spearman rank-order correlation.

3. Results

3.1 Overview of dog skin samples

All control and non-lesional AD skin samples were taken from the trunk area and the local CADESI scores were zero for each of these (**Table 2**). Lesional AD skin samples were mostly taken from the axilla and inguinal region, well-defined predilection sites according to the study of Favrot et al.[46] clearly presenting the highest local AD severity scores in our study as well (**Table 2**). One of the AD dogs also presented clear lesions at the trunk, these data were included in analyses of the lesional tissue group of this study.

3.2 Lamellar organization in SC of dog skin analyzed by SAXD

To determine whether changes occurred in the lamellar organization, diffraction patterns of SC of AD dogs and those of control animals were measured. Focusing on the diffraction patterns of control SC, a strong peak (II) is located at a q-value of 1.0 nm^{-1} corresponding to a spacing of 6.4 nm (**Figure 1A**). This peak is attributable to the 1st order diffraction of the SPP and the 2nd order diffraction peak of the LPP, as explained for human SC previously[9]. On the right-hand-side of this strong peak, a shoulder (III) is located at a q-value of approximately 1.3 nm^{-1} corresponding to a spacing of 4.7 nm, attributed to the LPP being the 3rd order diffraction peak of this phase. A shoulder (I) is located at 0.5 nm^{-1} (not shown in **Figure 1A**) and it is attributed to the LPP as the 1st order diffraction peak. A sharp peak (*) at a q-value of approximately 1.88 nm^{-1} (spacing of 3.34 nm) is due to the phase separated crystalline CHOL. On the right-hand side of this sharp peak a weak shoulder is located, which can be attributed to the 2nd order diffraction peak of the SPP and/or the 4th order diffraction peak of the LPP. When comparing the curves of the three control samples, the peak positions do not vary. However, a variation is observed in intensity of the shoulder (peak III) and of the peak attributed to phase separated CHOL (*).

Table 2. Skin sample characteristics. *An overview of skin samples (CT: control; NLS: non-lesional AD; LS: lesional AD) used in the present study and their characteristics.*

Skin Group	Dog	Sampling Site	Local CADESI-score		Total CADESI-score		Duration of clinical signs
			Local score**	Severity	Total score*	Severity	
Control1 (CT1)	D1	Trunk	0	None	0	none	-
Control2 (CT2)	D2	Trunk	0	None	0	none	-
Control3 (CT3)	D3	Trunk	0	None	0	none	-
Non-lesional1 (NLS1)	D4	Trunk	0	None	< 16	remission	-
Non-lesional2 (NLS2)	D5	Trunk	0	None	< 16	remission	-
Non-lesional3 (NLS3)	D6	Trunk	0	None	37	mild	-
Non-lesional4 (NLS4)	D7	Trunk	0	None	61	moderate	-
Lesional1 (LS1)	D6	Axilla	1	Mild	37	mild	2 mo.
Lesional2 (LS2)	D7	Axilla	2	moderate	61	moderate	2 mo.
Lesional3 (LS3)	D8	Axilla	3	moderate	116	moderate	3 mo.
Lesional4 (LS4)	D6	Inguinal	2	moderate	37	mild	2 mo.
Lesional5 (LS5)	D7	Inguinal	2	moderate	61	moderate	2 mo.
Lesional6 (LS6)	D8	Inguinal	3	moderate	116	moderate	3 mo.
Lesional7 (LS7)	D8	Trunk	3	moderate	116	moderate	3 mo.

**Total score (1240 maximum):0-15: remission; 16-59: mild AD; 60-119: moderate AD; ≥ 120: severe AD.*
***Local score: 0: none; 1: mild; 2-3: moderate; 4-5: severe.*

When focusing on the diffraction patterns of SC lipids from non-lesional AD samples (**Figure 1B**) slight variations were observed compared to the controls: i) the spacing corresponding to the position of the strongest peak (II) varies between 6.3 and 6.5 nm, ii) a weaker intensity of the shoulder indicated by III, iii) an additional peak (denoted by A) was shown in two out of four diffraction patterns (NLS1 and NLS4) at a q-value of approximately 0.75 nm^{-1} corresponding to a spacing of around 8.5 nm.

The diffraction patterns of lesional AD skin (**Figure 1C**) showed features also observed in the diffraction patterns of SC from non-lesional skin, though with weaker diffraction peaks. Differences in the diffraction patterns as compared to control skin are more pronounced. The most important being i) variation in the position of the strong diffraction peak (II), spacing varying between 6.2 and 6.5 nm, ii) a weaker intensity of both, the phase separated CHOL peak (denoted by *) and the shoulder denoted by III was observed, iii) an additional diffraction peak (denoted by A) corresponding to a spacing of

around 8.5 nm was observed in the patterns of LS2 and LS3, iv) a 2nd additional peak (denoted by B) was observed at a spacing of approximately 5.4 nm in the diffraction curve of LS7.

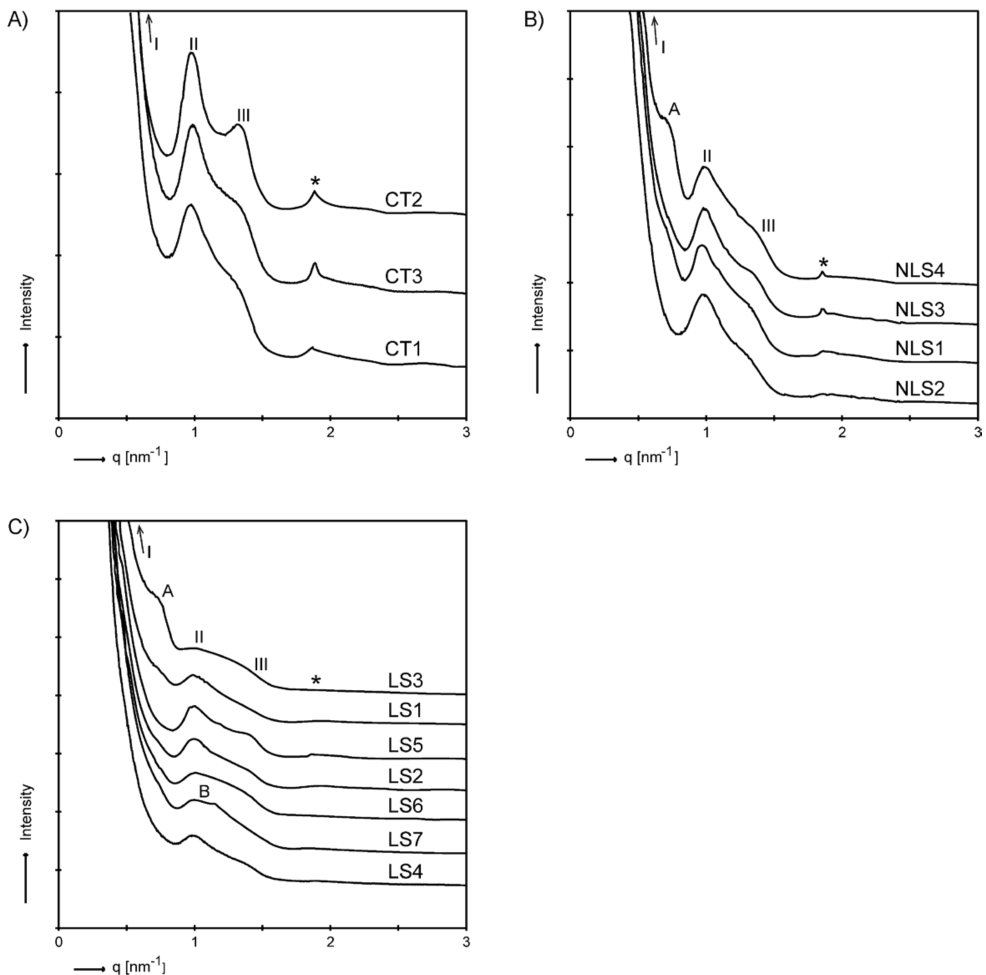


Figure 1. The diffraction patterns of SC from skin of control dogs, and non-lesional and lesional skin of AD dogs. *Small angle X-ray diffraction (SAXD) patterns of SC isolated from A) control (CT) skin (n=3); B) non-lesional (NLS) skin of AD dogs (n=4); and C) lesional (LS) skin of AD dogs (n=7), as described in detail in Table 2. Represented are I: the first order diffraction peak of LPP, II: the combination of the first order diffraction peak of the SPP and the second order diffraction peak of the LPP, III: the third order diffraction peak of LPP. The star (*) represents the diffraction peak of phase separated crystalline CHOL. See more detail of the typical SAXD profile in Janssens et al[9].*

3.3 Lateral organization in SC of dog skin analyzed by FTIR

To examine the lateral packing of the lipids, FTIR spectra from isolated SC of AD dogs were monitored and compared to controls. To determine the conformational

ordering, CH_2 symmetric stretching frequencies in the infrared spectra of SC were recorded between 0 °C and 90 °C with a continuous temperature increment. In SC of control skin, at 0 °C, the CH_2 symmetric stretching frequencies were 2848.6 cm^{-1} which gradually increased when increasing the temperature (**Figure 2A**). The thermotropic response curve of the CH_2 symmetric stretching vibrations of control SC showed prominent shifts at approximately 35-45 °C and 70-80 °C indicating two-phase transitions, the orthorhombic-hexagonal and hexagonal-liquid phase transitions, respectively (**Figure 2A**).

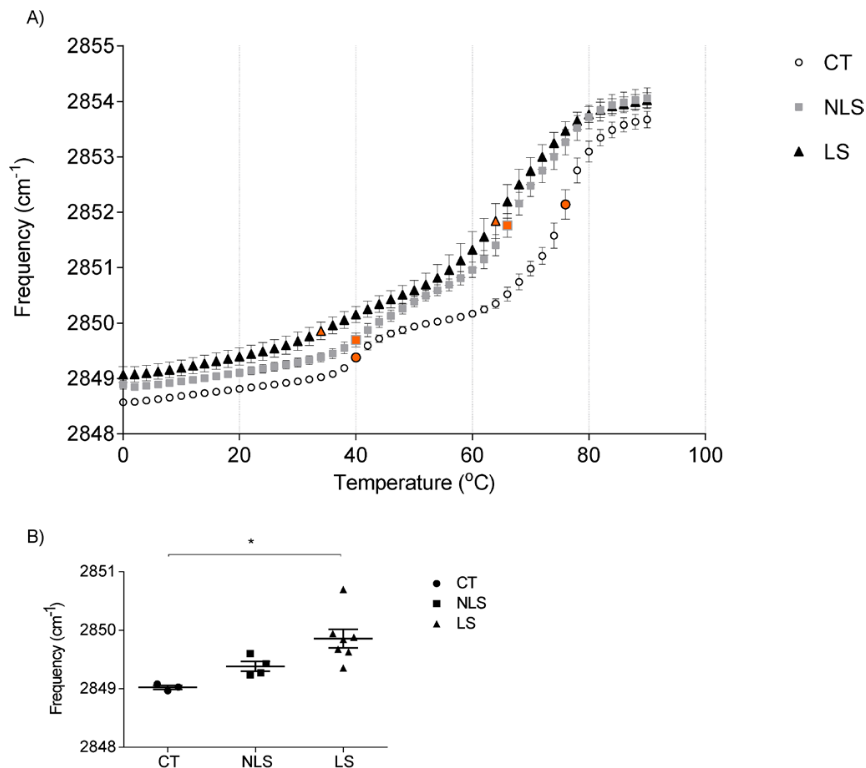


Figure 2. Conformational ordering of the hydrocarbon chains in the SC of dog skin as analyzed by FTIR. *Thermotropic response of the CH_2 symmetric stretching frequencies in a temperature range between 0 °C and 90 °C. A) Thermotropic response of the CH_2 stretching frequencies ± SEM cm^{-1} in SC isolated from control (CT, n=3), non-lesional AD (NLS, n=4) and lesional AD (LS, n=7) dog skin. The colored symbols represented the midpoints of the transition from orthorhombic to hexagonal or hexagonal to liquid phases. B) The CH_2 symmetric stretching frequencies at a selected temperature of 34 °C. A significant difference, $p<0.05$ (*) was observed between the average frequencies of CH_2 symmetric stretching vibrations of lesional skin (2849.9 cm^{-1}) and control skin (2849.0 cm^{-1}).*

The CH_2 symmetric stretching frequency in the spectrum of SC of non-lesional AD skin was 2848.9 cm^{-1} at 0 °C, slightly higher than the frequency of control skin at the same temperature (**Figure 2A**). The frequency gradually shifted to higher values at increasing

temperatures and remained higher than those of control skin in the whole temperature range. The curve of non-lesional AD also showed two prominent shifts in the CH_2 symmetric stretching frequencies at approximately 35-45 ^oC and 60-75 ^oC representing the two phase transitions but the shifts were less prominent than those observed in the thermotropic response curve of the control skin (**Figure 2A**).

The thermotropic response curve of SC of lesional AD skin started at 2849.1 cm^{-1} and when increasing the temperature frequencies were higher as compared to those of non-lesional AD skin and control skin in the temperature range of 0-80 ^oC (**Figure 2**). The first shift in the CH_2 symmetric stretching frequencies at approximately 35-45 ^oC was not very prominent (**Figure 2A)**. The second shift in CH_2 symmetric stretching frequencies was detected at approximately 60-70 ^oC with the average mid-point temperatures and frequencies shown in **Figure 2A**. The average mid-point temperatures and frequencies of the transition from the orthorhombic to hexagonal phase and of the transition from hexagonal to liquid phase in control samples (n=3), non-lesional samples (n=4) and lesional samples (n=7) are provided in **Table 3**. The CH_2 symmetric stretching frequency of control, non-lesional and lesional AD skin at 34 ^oC shown in **Figure 2B**, was chosen since dog skin temperature is reported to be approximately 34.5-35.5 ^oC[47,48].

Table 3. CH_2 stretching frequencies and midpoint transition temperatures. *The midpoint transition temperatures are based on the thermotropic response curves obtained from spectra of SC. Data shown in mean ± SEM. Significant differences between groups (P<0.05, P<0.01, P<0.005 denoted by *, **, ***, respectively) were tested by non-parametric Kruskal Wallis and Dunn's multiple comparison, N.S.: no statistical significance.*

Group		Control (CT, n=3)	Non-lesional (NLS, n=4)	Lesional (LS, n=7)	Comparison
CH_2 Stretching Orthorhombic-Hexagonal phase	Frequency (cm^{-1})	2849.4 ± 0.0	2849.8 ± 0.1	2849.8 ± 0.1	NLS vs CT* LS vs CT*
	Transitional temperature (^oC)	40.3 ± 0.3	41 ± 0.8	35 ± 1.5	NLS vs LS** LS vs CT*
CH_2 Stretching Hexagonal-Liquid phase	Frequency (cm^{-1})	2852.3 ± 0.1	2851.9 ± 0.2	2852 ± 0.2	N.S.
	Transitional temperature (^oC)	76.3 ± 0.7	66.5 ± 0.5	64.6 ± 1.4	NLS vs CT* LS vs. CT***

To obtain more detailed information about the orthorhombic and hexagonal lateral packing of lipids in SC, also the CH_2 scissoring frequencies in the FTIR spectra were analyzed between 0 and 60 °C. In control skin, two peaks in the scissoring vibrations were clearly visible below 40 ^oC at 1463 and 1473 cm^{-1} (**Figure 3A**), demonstrating that a large fraction of lipids was assembled in an orthorhombic packing. However, as a weak peak at 1467 cm^{-1} is also present, it cannot be excluded that a small fraction of lipids adopted a hexagonal packing at temperatures below 34 ^oC. Between 34 ^oC and 44 ^oC the intensity of

the scissoring frequency at 1467 cm^{-1} increased at the expense of the intensities of the frequencies at 1463 and 1473 cm^{-1}, representing the transition from the orthorhombic to the hexagonal phase. When focusing on the scissoring frequencies in the spectrum obtained from SC of non-lesional AD skin at low temperatures, the two peaks at 1463 and 1473 cm^{-1} are clearly present, indicating the presence of an orthorhombic phase (**Figure 3B**), similarly as in the spectrum of the control SC. In this example the orthorhombic to hexagonal phase transition occurred between 38 °C and 46 °C. The scissoring frequencies in the spectrum of lesional skin are clearly different. At low temperature, the two scissoring frequencies indicating the orthorhombic lateral packing are still present at 1463 and 1473 cm^{-1}, but the intensity is much weaker than in control and non-lesional SC (**Figure 3C**). This demonstrates that a substantial smaller fraction of lipids is assembled in an orthorhombic packing. Furthermore, at low temperatures, the scissoring vibration at 1467 cm^{-1} is prominently present, suggesting that a large fraction of lipids adopted a hexagonal packing. When increasing the temperature, the transition from an orthorhombic to hexagonal packing takes place at much lower average temperatures as compared to the control and non-lesional AD skin, the orthorhombic phase disappeared already at approximately 34 °C (**Figures 3D-F**).

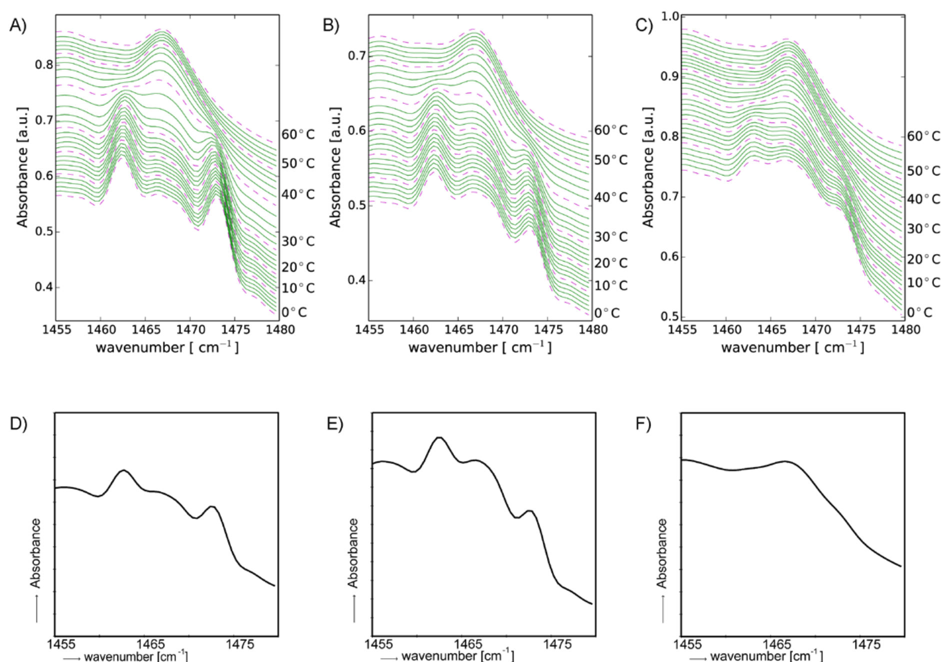


Figure 3. Representative examples of the scissoring vibrations (1460-1480 cm^{-1}) in the infrared spectra of SC. *Data shown are frequency spectra at temperatures between 0 °C and 60 °C (top row) and at 34 °C (bottom row) of control (A and D, respectively); non-lesional AD (B and E, respectively) and lesional AD (C and F, respectively) skin samples.*

3.4 Lipid composition in SC of dog skin analyzed by HPTLC and Mass spectrometry

Focusing on the individual lipid classes in SC, no significant differences were observed between AD (both non-lesional and lesional) and control samples in the relative abundance of CHOL, total CERs (**Figure 4A**) and CER subclass (**Figure 4B**). In contrast, the relative abundance of FFAs was significantly lower in lesional AD (9.6 ± 1.4%) compared to control samples (13.9 ± 1.0%) ($P<0.05$) (**Figure 4A**). The percentage of CER[EOS], CER[NS/NdS] and CER[AS/NH] subclasses were the most predominant of the total CERs content in control dog SC as well as in AD skin (**Figure 4B**).

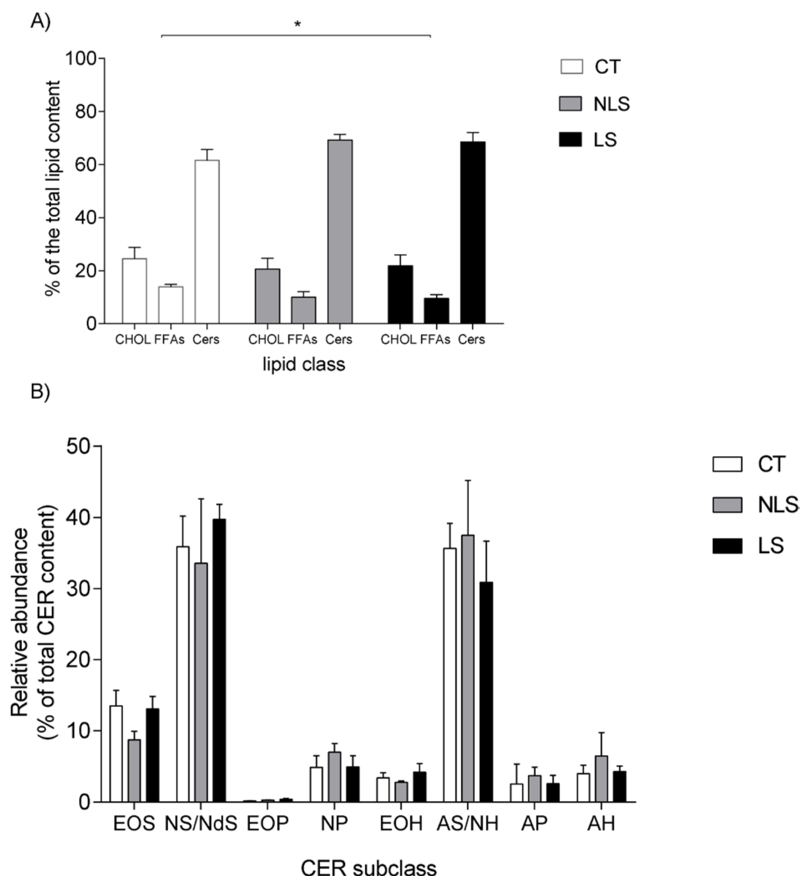


Figure 4. The lipid composition of dog SC. *The lipid composition of dog SC was analyzed by HPTLC. Represented are A) the percentages of major lipid classes: CHOL, FFAs and CERs (= sum of CER subclasses) and B) the abundance in percentage of each CER (sub) class: [EOS], [NS/NdS], [EOP], [NP], [EOH], [AS/NH], [AP], [AH] as compared to the total CER content. Data shown in mean ± SD of control (CT, n=3), non-lesional AD (NLS, n=3) and lesional AD (LS, n=7) skin samples. Significant differences compared to the control (P<0.05, denoted by *) were tested by non-parametric Kruskal Wallis and Dunn's multiple comparison.*

Using LC/MS the C44/C34 chain length of two CER subclasses, CER[NS] and CER[NdS] were examined as CER[NS] and CER[NdS] with a chain length of C34 were increased in AD human skin compared to controls[9,14]. The present analyses showed a significant reduction in the ratio of the peak areas of CER[NS] C44/C34 species in lesional SC of AD skin compared to control SC, while the peak area ratio of C44/C34 of CER[NdS] was not different between the groups (**Figure 5A**). In addition, we studied the association between the peak area ratio of C44/C34 of CER[NS] with the CADESI score (**Figure 5B**). A decreasing nonlinear relationship between the CER[NS] C44/C34 and the CADESI score was observed (Spearman rank test correlation at P=0.041, two-sided) (**Figure 5B**). The AD skin with the moderate CADESI score (non-lesional AD) showed a lower ratio (5-25) of C44/C34 CER[NS] compared to control skin and the ratio was even less (0-12) for AD skin with the high CADESI score (lesional AD).

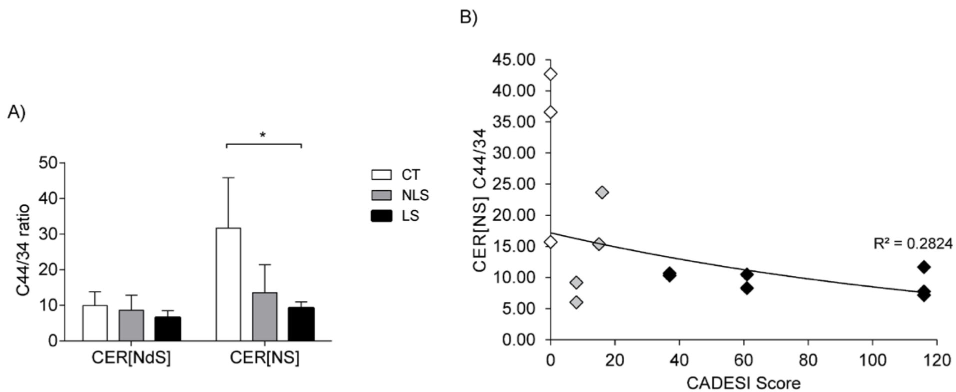


Figure 5. The correlation between CER chain length and AD dog SC. *A) The data shown in mean ± SD of C44/C34 ratio of CER subclasses in control (CT, n=3), non-lesional AD (NLS, n=4) and lesional AD (LS, n=7) dog skin samples. Significant differences compared to the control (P<0.05, denoted by *) were tested by non-parametric Kruskal Wallis and Dunn's multiple comparison. B) The correlation between the C44/C34 ratio of CER[NS] and the CADESI score of dog skin types: control indicated by ◇, non-lesional AD indicated by ◇ , lesional AD indicated by ◆; raw data were log-transformed and tested by Spearman rank-order correlation at P<0.05.*

4. Discussion

Since the exact pathogenesis of AD remains unclear and the primary cause is controversial, various hypotheses are under debate[4,5]. The present study is based on the paradigm that skin barrier abnormalities might be the etiological factors that facilitate triggering AD. Since lipids, besides proteins are crucial components of the skin barrier[8-10], alterations in lipid composition in the SC may change the lipid organization and thus the skin barrier function. Therefore, the focus of this study was to examine whether the lipid properties of the SC of non-lesional and lesional AD dog skin were different from those of SC of control dog skin.

In literature, only limited data is available about the lipid organization in the SC of AD dogs. In previous studies ultra-structural assessment of SC of canine AD skin suggested higher disordering in the lipid lamellae in both non-lesional and lesional samples, in the limited number of electron microscopic images analyzed[26-28]. In our study we used X-ray diffraction to examine the lamellar organization. This bulk technique measures general changes but is unable to monitor local disordering of the lamellar phases in SC of AD dog skin. In addition, lipid analysis using HPTLC and LC/MS revealed a reduced amount of total free CER in SC in lesional skin compared with control skin[6,49 50]. With respect to the two other major lipid classes FFAs and CHOL in SC, whereas one study reported no differences in the relative levels between lesional and control skin[50], another one reported lower amounts of FFAs and CHOL presented as weight per weight SC in AD skin[49]. Some of the observed discrepancies between literature and our current study can be due to differences within dog cohort, differences in analysis or due to the limited number of samples available for our analysis.

In the present study we examined both the lipid composition and the lipid organization in SC. We did not observe changes in the percentage of CHOL and CERs, including CER subclasses, but a reduced level of FFAs was observed in lesional AD compared to control skin. Furthermore, the ratio of C44/C34 of CER[NS] was drastically decreased in AD lesional skin. These changes in lipid composition have had consequences for the lipid organization as discussed below.

The lamellar organization in SC of non-lesional skin differed slightly from that in control animals, whereas lesional AD skin revealed a more prominent variation in the main peak position as well as a weaker shoulder in the SAXD patterns attributed to LPP, indicating changes in the lamellar phases and possibly decreased formation of LPP. The reduction in LPP formation results in an increased permeability of the skin barrier[8] and therefore the observed changes in the present study may contribute to a reduced skin barrier function. In previous studies it was observed that an increase in the level of CERs with a total chain length of C34 resulted in a reduction in the spacing[36,51]. Hence the observed drastic reduction in the ratio C44/C34 in CER[NS], the most abundant CER subclass in dog skin, may contribute to a change in the lamellar phases. The changes observed in the lamellar organization in SC of lesional skin of AD dogs are consistent with earlier studies in human AD skin[9,12]. Nonetheless, in those studies no additional peak corresponding to a spacing of 8.5 nm was observed. This peak may be due to dog hair that could not fully be removed since X-ray diffraction of only hairs revealed a diffraction peak at approximately 8.5 nm spacing.

The lateral packing of lipids in SC was examined by FTIR. Higher conformational disordering was indicated by an increase in the frequencies of CH_2 symmetric stretching vibrations in a large temperature range in lesional AD and non-lesional AD compared to control skin. In addition, measuring the CH_2 scissoring vibrations indicated that in SC of AD lesional skin the fraction of lipids assembled in an orthorhombic packing was reduced

compared to that in SC of control dog skin. This was confirmed by analyses of rocking vibrations (not shown, frequencies 728 and 720 cm^{-1}). Studies performed in lipid model systems, showed that a reduction in FFAs level and a reduction in chain length of CERs resulted in the fraction of lipids assembled in a hexagonal lateral packing rather than orthorhombic packing[37,52]. Therefore, the observed reduction in FFAs level and/or ratio CER[NS] C44/C34 may have contributed to hexagonal lipid organization in lesional skin in AD dog.

The increase in conformational disordering and the more prominent presence of lipids assembled in a hexagonal packing observed in SC of lesional skin of AD dogs are in agreement with findings in human AD skin as described previously[9,14,22]. Moreover, a correlation between the level of C34 and the severity of AD (SCORAD) in humans[9] was also indicated in the present study in canine AD (CADESI score). The latter indicates that the reduced chain length of CER are relevant not only in the lipid organization, hence barrier integrity, but as a consequence may also be correlated with the severity of the disease.

5. Conclusion

In the present study we examined the lipid composition, the lamellar organization and the lateral organization of the lipids in SC in lesional and non-lesional skin of AD dogs as well as in control dogs. Our data show that the alterations in lipid properties of SC in atopic skin of dogs are similar to changes in the lipid composition and organization observed in atopic dermatitis in humans[9,12,14,22], impair the physical integrity of SC, resulting in an impaired skin barrier. The loss of barrier function allows allergens and pathogens to easily penetrate the skin, which triggers the disease. Interestingly in humans as well as in dogs a correlation has been observed between the severity of the disease and the level of CERs with a total chain length of C34. A better understanding of the pathogenesis of canine AD will be beneficial to the improvement of therapeutic strategies of this skin disorder.

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Conflict of interest

The authors have no conflicts of interest.

References

1. Olivry, T. *et al*. The ACVD task force on canine atopic dermatitis: forewords and lexicon. *Vet. Immunol. Immunopathol.* **81**, 143-146 (2001).
2. Schlotter, Y. M., Rutten, V. P., Riemers, F. M., Knol, E. F. & Willemse, T. Lesional skin in atopic dogs shows a mixed Type-1 and Type-2 immune responsiveness. *Vet. Immunol. Immunopathol.* **143**, 20-26 (2011).
3. Griffin, C. E. & DeBoer, D. J. The ACVD task force on canine atopic dermatitis (XIV): clinical manifestations of canine atopic dermatitis. *Vet. Immunol. Immunopathol.* **81**, 255-269 (2001).
4. Novak, N. & Leung, D. Y. M. in *Pediatric Allergy: Principles and Practice* (eds Leung, D. Y. M. & Sampson, H.) 552-63 (W.B.Saunders, Edinburgh, 2010).
5. Elias, P. M. & Steinhoff, M. "Outside-to-inside" (and now back to "outside") pathogenic mechanisms in atopic dermatitis. *J. Invest. Dermatol.* **128**, 1067-1070 (2008).
6. Yoon, J. S. *et al*. Alteration of stratum corneum ceramide profiles in spontaneous canine model of atopic dermatitis. *Exp. Dermatol.* **20**, 732-736 (2011).
7. Chandrasekaran, S. K. & Shaw, J. E. Factors influencing the percutaneous absorption of drugs. *Curr. Probl. Dermatol.* **7**, 142-155 (1978).
8. de Jager, M. *et al*. A novel in vitro percutaneous penetration model: evaluation of barrier properties with p-aminobenzoic acid and two of its derivatives. *Pharm. Res.* **23**, 951-960 (2006).
9. Janssens, M. *et al*. Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. *J. Lipid Res.* **53**, 2755-2766 (2012).
10. Damien, F. & Boncheva, M. The extent of orthorhombic lipid phases in the stratum corneum determines the barrier efficiency of human skin in vivo. *J. Invest. Dermatol.* **130**, 611-614 (2010).
11. Groen, D., Poole, D. S., Gooris, G. S. & Bouwstra, J. A. Is an orthorhombic lateral packing and a proper lamellar organization important for the skin barrier function? *Biochim. Biophys. Acta* **1808**, 1529-1537 (2011).
12. Janssens, M. *et al*. Lamellar lipid organization and ceramide composition in the stratum corneum of patients with atopic eczema. *J. Invest. Dermatol.* **131**, 2136-2138 (2011).
13. Imokawa, G. *et al*. Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *J. Invest. Dermatol.* **96**, 523-526 (1991).
14. van Smeden, J. *et al*. The importance of free fatty acid chain length for the skin barrier function in atopic eczema patients. *Exp. Dermatol.* **23**, 45-52 (2014).
15. Jungersted, J. M., Hogh, J. K., Hellgren, L. I., Jemec, G. B. & Agner, T. Ethnicity and stratum corneum ceramides. *Br. J. Dermatol.* **163**, 1169-1173 (2010).
16. Ishikawa, J. *et al*. Changes in the ceramide profile of atopic dermatitis patients. *J. Invest. Dermatol.* **130**, 2511-2514 (2010).
17. Bouwstra, J. A. *et al*. Phase behavior of isolated skin lipids. *J. Lipid Res.* **37**, 999-1011 (1996).
18. Holleran, W. M., Takagi, Y. & Uchida, Y. Epidermal sphingolipids: metabolism, function, and roles in skin disorders. *FEBS Lett.* **580**, 5456-5466 (2006).
19. Wertz, P. W. & van den Bergh, B. The physical, chemical and functional properties of lipids in the skin and other biological barriers. *Chem. Phys. Lipids* **91**, 85-96 (1998).
20. Bouwstra, J. A., Gooris, G. S., van der Spek, J. A. & Bras, W. Structural investigations of human stratum corneum by small-angle X-ray scattering. *J. Invest. Dermatol.* **97**, 1005-1012 (1991).
21. Bouwstra, J. A., Gooris, G. S., Bras, W. & Downing, D. T. Lipid organization in pig stratum corneum. *J. Lipid Res.* **36**, 685-695 (1995).
22. Pilgram, G. S. *et al*. Aberrant lipid organization in stratum corneum of patients with atopic dermatitis and lamellar ichthyosis. *J. Invest. Dermatol.* **117**, 710-717 (2001).
23. van Smeden, J. & Bouwstra, J. A. Stratum Corneum Lipids: Their Role for the Skin Barrier Function in Healthy Subjects and Atopic Dermatitis Patients. *Curr. Probl. Dermatol.* **49**, 8-26 (2016).
24. Janssens, M. *et al*. Lipid to protein ratio plays an important role in the skin barrier function in patients with atopic eczema. *Br. J. Dermatol.* **170**, 1248-1255 (2014).
25. Reiter, L. V., Torres, S. M. & Wertz, P. W. Characterization and quantification of ceramides in the nonlesional skin of canine patients with atopic dermatitis compared with controls. *Vet. Dermatol.* **20**, 260-266 (2009).
26. Inman, A. O., Olivry, T., Dunston, S. M., Monteiro-Riviere, N. A. & Gatto, H. Electron microscopic observations of stratum corneum intercellular lipids in normal and atopic dogs. *Vet. Pathol.* **38**, 720-723 (2001).
27. Piekutowska, A., Pin, D., Reme, C. A., Gatto, H. & Haftek, M. Effects of a topically applied preparation of epidermal lipids on the stratum corneum barrier of atopic dogs. *J. Comp. Pathol.* **138**, 197-203 (2008).
28. Marsella, R., Samuelson, D. & Doerr, K. Transmission electron microscopy studies in an experimental model of canine atopic dermatitis. *Vet. Dermatol.* **21**, 81-88 (2010).

29. Olivry, T. & International Task Force of Canine Atopic Dermatitis. New diagnostic criteria for canine atopic dermatitis. *Vet. Dermatol.* **21**, 123-126 (2010).
30. Olivry, T. *et al*. Determination of CADESI-03 thresholds for increasing severity levels of canine atopic dermatitis. *Vet. Dermatol.* **19**, 115-119 (2008).
31. Olivry, T., Marsella, R., Iwasaki, T., Mueller, R. & International Task Force On Canine Atopic Dermatitis. Validation of CADESI-03, a severity scale for clinical trials enrolling dogs with atopic dermatitis. *Vet. Dermatol.* **18**, 78-86 (2007).
32. Tanojo, H., Bos-van Geest, A., Bouwstra, J. A., Junginger, H. E. & Boodé, H. E. In vitro human skin barrier perturbation by oleic acid: Thermal analysis and freeze fracture electron microscopy studies. *Thermochimica Acta* **293**, 77-85 (1997).
33. Groen, D., Gooris, G. S. & Bouwstra, J. A. New insights into the stratum corneum lipid organization by X-ray diffraction analysis. *Biophys. J.* **97**, 2242-2249 (2009).
34. de Sousa Neto, D., Gooris, G. & Bouwstra, J. Effect of the omega-acylceramides on the lipid organization of stratum corneum model membranes evaluated by X-ray diffraction and FTIR studies (Part I). *Chem. Phys. Lipids* **164**, 184-195 (2011).
35. Oguri, M., Gooris, G. S., Bito, K. & Bouwstra, J. A. The effect of the chain length distribution of free fatty acids on the mixing properties of stratum corneum model membranes. *Biochim. Biophys. Acta* **1838**, 1851-1861 (2014).
36. Mojumdar, E. H., Kariman, Z., van Kerckhove, L., Gooris, G. S. & Bouwstra, J. A. The role of ceramide chain length distribution on the barrier properties of the skin lipid membranes. *Biochim. Biophys. Acta* **1838**, 2473-2483 (2014).
37. Uchiyama, M., Oguri, M., Mojumdar, E. H., Gooris, G. S. & Bouwstra, J. A. Free fatty acids chain length distribution affects the permeability of skin lipid model membranes. *Biochim. Biophys. Acta* **1858**, 2050-2059 (2016).
38. Boncheva, M., Damien, F. & Normand, V. Molecular organization of the lipid matrix in intact Stratum corneum using ATR-FTIR spectroscopy. *Biochim. Biophys. Acta* **1778**, 1344-1355 (2008).
39. Lewis, R. N. & McElhaney, R. N. Fourier transform infrared spectroscopy in the study of lipid phase transitions in model and biological membranes: practical considerations. *Methods Mol. Biol.* **400**, 207-226 (2007).
40. Thakoersing, V. S., Ponec, M. & Bouwstra, J. A. Generation of human skin equivalents under submerged conditions-mimicking the in utero environment. *Tissue Eng. Part A.* **16**, 1433-1441 (2010).
41. Motta, S. *et al*. Ceramide composition of the psoriatic scale. *Biochim. Biophys. Acta* **1182**, 147-151 (1993).
42. van Smeden, J. *et al*. LC/MS analysis of stratum corneum lipids: ceramide profiling and discovery. *J. Lipid Res.* **52**, 1211-1221 (2011).
43. Masukawa, Y. *et al*. Characterization of overall ceramide species in human stratum corneum. *J. Lipid Res.* **49**, 1466-1476 (2008).
44. Ponec, M., Weerheim, A., Lankhorst, P. & Wertz, P. New acylceramide in native and reconstructed epidermis. *J. Invest. Dermatol.* **120**, 581-588 (2003).
45. Boiten, W., Absalah, S., Vreeken, R., Bouwstra, J. & van Smeden, J. Quantitative analysis of ceramides using a novel lipidomics approach with three dimensional response modelling. *Biochim. Biophys. Acta* **1861**, 1652-1661 (2016).
46. Favrot, C., Steffan, J., Seewald, W. & Picco, F. A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. *Vet. Dermatol.* **21**, 23-31 (2010).
47. Breathnach, R. M. *et al*. Association between skin surface pH, temperature and Staphylococcus pseudintermedius in dogs with immunomodulatory-responsive lymphocytic-plasmacytic pododermatitis. *Vet. Dermatol.* **22**, 312-318 (2011).
48. Hoffmann, M. V., Kastner, S. B., Kietzmann, M. & Kramer, S. Contact heat thermal threshold testing in beagle dogs: baseline reproducibility and the effect of acepromazine, levomethadone and fenpipramide. *BMC Vet. Res.* **8**, 206-6148-8-206 (2012).
49. Popa, I. *et al*. The lipid alterations in the stratum corneum of dogs with atopic dermatitis are alleviated by topical application of a sphingolipid-containing emulsion. *Clin. Exp. Dermatol.* **37**, 665-671 (2012).
50. Shimada, K., Yoon, J. S., Yoshihara, T., Iwasaki, T. & Nishifuji, K. Increased transepidermal water loss and decreased ceramide content in lesional and non-lesional skin of dogs with atopic dermatitis. *Vet. Dermatol.* **20**, 541-546 (2009).
51. Thakoersing, V. S. *et al*. Nature versus nurture: does human skin maintain its stratum corneum lipid properties in vitro? *Exp. Dermatol.* **21**, 865-870 (2012).
52. Mojumdar, E. H., Gooris, G. S. & Bouwstra, J. A. Phase behavior of skin lipid mixtures: the effect of cholesterol on lipid organization. *Soft Matter* **11**, 4326-4336 (2015).

Chapter 4

The bacterial and fungal microbiome of the skin of healthy dogs and dogs with atopic dermatitis and the impact of topical antimicrobial therapy, an exploratory study

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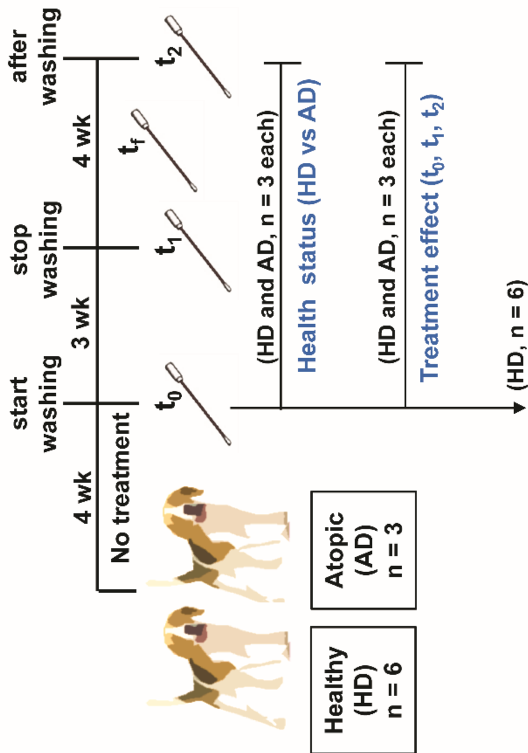
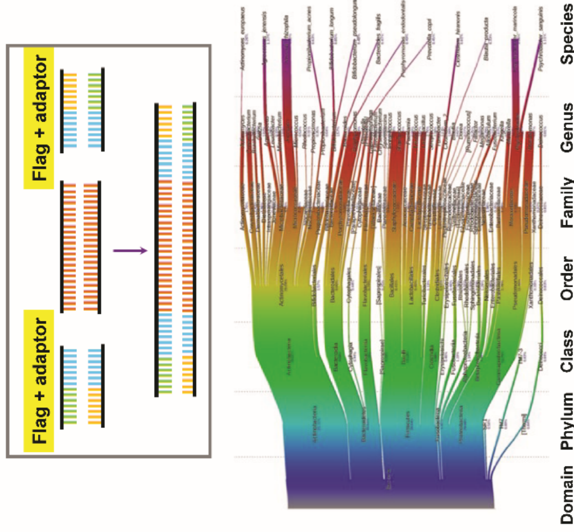
Graphical abstract





Abstract

Canine atopic dermatitis is a genetically predisposed inflammatory and pruritic allergic skin disease that is often complicated by (secondary) bacterial and fungal (yeast) infections. High-throughput DNA sequencing was used to characterize the composition of the microbiome (bacteria and fungi) inhabiting specific sites of skin in healthy dogs and dogs with atopic dermatitis (AD) before and after topical antimicrobial treatment. Skin microbiome samples were collected from six healthy control dogs and three dogs spontaneously affected by AD by swabbing at (non-) predilection sites before, during and after treatment. Bacteria and fungi were profiled by Illumina sequencing of the 16S ribosomal RNA gene of bacteria (16S) and the internally transcribed spacer of the ribosomal gene cassette in fungi (ITS). The total cohort of dogs showed a high diversity of microbes on skin with a strong individual variability of both 16S and ITS profiles. The genera of *Staphylococcus* and *Porphyromonas* were dominantly present both on atopic and healthy skin and across all skin sites studied. In addition, bacterial and fungal alpha diversity were similar at the different skin sites. The topical antimicrobial treatment increased the diversity of bacterial and fungal compositions in course of time on both AD and healthy skin.

Keywords: microbiome composition; 16S; ITS; canine; atopic dermatitis; topical treatment.

1. Introduction

Atopic dermatitis (AD) in dogs is a genetically predisposed inflammatory and pruritic allergic skin disease[1]. The skin microbiome may be the source of secondary infections that can influence the severity of canine AD[2]. Microbial culture-based studies showed that the most prominent bacterium on lesional skin of dogs with AD is *Staphylococcus pseudintermedius*, whereas *Malassezia pachydermatis* is the main fungal representative[3]. Topical antimicrobial therapy is widely used to relieve symptoms of AD partly caused by secondary infections[4].

Compared to human fewer studies focused on the canine skin microbiome. The relative abundance of the *Staphylococcus* genus was increased on the skin of AD dogs compared to healthy controls[5,6], and lower bacterial diversity was observed at skin sites affected by AD flares in dogs[5,7]. Likewise, fungal marker-gene sequences (ITS) revealed that the fungal diversity of lesional skin of atopic dogs was lower than that of healthy skin[8].

In dogs, specific areas of the skin are prone to be affected by AD[9]. In the present study, we aimed at characterization by high-throughput sequencing of the DNA of bacteria and fungi inhabiting the canine skin and compared the differences in microbiome composition i) at three predilection sites of canine AD (axilla, inguinal, periocular regions) and a non-predilection site (the trunk), ii) in health and AD and iii) before, during and after topical treatment with Malaseb® shampoo containing chlorhexidine 2% and miconazole 2%, proven to be effective for prevention and control of (secondary) bacterial and yeast growth on AD skin and consequent reduction of clinical symptoms of affected dogs[4,10]. With this exploratory study we intended to report initial descriptive data before embarking on a large survey.

2. Material and Methods

2.1 Dogs and sampling

Three atopic (AD, females, mean age 4.3 ± 4.0 SD years) and six healthy (HD, 4 females and 2 males; mean age: 4.75 ± 2.0 SD years) dogs of different breeds (**Table S1**) housed in the premises of the Department of Clinical Sciences of Companion Animals, Utrecht University were included. The three AD dogs were Bedlington-Beagle crossbreeds whereas the healthy dogs included three Bedlington-Beagle crossbreeds, two Beagles and one Greyhound. The AD dogs met the Favrot's diagnostic criteria for AD, and other causes of pruritus were ruled out by direct examination of the presence of fleas and flea faeces, coat brushing and skin scrapings[1]. Anti-parasitic control was achieved by monthly spot-on treatment with selamectin. Skin cytology was performed to rule out pyoderma and Malassezia dermatitis. Finally, the animals were fed an elimination diet for at least 8 weeks where food allergy was ruled out when no improvement on the diet was observed. For all nine dogs enrolled (**Figure 1**), hair was clipped at three lesional sites (axillae, inguinal and periocular) and one non-lesional site (craniolateral trunk), and skin microbiome samples

were collected by swabbing. A new pair of gloves was used for swabbing each sample. Sterile swabs (Isohelix DNA Buccal Swabs, Cell Projects Ltd, UK) were pre-moistened with either a sterile solution of 50 mM Tris buffer [pH 8], 1 mM EDTA, and 0.5% Tween-20 or a sterile phosphate buffered saline (PBS), for bacterial and fungal DNA isolation respectively. Swabs were placed parallel to the skin surface and rubbed back and forth for approximately 30 s. Initially, all healthy control dogs (n=6) were enrolled in the comparison of skin microbiome compositions of different AD predilection sites. Thereafter, sex- and breed-matched healthy control (HD, n=3) and AD (n=3) dogs were kept untreated for four weeks and were subsequently subjected to topical antimicrobial therapy with Malaseb® shampoo (Dermcare-vet Pty Ltd, Brisbane, Australia) twice weekly for three weeks. Finally, they were again kept untreated for four weeks. Sampling was conducted as follows: t_0: before start of treatment; t_1: after three weeks of treatment; t_2: four weeks after finalizing the 3-week treatment cycle; t_f: upon occurrence of flare-up symptoms in the period between t_1 and t_2 (**Figure 1**). Three AD dogs had AD lesions (erythema, lichenification, excoriations, self-induced alopecia) when the diagnosis was made, and in one of the three AD dogs AD lesions flared mainly at the inguinal area, axillae, forelimbs and hindlimbs after discontinuation of topical treatment (t_2). CADESI-03 scores of the AD dogs were between 0 and 116 at the start of the experiment and as a result of bathing the scores improved to between 0 and 33. Healthy dogs enrolled in this study did not have any skin lesions throughout the experiment. The Malaseb® shampoo was applied both on AD and healthy dogs to create similar treatment conditions for comparison of microbiome changes. All experimental procedures were approved by the Utrecht University Animal Ethic committee as required under Dutch legislation (DEC 2013.II.06.069 and DEC 2013.II.07.83)

2.2 Microbial DNA extraction for marker gene sequencing.

To release bacteria (16S), the cotton tips of swabs were repeatedly pressed against the wall of tubes containing 300 µl Microbead solution (MO BIO Ultraclean™ Microbial DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, USA). Each tube was vortexed at maximum speed with the MO BIO Vortex Adapter tube holder (MO BIO Laboratories Inc., Carlsbad, USA) for 10 minutes and microbial DNA was isolated according to the manufacturer's description. The microbial DNA samples were stored at -20 °C until further use.

For analysis of fungi (ITS) the cotton tips of the swabs were individually stored in 300 µl lysis solution (MasterPure™ Yeast DNA Purification kit, Epicentre, Madison, USA) at 4 °C. Then, 20 mg/ml lysozyme (Sigma-Aldrich, St. Louis, USA) was added to the samples and incubated for 1 h while shaking (220 rpm) at 37 °C, finally, the cotton tips were removed using sterile forceps. Next, a 5mm steel bead was added to mechanically disrupt fungal cell walls in samples using a Tissuelyser (Qiagen, Valencia, USA) for 2 min at 30 Hz. The Invitrogen PureLink Genomic DNA Kit (Invitrogen, Carlsbad, USA) was utilized for all subsequent steps. The microbial DNA samples were stored at -20 °C until further use.

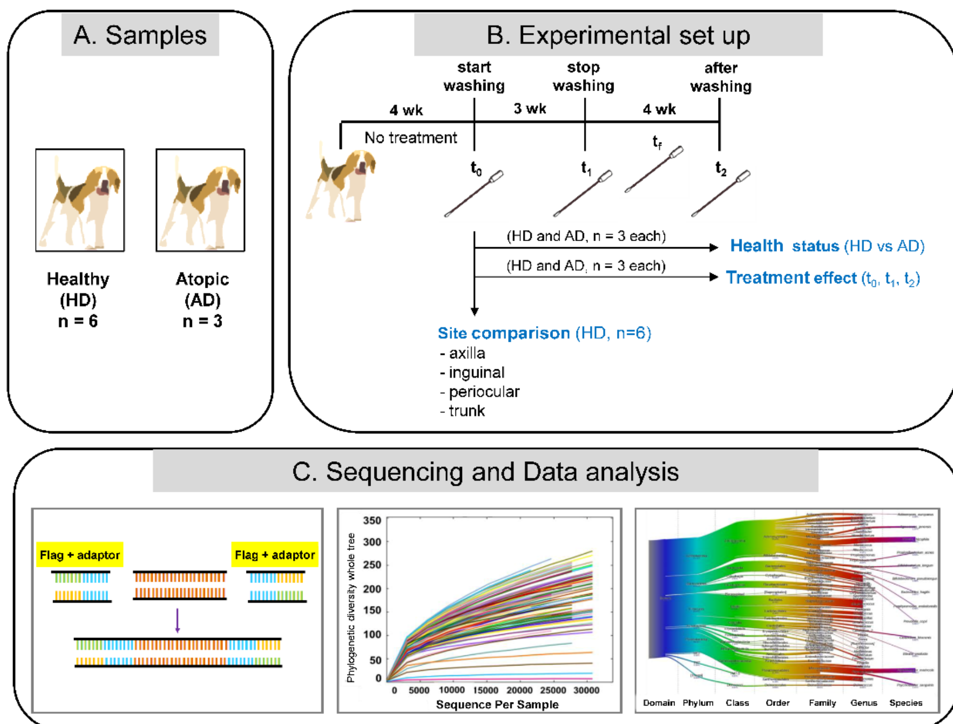


Figure 1. Study design and timeline of topical antimicrobial treatment.

2.3 Microbial DNA amplification for marker gene sequencing

Since according to a previous study in humans[11], only low amounts of microorganism DNA was found on skin as analyzed by PCR, a nested PCR was applied in the present study. In the first round of PCR, universal primers (16S: 200 nM forward primer (338F), 5’-ACT CCT ACG GGA GGC AGC AG-3’, 200 nM reverse primer (1061R), 5’CRR CAC GAG CTG ACG AC-3’; ITS: 400 nM forward primer (ITS1F), 5’-CTT GGT CAT TTA GAG GAA GTA A-3’, 400 nM reverse primer (ITS4R), 5’-TCC TCC GCT TAT TGA TAT GC-3’) were used to generally amplify the genomic DNA of the V3-V6 region of the 16S rRNA genes, and the ITS1-ITS2 region of the ITS genes, respectively. In the second round of PCR, flagged specific primers were used covering V3-V4 (16S) and ITS1-ITS2 (ITS) regions, resulting in barcoded products; for 16S: 200 nM forward primer (357F), 5’-*TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNN NNN* **CCT ACG GGA GGC AGC AG**-3’, 200 nM reverse primer (802RV2), 5’-*GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G***TA CNV GGG TAT CTA AKC C**-3’; and for ITS: 40 nM forward primer (ITS86F), 5’- *TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNN NNN NN***G TGA ATC ATC GAA TCT TTG AAC**-3’, 40 nM reverse primer (ITS4R), 5’ *GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G***TC CTC CGC TTA TTG ATA TGC**-3’; the italicized sequence is the flag sequence, *N* is the designated barcode (adaptor) to tag each product individually and the bold sequence is the universal primer sequence. Optimal

PCR conditions (50 µl reaction volume) were as follows: 5 µl of 10xKOD buffer, 5 µl of KOD dNTPs, 3 µl of KOD $MgSO_4$, forward and reverse primer, 1 µl of KOD hot start DNA polymerase (Novagen®, Osaka, Japan) and ultrapure water (Milli-Q™) for each reaction. All reactions were initiated at 95 °C for 2 min; followed by cycles of 95 °C for 20 s, 55 °C for 10 s, and 70 °C for 15 s for both 1st and 2nd rounds of PCR. Numbers of cycles for 1st and 2nd PCR were 30 and 35 for 16S respectively 25 and 25 for ITS. Each PCR product was examined for product size by electrophoresis on a 1% agarose gel and purified using a MSB Spin PCRapace kit (Invitek Inc., Carlsbad, USA) and a PureLink PCR purification kit (Invitrogen, Carlsbad, USA) for the initial and second (barcoding) PCR, respectively.

2.4 16S and ITS marker gene sequencing

The skin microbiome compositions were determined by sequencing of the PCR products of the bacterial and fungal marker genes 16S ribosomal RNA gene (16S) and the internal transcribed spacer (ITS). For this purpose, Illumina 16S rRNA and ITS amplicon libraries were generated from the microbial DNA isolated and barcoded as described above. Aliquots of minimally 100 ng per sample and a 260/280 ratio ranging between 1.8 and 2.0 were pooled and multiplexed libraries were sequenced by BaseClear BV (Leiden, The Netherlands), on an Illumina MiSeq system with a paired-end 300 cycles protocol and indexing. The sequencing run was analyzed with the Illumina CASAVA pipeline (v1.8.3) with demultiplexing based on sample-specific barcodes. The raw sequencing data produced were processed removing the sequence reads of too low quality (only "passing filter" reads were selected) and discarding reads containing adaptor sequences or failing PhiX Control with an in-house filtering protocol. A quality assessment on the remaining reads was performed using the FASTQC quality control tool version 0.10.0.

2.5 Illumina sequence data analysis

Demultiplexed FASTQ files as provided by BaseClear were first used to generate Illumina paired-end sequence pseudoreads by PEAR[12], using the default settings. For gene sequencing analysis, a customized Python workflow based on Quantitative Insights Into Microbial Ecology (QIIME version 1.8)[13] was adopted (http://qiime.org). Reads were filtered for chimeric sequences using the UCHIME algorithm version 4[14]. Open reference OTU calling for taxonomic classification of sequencing reads was performed with USEARCH version 6.1[15] as implemented in QIIME against the Greengenes database version 13_8 for bacterial sequences[16], and against the UNITE database version 12_11 for fungal sequences[17]. Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure as implemented in QIIME. Figures resulting from these clustering analyses were generated using the interactive tree of life (iTOL) tool[18]. Alpha diversity metrics (phylogenetic diversity whole tree (PDWT), Chao1, Observed Species and Shannon) were calculated by bootstrapping 10 reads per sample, and taking the average over ten trials. For

visualization of the differential microbiome, Cytoscape software version 3.1 3[19] was used together with in-house developed Python scripts for generating the appropriate input data deriving from the QIIME analysis. Fold changes in abundances of bacterial and fungal genera between healthy and AD samples of the four skin sites (the 2-log of the ratio Healthy/AD) are shown in **Table 1** and **Table 2** respectively, as heatmaps, created by the Excel version 2010 using the color scales of conformational formatting. Individual microbiota were ranked according to averaged relative abundances in the AD skin samples.

The average sequence read count per sample of this study was 31182 ± 5834 SD and 49064 ± 11846 SD, for 16S and ITS respectively, and likewise, the average number of operational taxonomic units (OTUs) per sample was 3053 ± 881 SD and 341 ± 158 SD. Three 16S samples were excluded from further analyses, one due to a low number of reads (44) and two others due to low OTU counts (148 and 169) (see more details in **Table S2** and **Table S3**). For an overview of the exact (16S and ITS) microbiome composition for each study sample we refer to **Table S4** and **Table S5.** Note that due to technical limitations in the resolution of 16S and ITS marker gene sequencing, OTUs calling on the level of species should be interpreted with caution.

2.6 Statistics

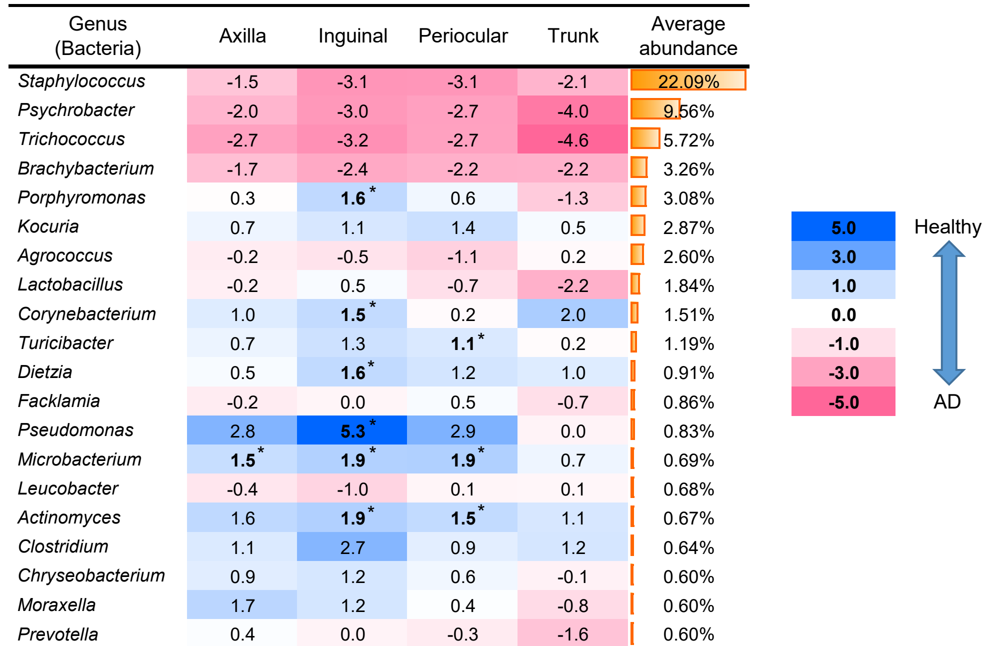
For the microbiome data in this manuscript, statistical significance between contrasts with regard to taxonomy abundances was tested by a non-parametric (unpaired) Mann-Whitney *U* test (MWU), uncorrected for multiple testing; unless stated otherwise. Note that in the case of uncorrected p-values, the p-value threshold that needs to be met in order to be considered significant is adapted by dividing the classic threshold of $P = 0.05$ by the number of taxa observed. Statistical tests were performed by custom, in-house Python scripts (SciPy module version 0.17.0; https://www.scipy.org/) downstream of QIIME, as described above. Principal component analysis (PCA) as well as multivariate Redundancy Analysis (RDA) were done using Canoco 5.04[20] using default settings of the analysis type 'Unconstrained' or 'Constrained', respectively. Canoco reported variance explained by particular variables (e.g. breed and site) by applying said variables as single supplementary variables in a PCA analysis. Relative abundance values for taxa were used as response data, and for RDA, the sample classes as explanatory variables. RDA calculates *P* values by permuting the sample classes. Significances mentioned in figures are as follows: n.s. (not significant), * P <0.05.

2.7 Accession numbers

The raw, unprocessed 16S rRNA and ITS marker gene Illumina sequence reads are publicly available for download at the European Nucleotide Archive (ENA) database[21] under study accession number: PRJEB20808 (http://www.ebi.ac.uk/ena/data/view/ PRJEB20808) or secondary accession number: ERP022994 (http://www.ebi.ac.uk/ena/data/view/

ERP022994). The sequencing data are available in FASTQ-format, including corresponding metadata for each sample. For additional information on sample characteristics we refer to **Table S1**.

Table 1. Differential presence of bacteria (16S) in the skin microbiome on different body sites of AD and healthy skin.



Genus (Bacteria)	Axilla	Inguinal	Periocular	Trunk	Average abundance
Staphylococcus	-1.5	-3.1	-3.1	-2.1	22.09%
Psychrobacter	-2.0	-3.0	-2.7	-4.0	9.56%
Trichococcus	-2.7	-3.2	-2.7	-4.6	5.72%
Brachybacterium	-1.7	-2.4	-2.2	-2.2	3.26%
Porphyromonas	0.3	**1.6** *	0.6	-1.3	3.08%
Kocuria	0.7	1.1	1.4	0.5	2.87%
Agrococcus	-0.2	-0.5	-1.1	0.2	2.60%
Lactobacillus	-0.2	0.5	-0.7	-2.2	1.84%
Corynebacterium	1.0	**1.5** *	0.2	2.0	1.51%
Turicibacter	0.7	1.3	**1.1** *	0.2	1.19%
Dietzia	0.5	**1.6** *	1.2	1.0	0.91%
Facklamia	-0.2	0.0	0.5	-0.7	0.86%
Pseudomonas	2.8	**5.3** *	2.9	0.0	0.83%
Microbacterium	**1.5** *	**1.9** *	**1.9** *	0.7	0.69%
Leucobacter	-0.4	-1.0	0.1	0.1	0.68%
Actinomyces	1.6	**1.9** *	**1.5** *	1.1	0.67%
Clostridium	1.1	2.7	0.9	1.2	0.64%
Chryseobacterium	0.9	1.2	0.6	-0.1	0.60%
Moraxella	1.7	1.2	0.4	-0.8	0.60%
Prevotella	0.4	0.0	-0.3	-1.6	0.60%

Listed are the 20 (out of 496) on average most abundant bacterial genera of atopic dermatitis dogs from three predilection sites: axilla, inguinal region and periocular, and one non-predilection site: trunk. Ranking is based on the averaged relative abundances of bacteria of the four skin sample sites in the three AD dogs. The heatmap presents the fold changes in abundance (the 2-log of the ratio Healthy/AD): 1 means two times more abundant in healthy samples, -1 means two times more abundant in AD samples, 0 means no change, etc. Significant differences (* P <0.05) in relative abundance of genera in AD compared to healthy skin at the same sampling site were determined by MWU. A Bonferroni-corrected P value threshold for significance is the genus-level P value divided by the number of total genera identified, $0.05/496 = P < 1.0*10^{-4}$. For abundances of the remaining genera and other taxon levels, see **Table S4**.

Table 2. Differential presence of fungi (ITS) in the skin microbiome on different body sites of AD and healthy skin.

Genus (Fungi)	Axilla	Inguinal	Periocular	Trunk	Average abundance
"Unidentified#01"	0.5	-0.3	-0.3	0.3	15.87%
Blumeria	-1.7*	-1.7*	-1.5	-1.8*	8.59%
"Unidentified#12"	-0.1	0.0	-0.4	0.6	7.72%
Epicoccum	-0.3	0.2	-0.2	0.4	3.83%
Fusarium	-0.2	-0.7	-2.1	0.6	2.48%
Cryptococcus	-0.9	-0.3	-2.5*	-0.3	1.43%
Pilidium	-2.4	0.0	0.0	-1.7	1.00%
Sporobolomyces	0.8	2.2*	0.1	-1.8*	0.80%
Rhodotorula	1.8*	0.3	1.1*	0.0	0.69%
Gibberella	-0.1	1.9	-2.3*	3.2	0.51%
"Unidentified#13"	1.5	-2.0	0.0*	2.8	0.49%
"Unidentified#6"	-4.0*	0.1	-5.7	-0.6	0.33%
Ramularia	0.6	5.4*	0.0	0.4	0.27%
Alternaria	3.5	-0.6	-1.1	-2.0	0.24%
Cystofilobasidium	-2.3	-2.9	0.2	0.0	0.17%
"Unidentified#45"	0.0*	-1.4	0.0	-1.1	0.15%
"Unidentified#23"	0.0	-5.4	0.0	0.0	0.13%
Dinemasporium	-2.0	-1.6	2.0	1.8	0.13%
Microdochium	1.0	3.6	-1.6	0.4	0.13%
Claviceps	0.0	2.3	0.0	-1.8	0.12%

Scale	
5.0	Healthy
3.0	
1.0	
0.0	
-1.0	
-3.0	
-5.0	AD

Listed are the 20 (out of 314) on average most abundant fungal genera of atopic dermatitis dogs from three predilection sites: axilla, inguinal region and periocular, and one non-predilection site: trunk. Ranking is based on the averaged relative abundances of fungi of the four skin sample sites in the three AD dogs. The heatmap presents the fold changes in abundance (the 2-log of the ratio Healthy/AD): 1 means two times more abundant in healthy samples, -1 means two times more abundant in AD samples, 0 means no change, etc. Note that "*Unidentified*" genera represent known classifiable taxa but currently without assigned nomenclature. Significant differences (* P <0.05) in relative abundance of genera in AD compared to healthy skin at the same sampling site were determined by MWU. A Bonferroni-corrected P value threshold for significance is the genus-level P value divided by the number of total genera identified, 0.05/314 = $P < 1.6*10^{-4}$. For abundances of the remaining genera and other taxon levels, see **Table S5**.

3. Results

3.1 Canine skin microbiome: general aspects

3.1.1 Study demographics and overview of canine skin microbiome.

Bacterial (16S) and fungal (ITS) microbiome compositions of both healthy and AD dog skin at four different sites (axilla, inguinal, periocular and trunk) and at three different time-points (before, during and after Malaseb® treatment) were assessed by a PCA at the genus-level. PCA showed clear separation (variance) between sample groups (**Figure 2**). Most notably, distances between sample sites were less than those between health and AD states or between time-points around treatment (Malaseb® treatment effect) for both 16S and ITS, indicating that there is more overlap between microbiome composition and

abundance between different skin niches than between health states and between stages (i.e. effect) of treatment.

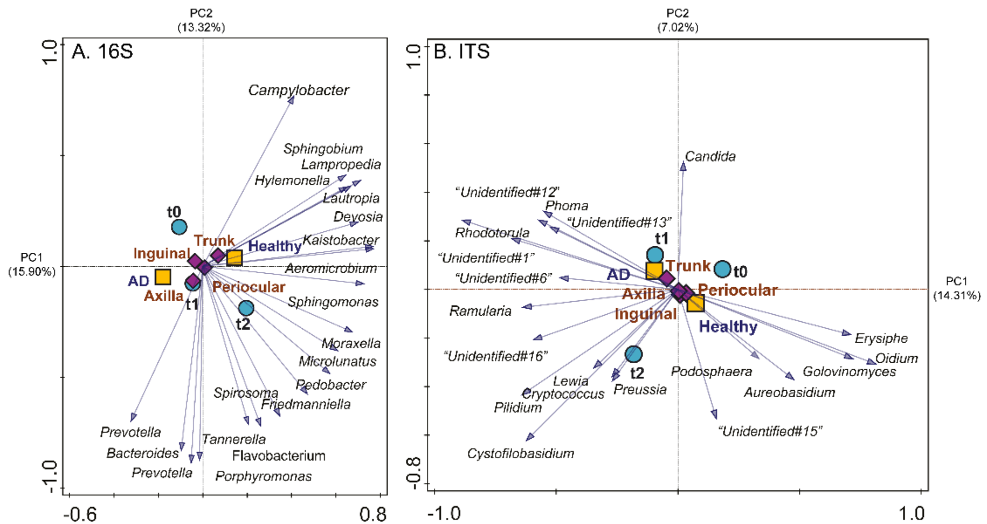


Figure 2. Bacterial and fungal genus-level compositions of all study samples indicate a higher microbial similarity between sample sites than between health states or treatment stages. These representations of a principal component analysis (PCA) of both 16S (**A**) and ITS (**B**) datasets reflect the distances between study samples based on genus-level microbial composition and abundances for each sample. Centroids representing samples of the same sample group are shown as colored symbols accompanied by their respective labels (◆ skin site: axilla, inguinal, periocular, trunk; □ health status: AD, healthy; ● time-point during Malaseb® treatment: t_0 before washing, t_1 after three weeks of washing, t_2 four weeks after termination of washing). The blue arrows point to the genera (names shown in italics) explaining compositional differences between the samples. Note that "*Unidentified*" genera represent known classifiable taxa but currently without assigned nomenclature.

3.1.2 Comparison of microbiome composition on different (non-)predilection skin sites within healthy or AD dogs

In RDA, the bacterial and fungal skin microbiome composition on genus-level in healthy dogs did not differ significantly between skin niches defined as AD predilection (axilla, inguinal and periocular) or non-predilection (trunk) sites, for 16S (P = 0.2) nor ITS (P = 0.3). Accordingly, a clear clustering of samples collected from similar body sites based on microbiome composition was not observed (**Figures S1-S4**). However, the samples collected from different skin sites of the same individual did show a certain degree of variation in microbial composition (both 16S and ITS) but samples from the same breed were at relatively close distance. For 16S, 47.0% of the variation was explained by individual differences, 30.7% by breed and 11.6% by sampling site (**Figure S5A**). For ITS, these figures were 40.0%, 28.0% and 10.8% respectively (**Figure S5B**). Notably, separation based on breed is more convincing for ITS than for 16S, supported by (beta diversity) clustering of samples based on full community composition (**Figures S1-S2**).

3.2 Comparison of skin microbiome composition between healthy and AD skin

3.2.1 Alpha diversity

The differences in alpha diversity of bacteria (**Figure 3A**) nor fungi (**Figure 3B**) were statistically significant between corresponding AD and healthy skin sites; nevertheless, alpha diversity tended to be lower in AD skin at most sites studied (**Figures 3A-B**).

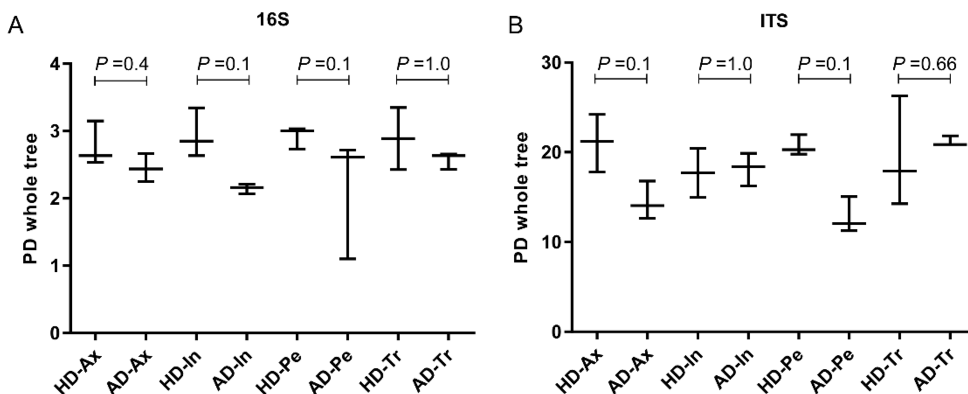


Figure 3. Phylogenetic Diversity Whole tree (PDWT) analysis of 16S and ITS data. The diversity analyzed by PDWT of 16S (**A**) and ITS (**B**) microbiome profiles observed in atopic dermatitis (AD) and healthy (HD) skin at the four sites studied (Ax: axilla, In: inguinal, Pe: periocular, Tr: trunk).

3.2.2 *Differential composition of the microbiome on different body sites between AD and healthy skin.*

Based on RDA, differences in genera contributing to the microbiome composition of both 16S and ITS associated to disease status (AD versus healthy) were not statistically significant (data not shown). The predominant genera based on body sites and health states are shown in **Table 1** (16S) and **Table 2** (ITS)**.**

In both AD and healthy dog skin, the bacterial microbiome composition differed only slightly among skin sites studied (**Table 1**)**.** The, on average (across all skin sites), most abundant bacteria observed in AD skin were *Staphylococcus*, *Psychrobacter*, *Trichococcus*, *Brachybacterium* and *Porphyromonas.* These genera were also present on healthy skin but varied in the relative abundance. The most dominant bacterial (genus-level) taxa shared between all four body sites in healthy skin are *Pseudomonas* (5.61% ± 1.96% SEM), *Kocuria* (5.29% ± 0.62%), *Porphyromonas* (4.31% ± 1.52%), *Staphylococcus* (3.65% ± 0.72%) and *Corynebacterium* (3.31% ± 1.08%) (**Table S4)**. Differences were observed in the order of the top abundant taxa among the sample sites of dogs with AD (**Table 1**). *Staphylococcus* was always more abundant in AD skin than in controls at the same sites (axilla: 14.46% versus 5.02%; inguinal 36.50% versus 4.26%; periocular 30.46% versus 3.66%; trunk 6.95% versus

1.66%) (**Figure S6***,* t_0 results), although this did not reach statistical significances. In samples of lesional sites of AD dogs during flare-up the presence of *Staphylococcus* was the most abundant among the bacterial microbiome community (21.74 ± 8.29% SEM). Interestingly, the relative abundances of the lowly dominant *Microbacterium* were significantly lower at each of the three predilection sites in AD dogs as compared to those in healthy ones (**Table 1**). In contrast, for none of the 496 bacterial genera significant differences in relative abundance were found between AD and healthy trunk skin, considered to be a non-predilection site.

The most abundant fungal genera, found on the healthy skin across all sites studied were "*Unidentified#01",* a known classifiable fungal taxon but currently without assigned nomenclature, (17.28% ± 3.26% SEM)*, "Unidentified#12"* (7.97% ± 1.01%)*, Epicoccum* (4.02% ± 0.63%)*, Blumeria* (2.68% ± 0.32%) and *Ramularia* (2.66% ± 2.31%). Like in healthy skin, "*Unidentified#01"* was the most abundant at every AD skin site studied (**Table 2**), and this genus was also the most dominant taxon of fungi presenting during flare-up of AD (15.46% ± 1.79% SEM).

The largest differences in relative abundances at fungal genus-level between AD and healthy skin were those observed for ***Blumeria.*** These were statistically significant increases at axilla, inguinal and trunk of AD skin (**Table 2** and **Figure S7***,* t_0).

3.3 Topical antimicrobial treatment with Malaseb® shampoo

3.3.1 The effect of treatment on the diversity of the microbiome on canine skin

Bacterial and fungal diversities on healthy as well as AD skin, did not differ significantly at the three different time-points, before, during and after treatment (see **Figure S8**). Interestingly, however, the trend observed in the bacterial diversity in AD skin was an initial increase from t_0 (start of treatment after at least 4 weeks without intervention) to t_1 (termination of treatment) and a subsequent decrease to the level of t_0 in the four weeks towards t_2. The fungal diversity at both AD and healthy skin decreased towards t_1 and had increased at t_2, close to that at t_0. In addition, although not statistically significant, a lower diversity was observed in AD skin as compared to healthy skin at all time-points.

3.3.2 The effect of topical treatment on 16S and ITS microbiome composition

Topical treatment of dog skin caused changes in 16S microbiome composition (AD and healthy skin data combined) in course of time. A clear separation between time-points, as a result of treatment, (3 different colored ovals) was seen in axilla (**Figure 4A**; $P = 0.026$), inguinal (**Figure 4B**; $P = 0.032$), periocular region (**Figure 4C**; $P = 0.004$) and trunk (**Figure 4D**; $P = 0.028$).

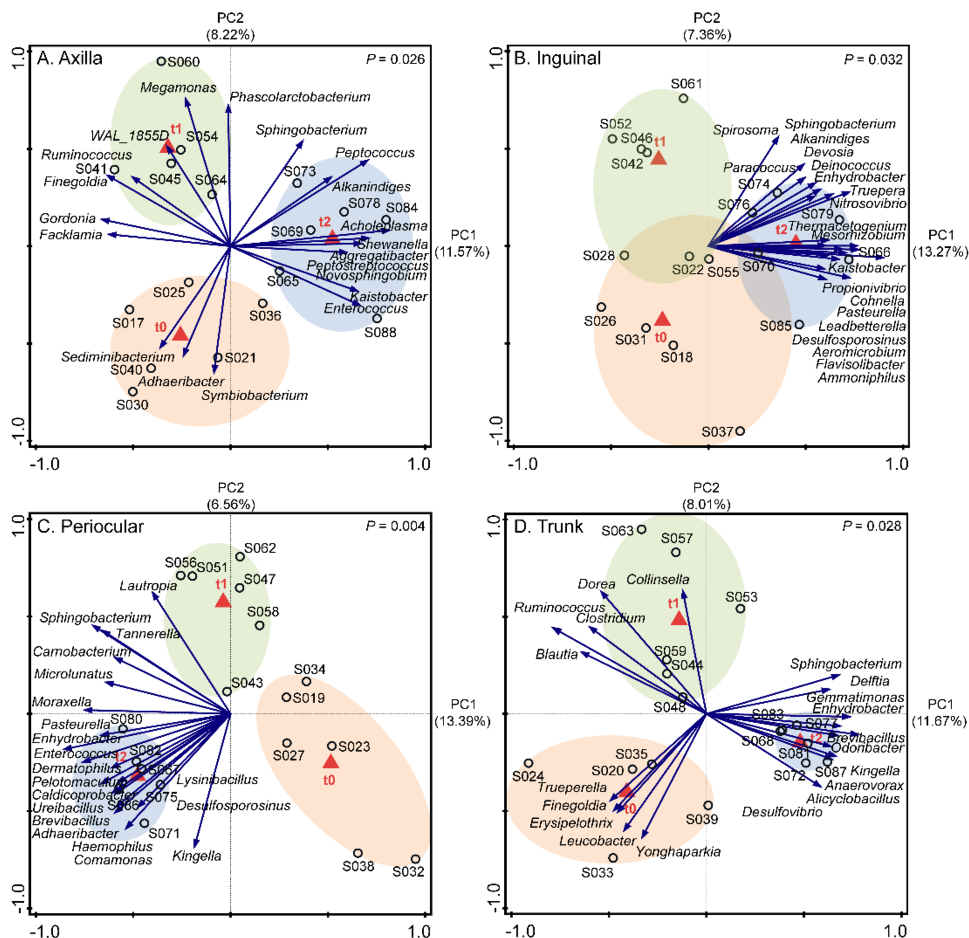


Figure 4. Treatment effect on 16S profiles at different body sites. The16S microbiome composition associated to Malaseb® treatment at axilla (**A**), inguinal (**B**), periocular (**C**) and trunk (**D**), based on all data (irrespective of health status) visualized in a redundancy analysis (RDA) plot (Treatment was used as explanatory variable, corrected for Health Condition). Each small open circle represents the skin genus-level composition of one sample. The red triangles are the centroids of the sample groups for each time point t_0, t_1 and t_2, whereas the colored sample overlays represent the sample clusters per time-point; orange: t_0, green: t_1 and blue: t_2). Blue arrows: taxa that most contribute to the separation on samples in the respective directions. Significance level at $P < 0.05$.

Likewise, the ITS microbiome composition (AD and healthy skin data combined) at all sample sites changed significantly in course of time (axilla and trunk, **Figure 5A** and **Figure 5D** at $P = 0.001$; inguinal, **Figure 5B** at $P = 0.019$; periocular, **Figure 5C** at $P = 0.049$).

Hence, both the differences in bacterial (16S, **Figure 4**) and fungal (ITS, **Figure 5**) skin composition in course of time (treatment effect) were statistically significant if samples were not stratified on health condition (i.e. analysis irrespective of AD).

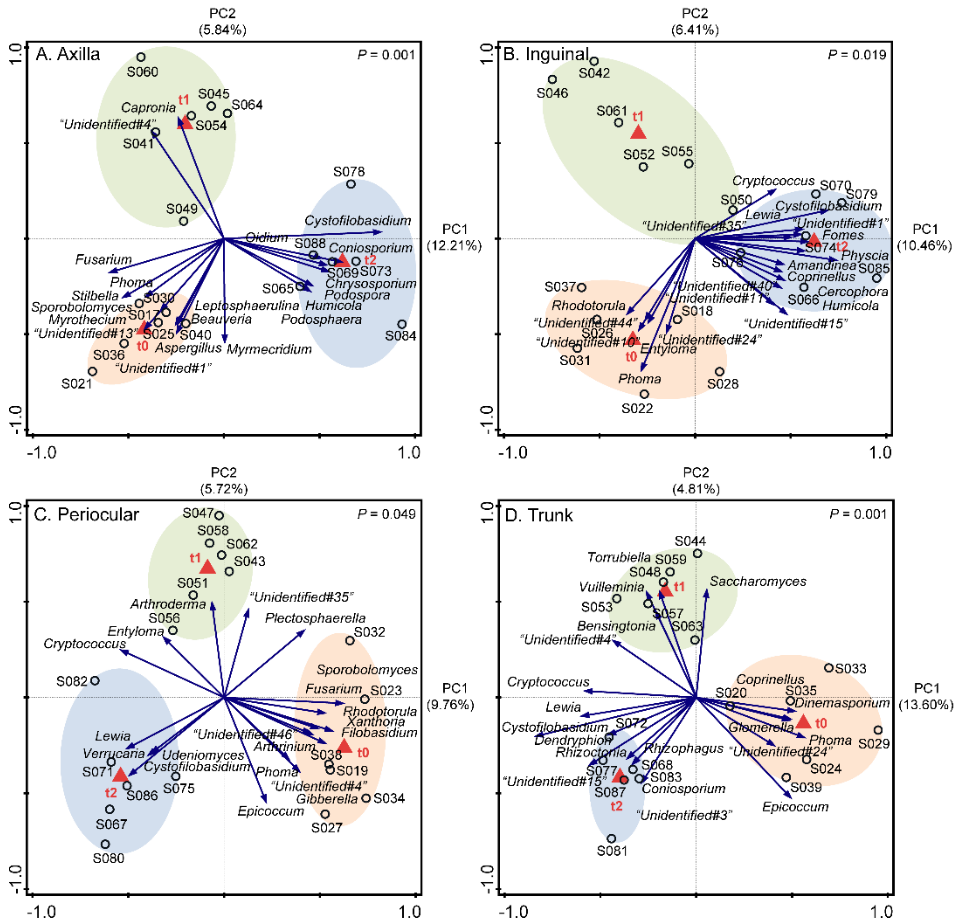


Figure 5. Treatment effect on ITS profiles at different body sites. The ITS microbiome composition associated to Malaseb® treatment at axilla (**A**), inguinal (**B**), periocular (**C**) and trunk (**D**), based on all data (irrespective of health status) visualized in a redundancy analysis (RDA) plot (Treatment was used as explanatory variable, corrected for Health Condition). Each small open circle represents the skin genus-level composition of one sample. The red triangles are the centroids of the sample groups for each time point t_0, t_1 and t_2, whereas the colored sample overlays represent the sample clusters per time-point; orange: t_0, green: t_1 and blue: t_2). Blue arrows: taxa that most contribute to the separation on samples in the respective directions. Significant level at P <0.05.

3.3.3 The most notable changes as a result of topical treatment

The most notable changes in the microbiome, as a result of treatment were observed in the genus *Epicoccum (*ITS) (**Figure S9**). In AD dogs, the genus of *Blumeria* revealed the largest changes in the relative abundance determined by time-points of sampling (treatment effect) in comparison to the other fungal genera identified (**Figure S7**). Its abundance was high in untreated AD skin (t_0), decreased after treatment (t_1) and remained low until four weeks after treatment withdrawal (t_2) (**Figure S7;** for axilla, trunk

and inguinal). The abundance of *Blumeria* on healthy skin was constantly low at all time-points and clearly lower than that on AD skin.

4. **Discussion**

This study aimed to investigate both the bacterial (16S) and fungal (ITS) skin microbiome compositions, both in different individuals, at different predilection sites and with respect to the health status (AD versus healthy). Furthermore, changes were recorded in the microbiome composition after topical treatment with Malaseb® shampoo, a common choice of treatment known to reduce the clinical severity of AD most likely partly due to (secondary) infection.

Analysis of the skin 16S and ITS microbiome composition in the present cohort of dogs revealed a strong inter-dog variability (individual), whereas breed effects were considerable, but less. In samples collected from different body sites both from AD and healthy dogs, no significant differences in microbiome composition (both 16S and ITS) were found. This confirms findings of another study on fungal composition of canine skin[8]. In contrast, on human skin the microbiome composition was shown to differ depending on body sites[22,23], however comparison of microbiome compositions between human and canine skin is complicated due to strongly differing microbiomes. Moreover in dogs alpha diversity of skin microbiome was considerably higher than that in humans[11,24].

Comparing AD and healthy skin microbiome profiles, no significant differences in the alpha diversity were found, only a trend of reduced 16S and ITS diversity in AD compared to control skin. A similar trend was reported in other canine studies[5,7,8]. The topical treatment increases the diversity of bacteria, but the opposite effect was observed for fungal diversity. Those non-significant changes, that may be explained by difference between broad antibacterial and antifungal effects of the shampoo, and in potential differences in population dynamics amongst bacteria and fungi after treatment, need to be confirmed in larger studies. However, treatment with an antimicrobial shampoo, known to successfully alleviate clinic symptoms of AD, significantly changed both 16S and ITS profiles of the microbiome in course of time and at every site studied but irrespective of AD skin status. Only the genus, *Blumeria* (ITS), showed considerable changes in relative abundance determined by both health status and treatment effect. Whether these changes are affected by the atopic dermatitis directly or the associated inflammation, or by both, is unclear. The significant increase of this genus ($P < 0.05$; uncorrected) observed on AD as compared to healthy skin in the present study, even though it contrasts findings in an earlier study of fungi on canine skin[8], warrants further studies on the relationship between skin health status and *Blumeria*.

In the present study, the bacterial genera *Staphylococcus* and *Porphyromonas* were dominantly present in both AD and healthy skin. The finding of *Staphylococcus* as the predominant colonizer of the 16S profile on AD skin is consistent with many other studies in canine[5,7], feline[25] and human skin[22,26]. Staphylococci are common commensals on the

canine skin[27]. *Porphyromonas*, commonly found in the canine oral cavity[28], may have been transferred to skin by licking, presumably more frequently in AD dogs due to itch. Other genera present on healthy skin, *Kocuria*, *Pseudomonas* and *Corynebacterium* are common to dog skin[7,27]. The abundance of the low abundant *Microbacterium* was significantly different (*P* <0.05; uncorrected) when compared between AD and control skin on all predilection sites but not on the trunk (non-predilection). The observed significant differences in the lowly abundant genera might be due to the reduced alpha diversity observed in the AD skin microbiome.

The most abundant fungal genera on both canine AD and healthy were "*Unidentified#01*", "*Unidentified#12*", *Blumeria* and *Epicoccum*. Due to technical limitation of ITS marker gene analyses, "*Unidentified#01*" can only be qualified as not belonging to the phyla *Ascomycota* or *Basidiomycota,* and "*Unidentified#12*" as belonging to the phylum *Ascomycota. Malassezia* one of the most dominant yeasts (fungi) reported on canine skin[8], when it comes to identification of the microbiota composition by culture, was hardly detected in the present ITS marker gene analysis, that has shown to readily detect *Malassezia* if used as a positive control. Apparently, the skins of dogs in our study are virtually devoid of the *Malassezia* genus (presence of 0.02% on average). The ITS sequencing data was scrutinized by aligning the raw sequencing data by hand to the representative *Malassezia* ITS genus sequence to ensure that *Malassezia* reads were indeed not missed even in the raw sequencing data (results not shown). The apparent discrepancy between *Malassezia* presence as described in the literature and our study may be partially explained by housing condition and/or a small sample size of our study. The other limitations of our study include varied CADESI scores, mild secondary infections and low breed diversity.

Obviously additional factors giving rise to abnormalities as reflected in AD such as skin barrier function, immune responses, genetic background, etc., may affect the microbiome composition[2,29]. Differences in skin health caused by AD might alter its capacity to deal with changes in microbiome composition[2,5]. Interaction between microbiome and AD is likely to be a two-way street where microbiome and disease factors influence each other[2,6]. The predominant taxa and multiple factors potentially disturbing skin integrity in AD-related circumstances should be further investigated in a larger study.

Conclusion

Taken together, this study shows that the variation in microbiome composition of canine skin is likely to be dependent on individuality, and to a lesser extent is determined by skin site, health status or treatment effect. However, in some specific taxa significant differences could be observed by disease or treatment effect that need to be further investigated.

Declarations of interest

None.

Acknowledgements

We would like to thank Patrick Zeeuwen for support in DNA sampling, isolation and amplification. This work was supported by a scholarship from the Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand; and a ZonMw/NWO-ALW grant "Enabling Technologies" project number 40-43500-98-091. The funding sources had no involvement in study design; the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

References

1. Hensel, P., Santoro, D., Favrot, C., Hill, P. & Griffin, C. Canine atopic dermatitis: detailed guidelines for diagnosis and allergen identification. *BMC Vet. Res.* **11**, 196-015-0515-5 (2015).
2. Santoro, D. *et al*. Review: Pathogenesis of canine atopic dermatitis: skin barrier and host-micro-organism interaction. *Vet. Dermatol.* **26**, 84-e25 (2015).
3. Miller, W. H., Griffin, C. E., Campbell, K. L. & Muller, G. H. in *Muller and Kirk's Small Animal Dermatology7: Muller and Kirk's Small Animal Dermatology* (Elsevier/Mosby, 2013).
4. Olivry, T. *et al*. Treatment of canine atopic dermatitis: 2015 updated guidelines from the International Committee on Allergic Diseases of Animals (ICADA). *BMC Vet. Res.* **11**, 210-015-0514-6 (2015).
5. Bradley, C. W. *et al*. Longitudinal Evaluation of the Skin Microbiome and Association with Microenvironment and Treatment in Canine Atopic Dermatitis. *J. Invest. Dermatol.* **136**, 1182-1190 (2016).
6. Bjerre, R. D., Bandier, J., Skov, L., Engstrand, L. & Johansen, J. D. The role of the skin microbiome in atopic dermatitis: a systematic review. *Br. J. Dermatol.* **177**, 1272-1278 (2017).
7. Rodrigues Hoffmann, A. *et al*. The skin microbiome in healthy and allergic dogs. *PLoS One* **9**, e83197 (2014).
8. Meason-Smith, C. *et al*. What is living on your dog's skin? Characterization of the canine cutaneous mycobiota and fungal dysbiosis in canine allergic dermatitis. *FEMS Microbiol. Ecol.* **91**, 10.1093/femsec/fiv139. Epub 2015 Nov 4 (2015).
9. Favrot, C., Steffan, J., Seewald, W. & Picco, F. A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. *Vet. Dermatol.* **21**, 23-31 (2010).
10. Mueller, R. S., Bergvall, K., Bensignor, E. & Bond, R. A review of topical therapy for skin infections with bacteria and yeast. *Vet. Dermatol.* **23**, 330-41, e62 (2012).
11. Zeeuwen, P. L. *et al*. Microbiome dynamics of human epidermis following skin barrier disruption. *Genome Biol.* **13**, R101 (2012).
12. Zhang, J., Kobert, K., Flouri, T. & Stamatakis, A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **30**, 614-620 (2014).
13. Caporaso, J. G. *et al*. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**, 335-336 (2010).
14. Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194-2200 (2011).
15. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460-2461 (2010).
16. DeSantis, T. Z. *et al*. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* **72**, 5069-5072 (2006).
17. Koljalg, U. *et al*. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. *New Phytol.* **166**, 1063-1068 (2005).
18. Letunic, I. & Bork, P. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* **23**, 127-128 (2007).
19. Shannon, P. *et al*. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* **13**, 2498-2504 (2003).
20. Cajo J. F. ter Braak & Petr Smilauer. Canoco Reference Manual and User's Guide: Software for Ordination (version 5.0). (2012).
21. Leinonen, R. *et al*. The European Nucleotide Archive. *Nucleic Acids Res.* **39**, D28-31 (2011).
22. Grice, E. A. *et al*. Topographical and temporal diversity of the human skin microbiome. *Science* **324**, 1190-1192 (2009).
23. Findley, K. *et al*. Topographic diversity of fungal and bacterial communities in human skin. *Nature* **498**, 367-370 (2013).
24. Takemoto, A., Cho, O., Morohoshi, Y., Sugita, T. & Muto, M. Molecular characterization of the skin fungal microbiome in patients with psoriasis. *J. Dermatol.* **42**, 166-170 (2015).
25. Older, C. E. *et al*. The feline skin microbiota: The bacteria inhabiting the skin of healthy and allergic cats. *PLoS One* **12**, e0178555 (2017).
26. Kong, H. H. *et al*. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res.* **22**, 850-859 (2012).
27. Weese, J. S. The canine and feline skin microbiome in health and disease. *Vet. Dermatol.* **24**, 137-45.e31 (2013).
28. Falagas, M. E. & Siakavellas, E. Bacteroides, Prevotella, and Porphyromonas species: a review of antibiotic resistance and therapeutic options. *Int. J. Antimicrob. Agents* **15**, 1-9 (2000).
29. Chermprapai, S. *et al*. Altered lipid properties of the stratum corneum in Canine Atopic Dermatitis. *Biochim. Biophys. Acta* **1860**, 526-533 (2018).

30. Sundquist, A. *et al*. Bacterial flora-typing with targeted, chip-based Pyrosequencing. *BMC Microbiology* **7**, 108 (2007).

Supplemental material

1. Supplemental tables

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2. Supplemental figures

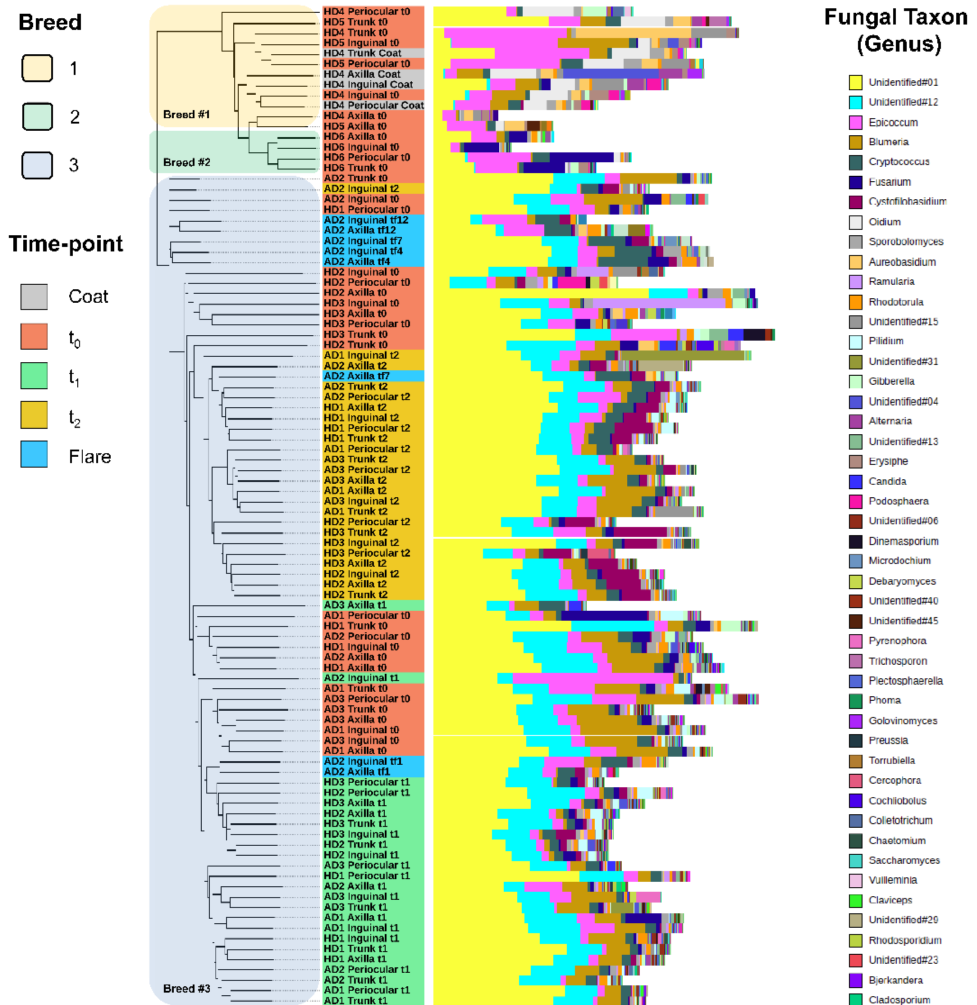


Figure S1. Clustering and microbial community composition of dog skin ITS samples, with visualization on genus-level. ITS samples are grouped according to breed as highlighted in the first column (orange: Beagle; green: Greyhound and blue: Bedlington-Beagle crossbreed). Within breed groups samples were clustered using UPGMA with weighted UniFrac (beta diversity metric) as a distance measure. Time-points of sampling are highlighted in the second column (red: t_0, green: t_1, yellow: t_2, blue: flare); coat samples are highlighted in grey. The full length of each stacked-bar represents 100% of genus-level classified reads per sample, colors represent relative abundances of genera as defined in the right column. The figure was generated with iTOL.

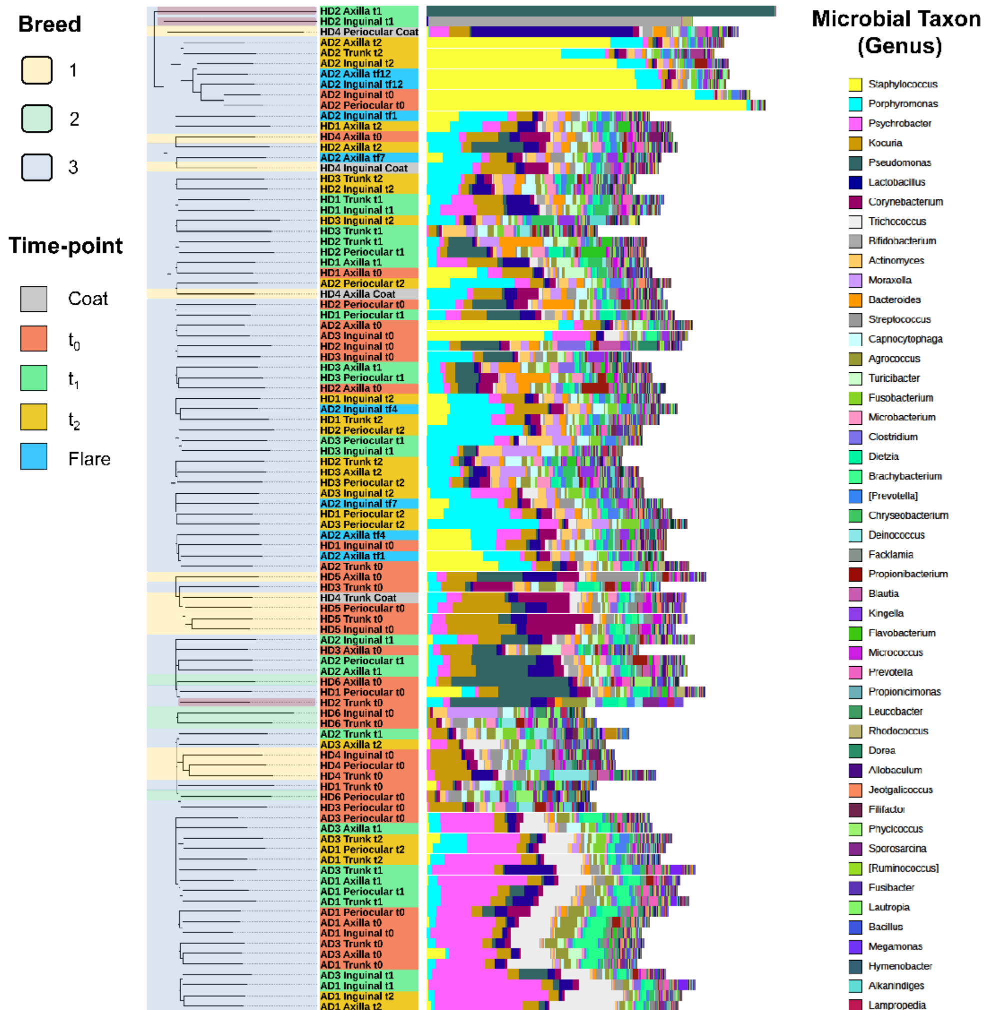
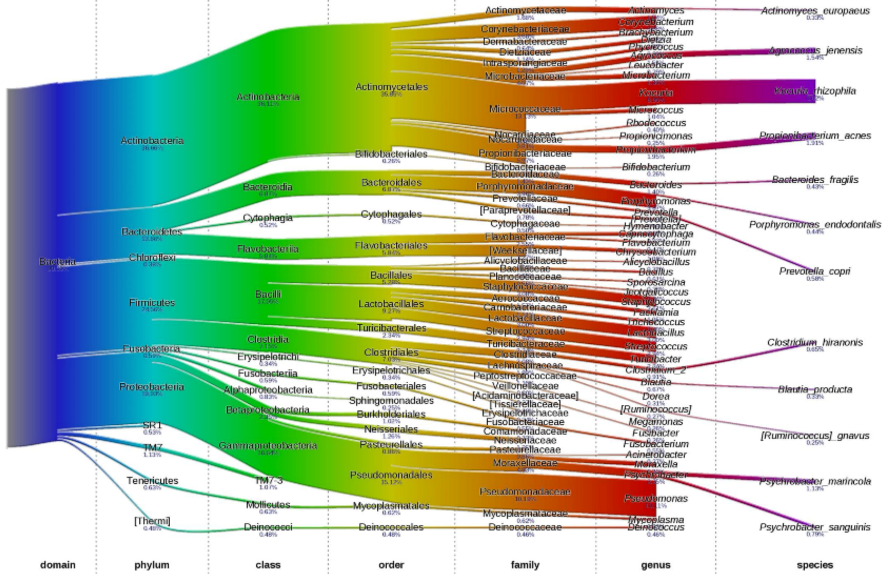


Figure S2. Clustering and microbial community composition of dog skin 16S samples, with visualization on genus-level. The 16S microbiome composition of atopic dermatitis (AD) and healthy (HD) dog skin samples were clustered using UPGMA with weighted UniFrac (beta diversity metric) as a distance measure. 16S samples belonging to the same breed are highlighted in the first column (orange: Beagle; green: Greyhound and blue: Bedlington-Beagle crossbreed), or time-point are highlighted in the second column (red: t_0, green: t_1, yellow: t_2, blue: flare); coat samples are highlighted in grey. The full length of each stacked-bar represents 100% of genus-level classified reads per sample, colors represent relative abundances of genera as defined in the right column. The figure was generated with iTOL.

A

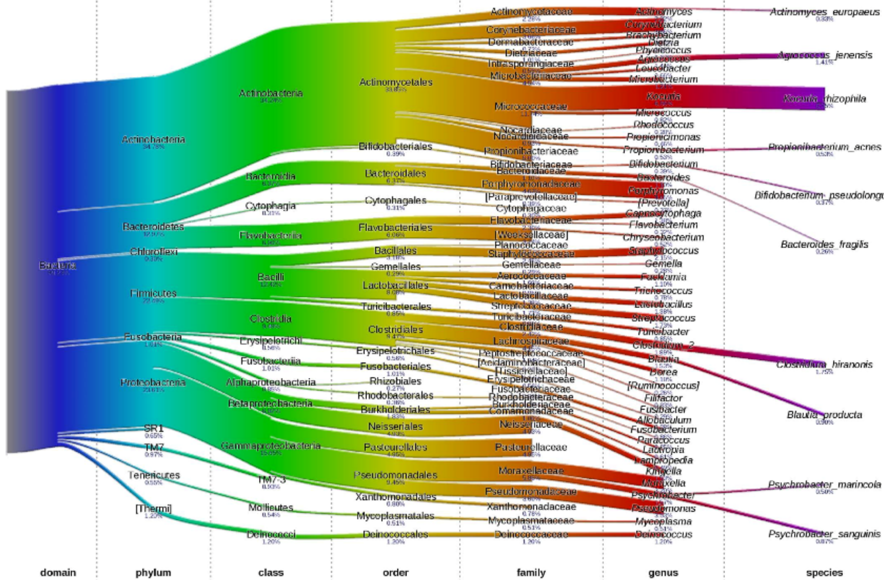
16S



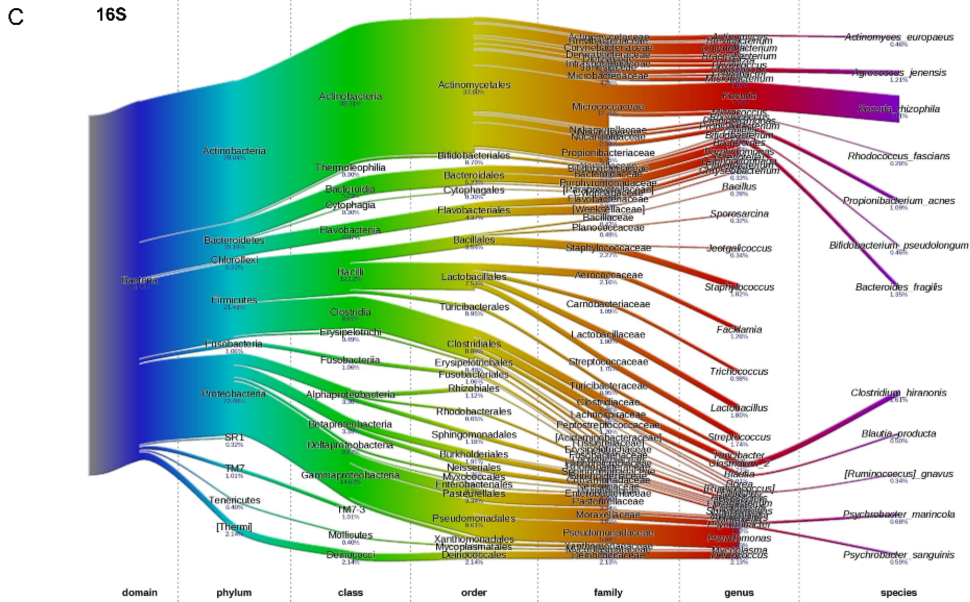
Axilla

B

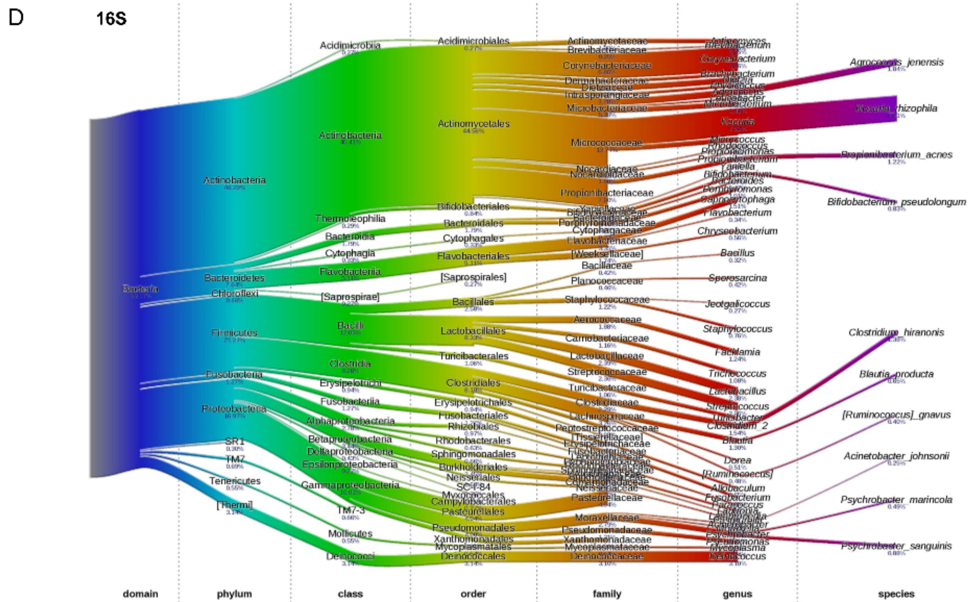
16S



Inguinal



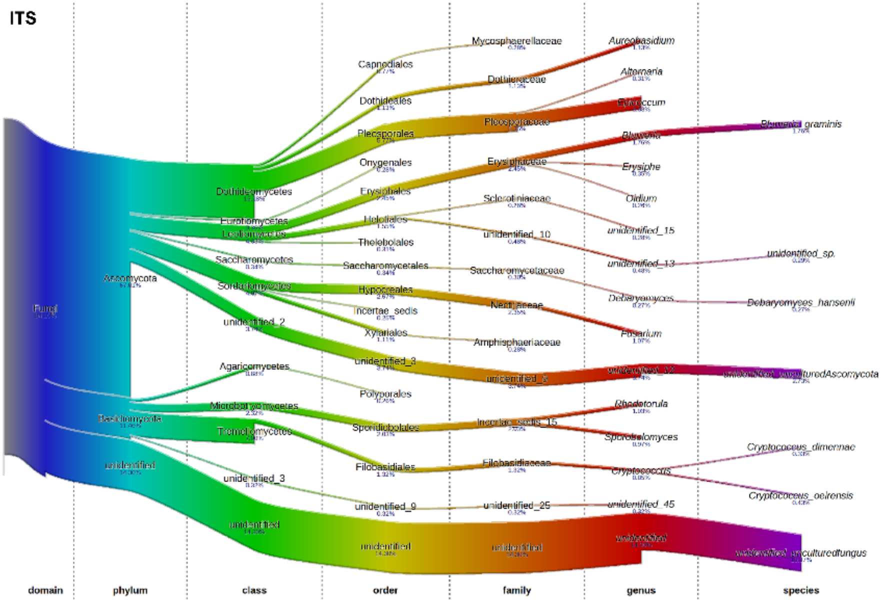
Periocular



Trunk

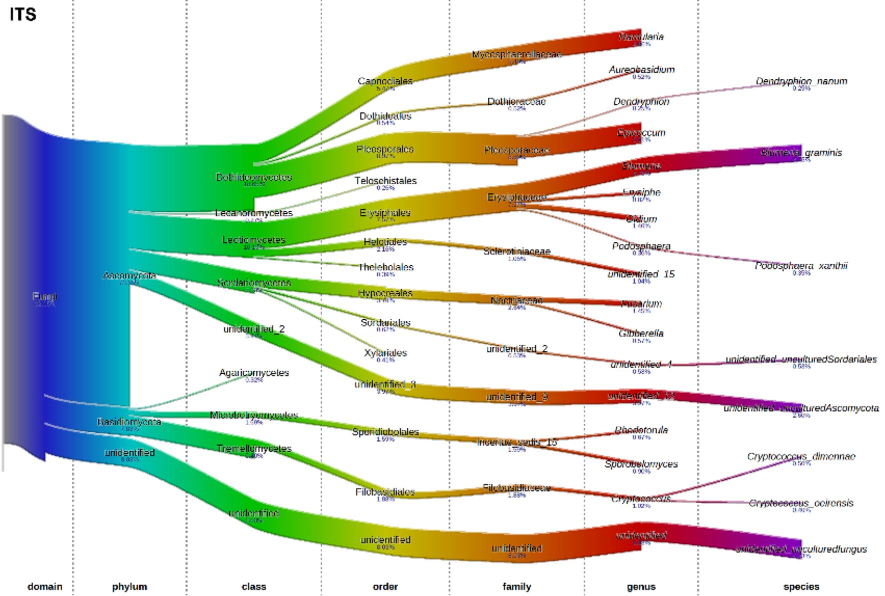
Figure S3. Phylogenetic trees of 16S-based microbiome composition in healthy dog skin at different sites. Bacterial compositions (averaged) in healthy dog skin at axilla (**A**), inguinal (**B**), periocular (**C**) and trunk (**D**). Figure generated using software described in Sundquist et al[30].

A

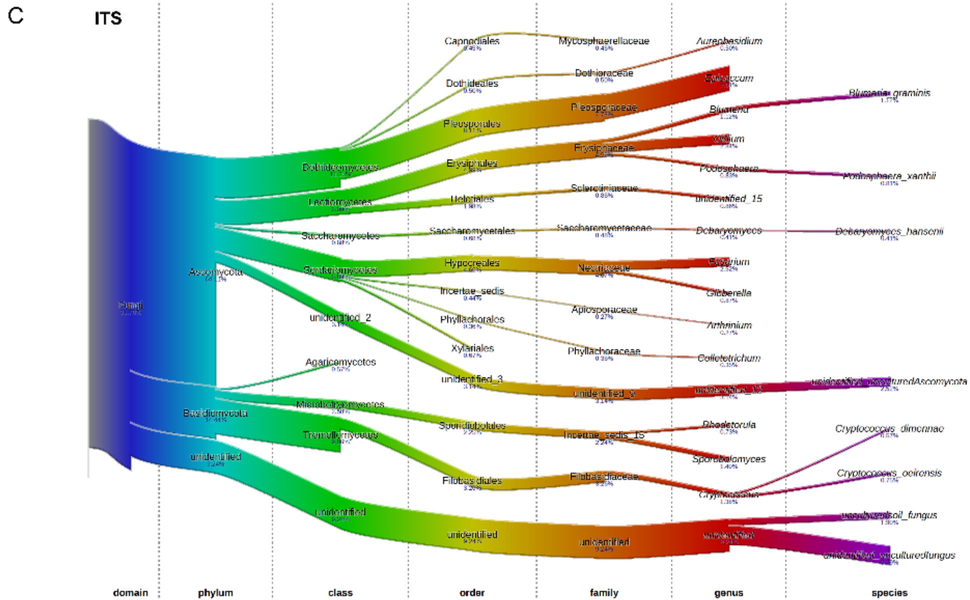


Axilla

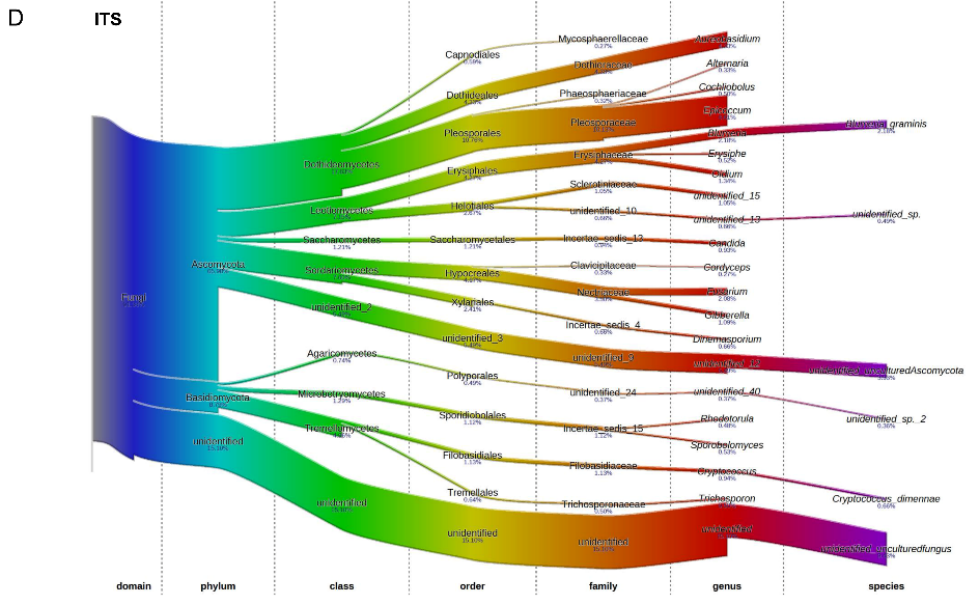
B



Inguinal



Periocular



Trunk

Figure S4. Phylogenetic trees of ITS-based microbiome composition in healthy dog skin at different sites. Fungal compositions (averaged) in healthy dog skin at axilla (**A**), inguinal (**B**), periocular (**C**) and trunk (**D**). Figure generated using software described in Sundquist et al[30].

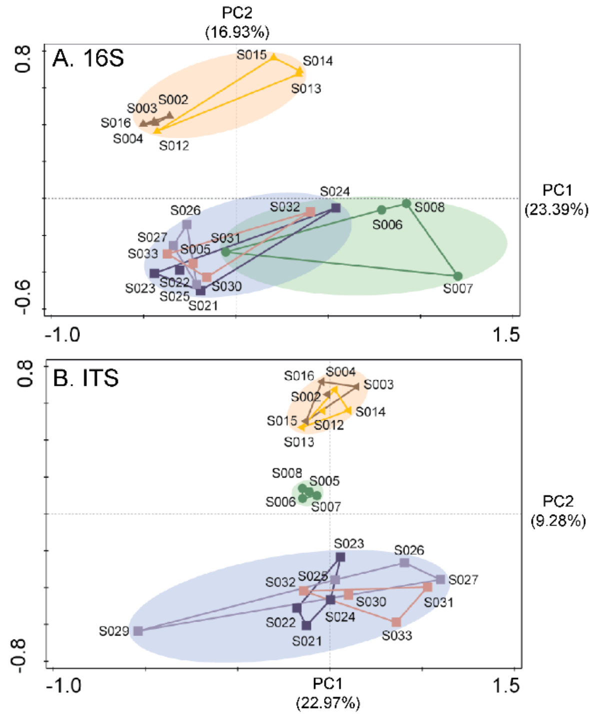


Figure S5. Canine skin is marked by breed-dependent differences in both bacterial and fungal genus-level composition. PCA reflecting the effect of individuality and breed on 16S (**A**) and ITS (**B**) microbiome composition and abundance at the genus-level. Each symbol represents one sample. Connected symbols of the same color and shape represent sample sites from the same dog (individual). Colored sample overlays represent sample clusters of dogs of the same breed; orange: Beagle, blue: Bedlington-Beagle crossbreed, green: Greyhound. For clarity taxa arrows were omitted.

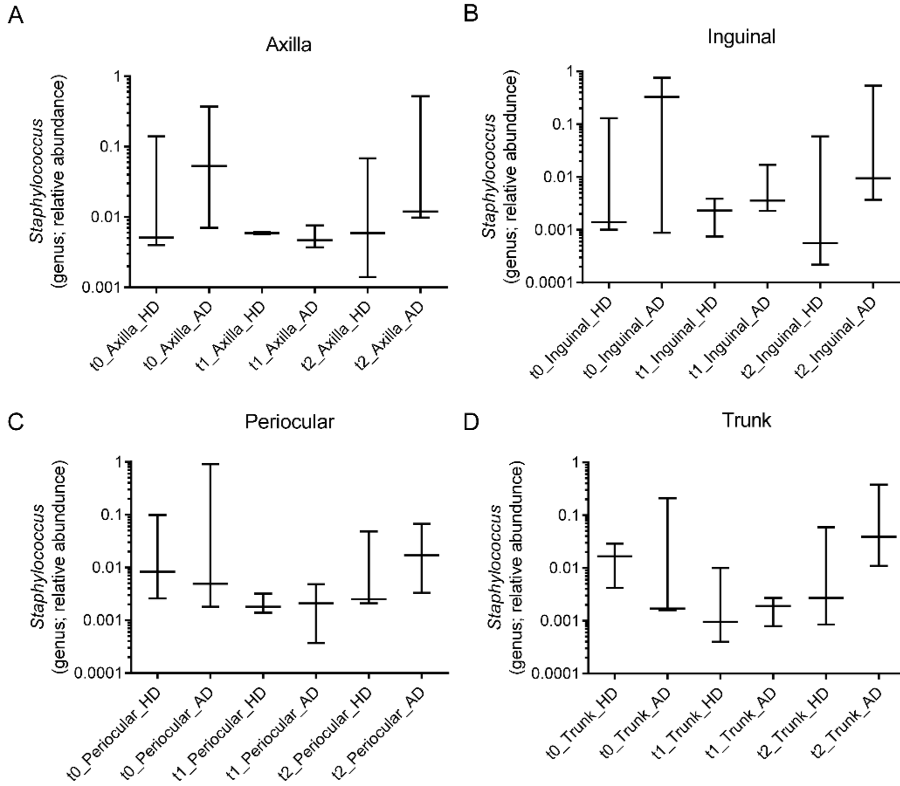


Figure S6. The most prominent genus of bacterial skin microbiome composition in AD versus healthy skin at different sites and time-points. The differences in relative abundance of the most prominent genus (*Staphylococcus*) of bacterial skin microbiome composition in atopic dermatitis (AD) versus healthy (HD) dog skin (disease effect) at axilla (**A**), inguinal (**B**), periocular (**C**) and trunk (**D**) in association with treatment (3 time-points: t_0, t_1 and t_2). No statistically significant differences between groups.

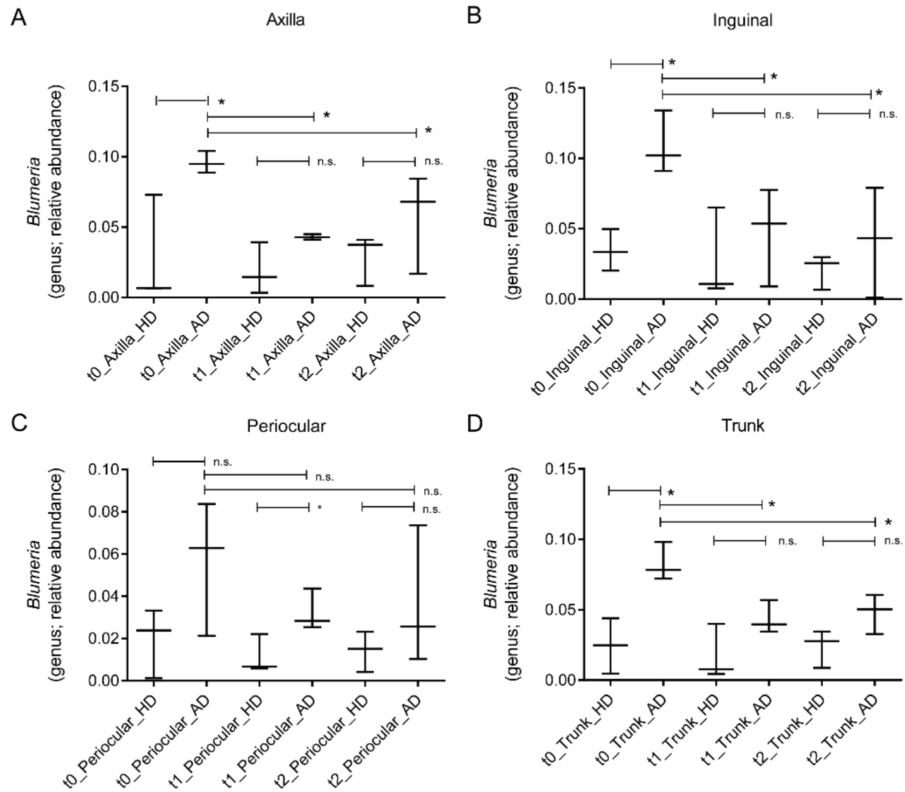


Figure S7. The most prominent genus of fungal skin microbiome composition in AD versus healthy skin at different sites and time-points. The differences in relative abundance of the most prominent genus (*Blumeria)* of fungal skin microbiome composition in atopic dermatitis (AD) versus healthy (HD) dog skin (disease effect) at axilla (**A**), inguinal (**B**), periocular (**C**) and trunk (**D**) in association with treatment (3 time-points: t_0, t_1 and t_2). Significances mentioned in figures are as follows: n.s. (not significant), * P <0.05.

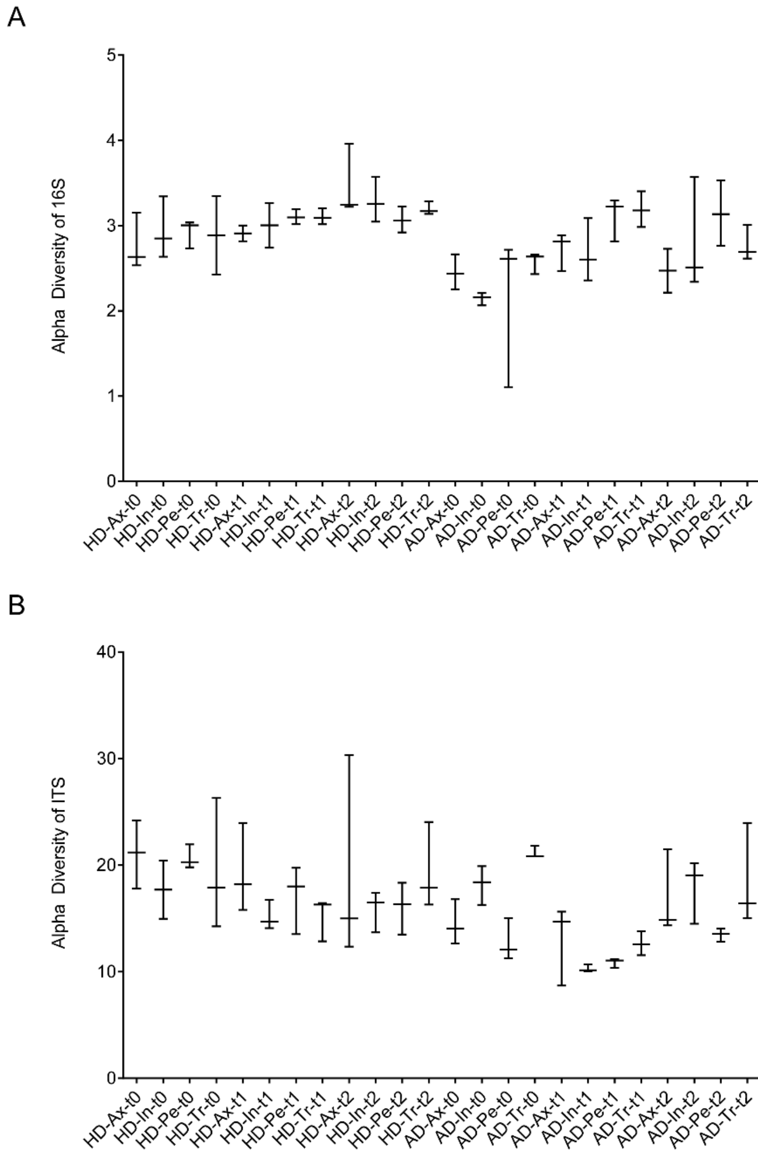


Figure S8. Phylogenetic Diversity Whole tree (PDWT) analysis of 16S and ITS data. The diversity analyzed by PDWT of 16S (**A**) and ITS (**B**) microbiome profiles observed in atopic dermatitis (AD) and healthy (HD) skin at four sites studied (Ax: axilla, In: inguinal, Pe: periocular, Tr: trunk) of three time-points (t_0, t_1 and t_2).

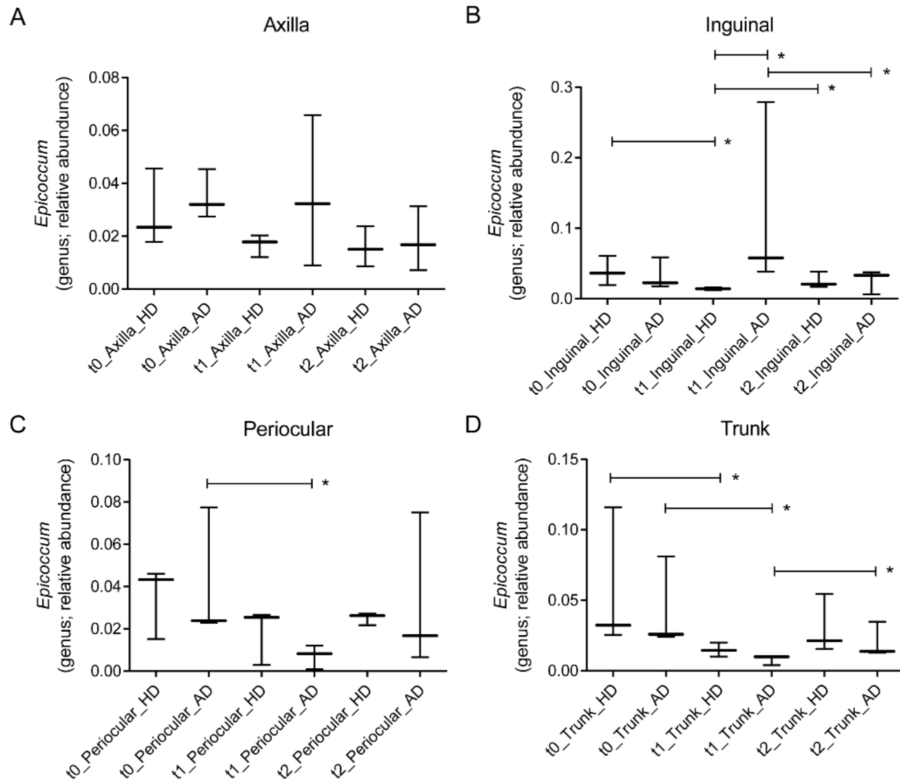


Figure S9. The most prominent genus of fungal skin microbiome composition in AD versus healthy skin as a result of treatment. The differences in relative abundance of the most prominent genus *Epicoccum* of fungal skin microbiome composition in atopic dermatitis (AD) versus healthy (HD) dog skin at axilla (**A**), inguinal (**B**), periocular (**C**) and trunk (**D**) in association with treatment (3 time-points: t_0, t_1 and t_2). Significances mentioned in figures are as follows: n.s. (not significant), * P <0.05.

Chapter 5

Expression of antimicrobial peptides and cytokines in the skin of healthy dogs and dogs with atopic dermatitis and the impact of topical antimicrobial treatment, an exploratory pilot study

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Abstract

Introduction: Atopic dermatitis (AD) is a complex skin disorder potentially involving both imbalanced immune responsiveness against allergen, and a disrupted skin barrier, often complicated by secondary infection. Alterations in the production of antimicrobial peptides (AMP) and cytokines in lesional skin may play an important role in this process. Treatment with antimicrobial shampoo usually alleviates clinical symptoms.

Hypothesis/objectives: Expression of AMP and (pro-)inflammatory cytokines in skin of healthy dogs and dogs with AD alter as a result of topical treatment (washing).

Animals: Three healthy control dogs and three dogs with spontaneous AD.

Methods: Dogs were treated with antimicrobial shampoo (Malaseb®) according to manufacturer's description. Skin biopsies were collected from multiple skin locations before, during and after topical treatment. Expression of AMP (cBD1, cBD103, K9CATH) and Th1 (IFN-γ), pro-inflammatory (TNF-α), Th2 (IL-4, IL-13), Th17 (IL-17, IL-22) and regulatory (IL-10), cytokines was assessed by RT-PCR.

Results: AMP expression in disease predilection sites at the onset of treatment tended to be lower than that at a non-predilection site, whereas the opposite was observed for cytokine expression. Treatment alleviates the severity of the lesions which coincided with reduction of AMP and cytokine expression in AD skin biopsies immediately after therapy compared to before and after therapy withdrawal.

Conclusion and clinical importance: Expression of AMP and (pro-)inflammatory cytokines in skin of healthy and atopic dogs varies according to location and as a result of topical treatment.

Keywords: Antimicrobial peptide, cytokine, skin, canine, atopic dermatitis.

1. Introduction

The skin barrier acts as the first line of defense against physical, chemical and microbial insults[1]. As part of the defense system keratinocytes (KC) and leukocytes, recruited during inflammation, possess the ability to secrete two major families of antimicrobial peptides (AMP), defensins and cathelicidins[2]. AMP have many beneficial properties to the host such as antimicrobial activity (Gram-negative and Gram-positive bacteria, fungi and viruses) and immunomodulatory activity, besides stimulation of wound healing and antitumor activity[2-5]. Defensins are categorized into three subfamilies α, β, and θ[2]. The β-defensins (BD) found to be expressed in skin of healthy controls and atopic dogs are cBD1, cBD102, cBD103, cBD122 and cBD127[6,7]. Only one cathelicidin gene is expressed in dogs (K9CATH)[8]. Skin inflammation and infection not only negatively impact skin barrier function but also influence the regulation of AMP production[9].

Atopic dermatitis (AD), is a complex disorder associated with environmental allergens in dogs that shares many characteristics with human AD, like involvement of a disrupted skin barrier, imbalanced immune responsiveness, and subsequently enhanced allergen invasion and developing disease severity[10]. In general, upon allergen penetration, KC are able to produce thymic stromal lymphopoietin, interleukin (IL)-1, IL-6, and transforming growth factor-β[11-13]. These factors may lead to migration of Langerhans cells and dendritic cells to the draining lymph node as well as influx of leukocytes resulting in increased production of inflammatory cytokines[14]. In lesional atopic skin of canine AD; Th1, Th2, and Th17 cytokines as well as regulatory cytokines may be produced subsequently[15-21]. It has been reported that due the impaired barrier function of KC[22] and imbalanced immune responsiveness in human AD, Th2 cytokines in particular, reduce AMP production[22-27]. In contrast IL-1, IL-17 and IL-22 seem to have the stimulatory effect on AMP production in human skin[25,26].

The skin microbiome contributes to stimulation of AMP production in healthy, steady state, skin as well as to altered expression in atopic skin and can play an important role in exacerbation of AD[28-30]. Invasion of opportunistic pathogens may be combatted by topical treatment with antimicrobial shampoo containing chlorhexidine 2% and miconazole 2% (Malaseb®) that has shown to be effective against bacteria and yeast growth[31-33]. We hypothesized that if the bacterial and fungi/yeast burden on the skin is diminished and skin inflammation reduced, AMP production may restore to normal. In addition, varied expression of AMP at different body sites of normal skin has been described[1], this may be related to the fact that some areas of the skin are more prone to be affected by the AD (predilection sites i.e. axilla and inguinal region)[34]. To explore whether AMP and inflammatory cytokine expression in the skin can differ both due to location and disease severity, hence due to topical antimicrobial treatment, we investigated differential expression of AMP and multiples cytokines involved in AD pathogenesis in both AD and control dogs, in association with predilection sites, different

disease severity and, before during and after topical treatment (**Figure 1**) with antimicrobial shampoo.

2. Material and Methods

2.1 Animals and experimental design

Three control dogs, two Beagles and one Greyhound, had no history of allergic disease or allergy-related clinical lesions. Three Bedlington-Beagle crossbreds with spontaneous AD were diagnosed according to Bizikova et al[35] and Hensel et al[36]; and the severity of AD lesions was evaluated by the CADESI-03 (Canine Atopic Dermatitis Extent and Severity Index)[37,38]. The animals were owned by the Department of Clinical sciences of Companion Animals, Faculty of Veterinary medicine, Utrecht University and all housed in the same premises.

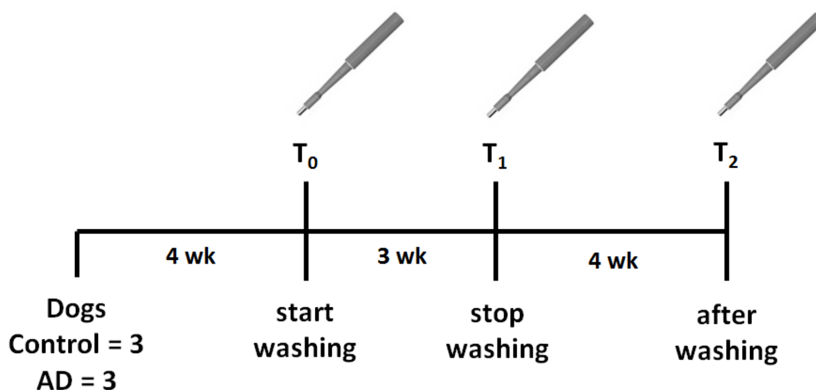

Figure 1. The experimental set up of this study. After inclusion AD dogs were withdrawn from any treatment during a period of 4 weeks, subsequently at T_0 healthy dogs were included and all animals washed twice weekly for 3 weeks until T_1 and next withdrawn from treatment for 4 weeks, until final sampling at T_2.

All animals were treated according to the following protocol approved by the animal ethical committee (DEC 2013.II.07.83) as required under Dutch legislation. No concurrent anti-inflammatory and anti-bacterial/fungal treatments were allowed during and 4 weeks prior to the study. The first timepoint T_0 (**Figure 1**), start of treatment refers to the situation at time of inclusion in control animals and in atopic dogs directly prior to topical treatment consisting of washing twice weekly for three weeks with topical antimicrobial shampoo (Malaseb®, Dermcare-vet Pty Ltd, Brisbane, Australia) according to the manufacturer's guidelines. At T_0, skin conditions of all dogs were assessed according to CADESI-03, and skin biopsies were taken at three different locations, respectively two designated AD predilection sites (axillae and inguinal region) and one designated non-predilection site (lateral thorax). This procedure was repeated at T_1 directly after treatment and at T_2, 4 weeks after withdrawal of treatment (**Figure 1**). After skin condition

assessment and removal of the haircoat with a clipper, two biopsies were taken under sedation with dexmedetomidine (intravenous, 20 µg/kg) from each designated skin location using a 4 mm biopsy punch. Skin biopsies were immediately snap-frozen in liquid nitrogen and stored at -80 ^{o}C until used for RNA isolation to study AMP and cytokine mRNA expression by Real-time qPCR**.**

2.2 Quantitative real-time PCR (qPCR) to assess expression of AMP and cytokines

Shortly before RNA isolation, frozen skin biopsies were thawed and subcutaneous fat was removed. The tissue was cut into small pieces and immersed in Trizol. A 5 mm bead was added to the suspension and the TissueLyzer (Retsch® MM301, Haan, Germany) was used to mechanically homogenize the skin in a tube containing the bead with Trizol. After centrifuging, the aqueous phase was transferred to a RNeasy® mini spin column (Qiagen, Austin, USA) and RNA isolated according to Manufacturer's recommendation. A quantity of 500 ng of RNA as measured by a Nanodrop-1000 spectrophotometer was used for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, USA) according to manufacturer's instructions.

The primer pair sequences shown in **Table 1,** were either previously published or designed by NCBI using Primer3 and BLAST*. Expression of selected (pro-)inflammatory cytokine genes, as well as those of genes of the two families of AMP (defensins and cathelicidins) and one reference gene, RPS19, in the biopsies was measured by SYBR® green (Bio-rad Laboratories Inc. licensed by Molecular Probes, Eugene, USA) in a MyiQ™ single-color real-time PCR detection system (Bio-Rad laboratories, Richmond, USA). Primers for cytokine PCR were used at pre-determined optimal concentrations of 400 nM, the primers for AMP PCR were used at the concentration of 300 nM. The PCR conditions consisted of 5 minutes of initial denaturation at 95 ^{o}C, followed by 40 cycles of 20s at 95 ^{o}C, 30s of T_m at the specific temperatures (**Table 1**) for each primer pair and 30s elongation at 72 ^{o}C (in case the $T_m \geq 58$ ^{o}C, no elongation step was performed as optimized earlier)[39]. All reactions were performed in duplicate and a melting curve analysis was done to determine the purity of the product amplified. The mRNA expression normalized to that of the reference gene RPS19[40] according to Pfaffl[41] was determined by the MyiQ software. The ratios of relative expressions of genes studied in AD (n=3) compared to those of control (n=3) samples at the indicated timepoints were calculated and described as ratio AD:CT in the figures. Ratios higher than 2 or less than 0.5 were considered as different.

Table.1 Primer sequences

Gene	Forward primer (5’-3’)	Reverse primer (5’-3’)	T_m (°C)
RPS19[39]	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG	61
cBD1[6]	AGCCTCTCTCCCTTCTCTGG	GCCATCGATCCTGGTAAAGA	57
cBD103[6]	GCCGCTGCTTACTTGTACCT	CCTCATGACCAACAGGCTTC	57
K9CATH*	TGTGTCCCAAGACGACACAG	ATGAGCTCTTTCAGCCGGTC	56
IFN-γ [42,43]	AGCGCAAGGCGATAAATG	GCGGCCTCGAAACAGATT	55.8
TNF-α[42,43]	CCCCGGGCTCCAGAAGGTG	GCAGCAGGCAGAAGAGTGTGGTG	64
IL-4[42,43]	CCAAAGAACACAAGCGATAAGGAA	GTTTGCCATGCTGCTGAGGTT	61
IL-10[42,43]	CCCGGGCTGAGAACCACGAC	AAATGCGCTCTTCACCTGCTCCAC	63
IL-13[42,43]	GAGGAGCTGGTCAACATCA	TGCAGTCGGAGACATTGA	59
IL-17[42,43]	CACTCCTTCCGGCTAGAGAA	CACATGGCGAACAATAGGG	61
IL-22[42,43]	GGCCAGGCTCAGCAACAAGCT	CGCAGGCATTTCTCAGGGCCAT	61

*see https://www.ncbi.nlm.nih.gov/tools/primer-blast/. Accession number: NM_001003359.1

3. Results

3.1 AD Clinical scores in course of time

CADESI-03 total scores of the AD dogs ranged between 0 and 116 at the onset of the experiment (T_0) before washing and improved as a result of three weeks washing (T_1) to 0 and 33. After discontinuation of treatment for four weeks (T_2) scores were between 0 and 37. Healthy dogs enrolled in this study did not have skin lesions throughout the experiment.

3.2 Differential expression of AMP genes

To address the differential expression of AMP at different skin sites and as a result of topical treatment we analyzed mRNA expression of cBD1, cBD103 and K9CATH orthologues to human HBD1, HBD3 and LL-37, respectively[2,44].

Canine BD1 mRNA expression in control skin biopsies was stable between T_0 and T_1 but tended to show a slight decrease at T_2 whereas cBD1 mRNA expression in AD skin biopsies was increased (predilection site) or stable (non-predilection) at T_2 (**Figure 2A**). These small differences are reflected by clearer differences in ratio of expression of cBD1 between samples of AD and control skin at T_2, that was 4.54 times higher in AD thorax skin compared to that in control dog thorax skin, 2.82 times in the axillary and 2.78 times in the inguinal region (**Figure 2D**).

The mRNA expression of cBD103 decreased in time, throughout, and at all skin sites of AD dogs (**Figure 2B**). In contrast the cBD103 mRNA expression in control skin was

stable between T_0 and T_1 and showed a later decline between T_1 and T_2. Differences in cBD103 expression in AD skin relative to that in controls in course of treatment were not observed (**Figure 2E**).

In general, K9CATH mRNA levels were stable, though low in all skin biopsies analyzed (**Figure 2C**). In AD skin biopsies taken from axilla and inguinal regions a slight non-significant increase in K9CATH mRNA expression in course of time was observed (**Figure 2C**). No differential expression of K9CATH was observed between AD and control samples (**Figure 2F**). In sum, AMP expression as measured by mRNA expression in skin biopsies showed only small changes over time.

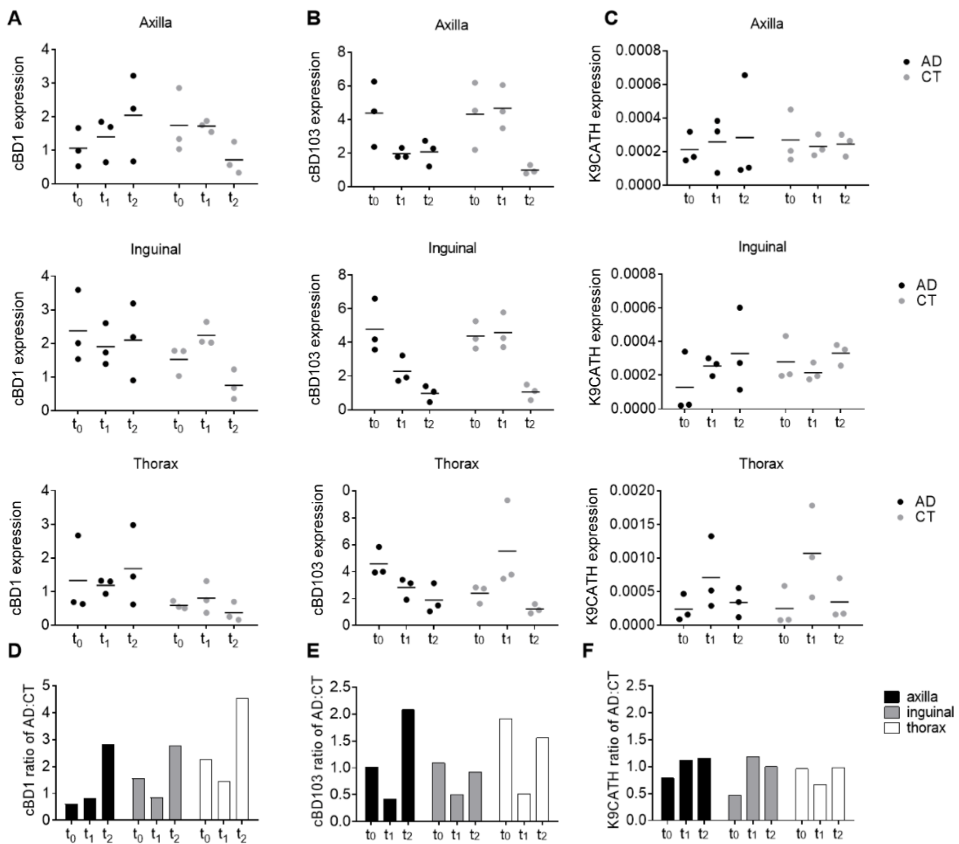

Figure 2. cBD1, cBD103 and K9CATH gene expression in the skin. *The mRNA expression of cBD1 (**A**), cBD103 (**B**) and K9CATH (**C**) in AD (●) and control (CT, ●) skin biopsies at different locations (axilla, inguinal region and lateral thorax) and stages of treatment (T_0, T_1, T_2) as determined by normalizing qRT-PCR values to those of the reference gene RPS19. Data are expressed as individual values and mean of 3 animals (horizontal line). The averaged expression of cBD1 (D) cBD103 (E) and K9CATH (F) in AD skin biopsies relative to that in CT biopsies at different locations and times of treatment is expressed as ratio AD:CT.*

3.3 Differential expression of Cytokine genes

Next to AMP expression we analyzed the mRNA expression of a Th1 cytokine (IFN-γ), a pro-inflammatory cytokine (TNF-α), Th2 cytokines (IL-4, IL-13), Th17 cytokines (IL-17, IL-22) and a regulatory cytokine (IL-10), described to be AD-related and influence AMP production[22-27].

Th1 and pro-inflammatory cytokines

In general, mRNA expression levels of both TNF-α and IFN-γ in the skin biopsies from both control and AD dogs were low (**Figures 3A-B**). The mRNA expression of IFN-γ in AD thorax skin tended to increase at T_2 compared to earlier timepoints, resulting in a 2.45 times higher expression in AD lateral thorax compared to the controls (**Figure 3C**). The mRNA expression of TNF-α in axilla, inguinal region and thorax of both AD and control skin biopsies were more or less the same at T_0 and T_1, and tended to be increased at T_2 (**Figure 3B**). The ratio of TNF-α expression in AD relative to control skin biopsies tended to increase at T_2 (**Figure 3D**).

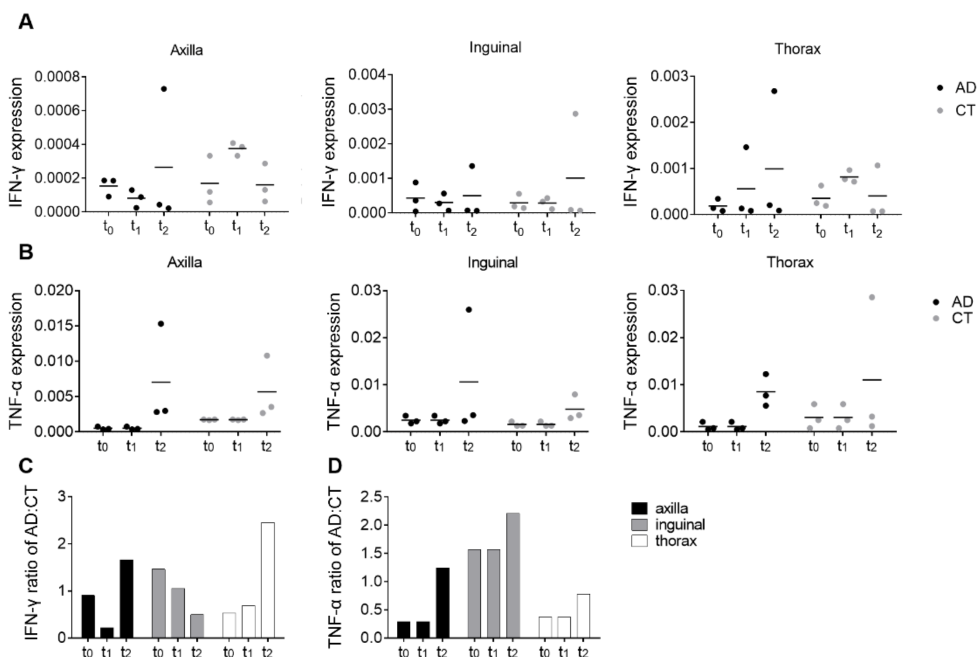

Figure 3. Th1 and pro-inflammatory cytokine gene expression. *The mRNA expression of IFN-γ (**A**) and TNF-α (**B**) in AD (●) and control (CT, ●) skin biopsies at different locations (axilla, inguinal region and lateral thorax) and stages of treatment (T_0, T_1, T_2) as determined normalizing qRT-PCR values to those of the reference gene RPS19. Data are expressed as individual values and mean of 3 animals (horizontal line). The averaged expression of IFN-γ (**C**) and TNF-α (**D**) in AD skin biopsies relative to that in CT biopsies at different locations and times of treatment is expressed as ratio AD:CT.*

The expression of IFN-γ and TNF-α was increased after treatment, especially in AD. However, variation in expression of IFN-γ during treatment was observed between predilection and non-predilection sites, that tended to increase in the former and decrease in the latter.

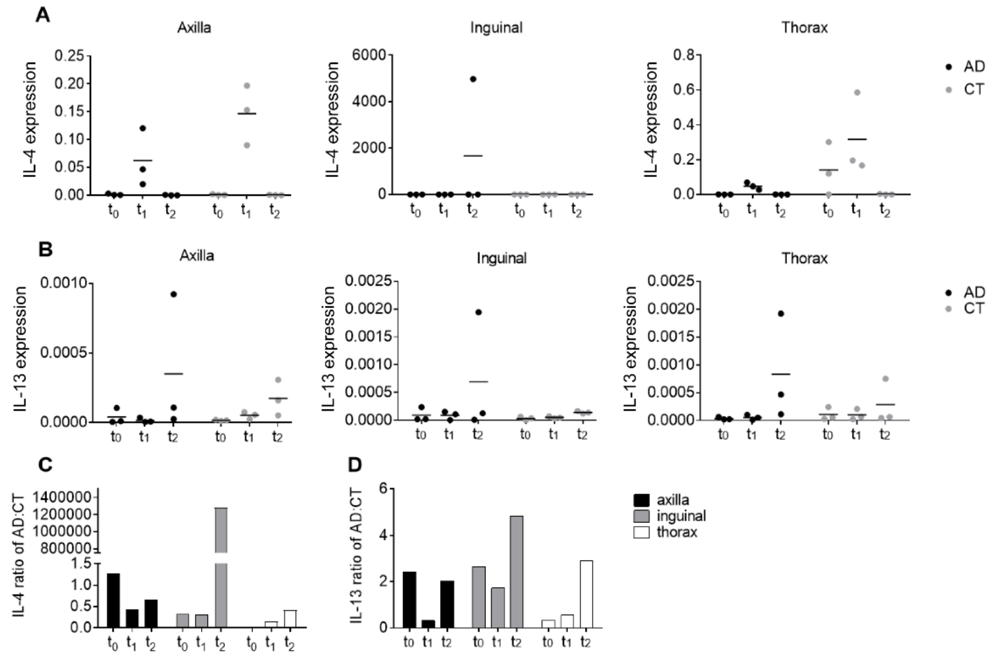

Figure 4. Th2 cytokine response. *The mRNA expression of IL-4 (**A**) and IL-13 (**B**) in AD (●) and control (CT, ●) skin biopsies at different locations (axilla, inguinal region and lateral thorax) and stages of treatment (T_0, T_1, T_2) as determined normalizing qRT-PCR values to those of the reference gene RPS19. Data are expressed as individual values and mean of 3 animals (horizontal line). The averaged relative expression of IL-4 (**C**) and IL-13 (**D**) in AD skin biopsies relative to that in CT biopsies at different locations and times of treatment is expressed as ratio AD:CT.*

Th17 cytokines

The mRNA expression of IL-17 in the control skin samples was highest at T_0 and gradually decreased in course of time (**Figure 5A**). The expression of IL-17 mRNA in AD samples decreased at T_1 compared to T_0, but had increased at T_2 (**Figure 5A**). The ratios of IL-17 expression in AD skin samples relative to control ones are elevated at T_2 in all locations (2, 58.7 and 46.8 times higher; in axilla, inguinal and thorax, respectively) (**Figure 5C**).

The mRNA expression levels of IL-22 were increased at T_2 in AD samples **(Figure 5B**). The ratio of IL-22 expression in AD samples relative to controls was close to 1 at all timepoints studied (**Figure 5D**).

In parallel to the mRNA levels of Th2 cytokines, IL-4 and IL-13, also those of the Th17 category, IL-17 and IL-22 mRNA levels were low in skin biopsies.

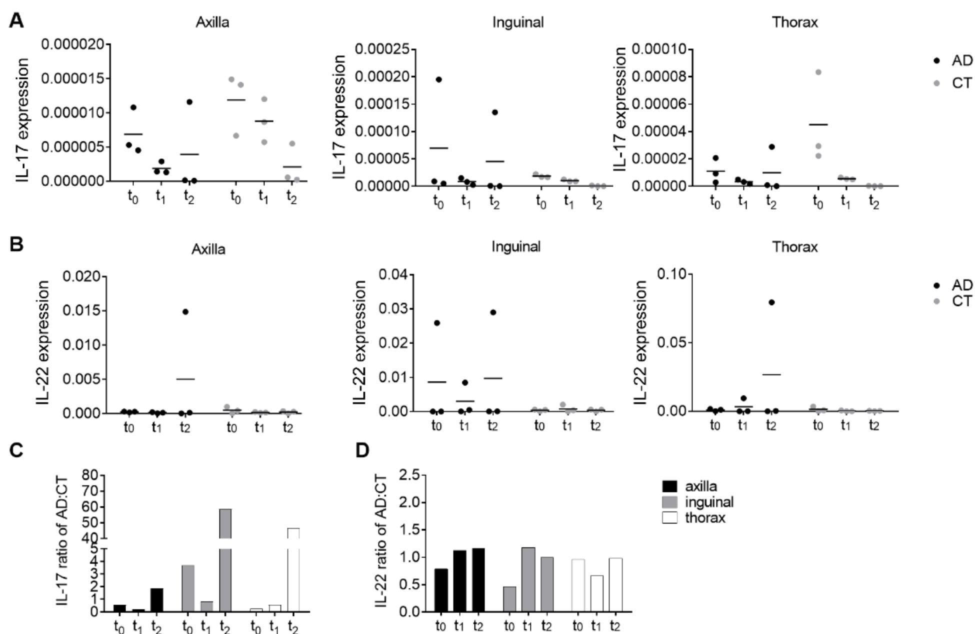

Figure 5. Th17 cytokine response. *The mRNA expression of IL-17 (**A**) and IL-22 (**B**) in AD (●) and control (CT, ●) skin biopsies at different locations (axilla, inguinal region and lateral thorax) and stages of treatment (T_0, T_1, T_2) as determined normalizing qRT-PCR values to those of the reference gene RPS19. Data are expressed as individual values and mean of 3 animals (horizontal line). The averaged relative expression of IL-17 (**C**) and IL-22 (**D**) in AD skin biopsies relative to that in CT biopsies at different locations and times of treatment is expressed as ratio AD:CT.*

A regulatory cytokine

A similar trend in the mRNA expression of IL-10 in course of time was observed between AD and control skin, in axilla and thorax, that it was gradually decreased in course of time in axilla, and in thorax the increased expression was observed at T_1 (**Figure 6A**). The IL-10 expression of AD samples at predilection sites (axilla and inguinal) tended to be higher than controls overtime (**Figures 6A-B**). The ratio of IL-10 expression in AD relative to controls tended to be higher at T_2 compared to both at T_0 and T_1 (**Figure 6B**).

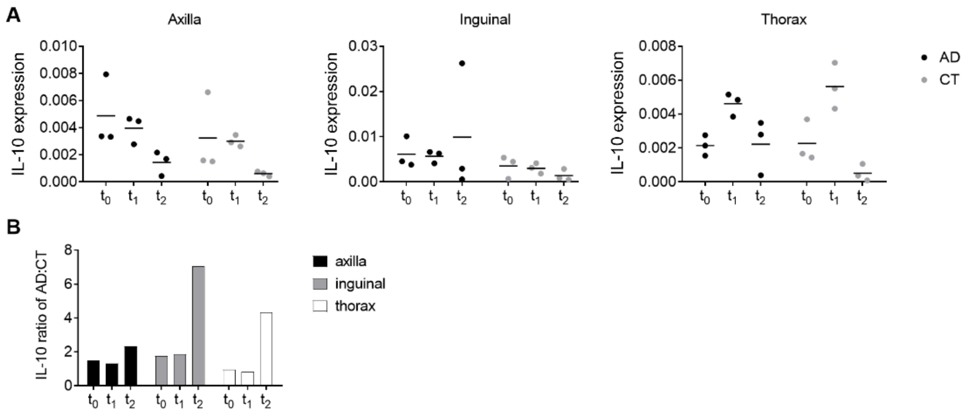

Figure 6. Regulatory cytokine response. *(**A**) The mRNA expression of IL-10 in AD (●) and control (CT, ●) skin biopsies at different locations (axilla, inguinal region and lateral thorax) and stages of treatment (T_0, T_1, T_2) as determined normalizing qRT-PCR values to those of the reference gene RPS19. Data are expressed as individual values and mean of 3 animals (horizontal line). (**B**) The averaged relative expression of IL-10 in AD skin biopsies relative to that in CT biopsies at different locations and times of treatment is expressed as ratio AD:CT.*

4. Discussions

AMP are functionally characterized by antimicrobial and immunomodulatory activities[2-5]. AMP expression by cells in the skin barrier is one of the many different factors contributing to skin integrity, that can be influenced by multiple internal and external stimuli[1]. Different skin characteristics e.g. pH, hair follicles, sebaceous glands, temperature, humidity, may vary by topographical location[45,46] and thus affect the load and diversity of skin microbiome[45,47,48]. Moreover, AD typically associated with skin inflammation or infection is likely to induce AMP expression in human skin[49,50] as well as dog skin[44,51] and anti-microbial topical therapy has been widely used to reduce the severity of AD lesions in dogs, caused by secondary infection[52].

Previous studies regarding AMP expression in atopic dermatitis showed contradictory results. Some observed no differences in mRNA expression of cBD103 between AD and healthy dog skin[53,54], others reported an increase in cBD103 in atopic dogs[44] and finally decreased expression of cBD103 in lesional atopic skin has been described[6]. In the latter study increased expression of cBD1 in AD was described. In the current study, expression of cBD1 and cBD103 in AD at non-predilection sites tended to be higher than predilection sites compared to healthy skin. The expression of cBD1 and cBD103 tended to decrease as a result of treatment (T_1) and increase after abstention of treatment (T_2) in all AD samples compared to the controls. Expression of K9CATH in skin was generally low compared to cBD1 and cBD103 expression, a finding similar to our observation for their expression, *in vitro,* by a canine KC cell line[55]. Yet, K9CATH expression in samples from predilection sites of AD in AD dogs was increased after treatment, which

contrasted expression in a non-predilection site. Overall, the expression of AMP in a non-predilection site (thorax) differs from that in predilection sites (axilla and inguinal). This deviation may be due to disease severity or disturbance of local skin tissue, prone to develop AD, resulting in decreased expression of AMP[8,56 57].

Reasons for the discrepancies between study results may have been differences in study approaches such as the stage of disease, the biopsy location and/or the treatment before or during investigation. In the present study, the biopsy locations were known (non-) predilection sites and followed up for changes in clinical characteristics before, during and after treatment.

As a result of treatment not only AMP expression but also that of inflammatory cytokines in the skin altered. The expressions of Th2 cytokines (IL-4 and IL-13) and the regulatory cytokine IL-10 tended to be higher in AD predilection sites before treatment than at non-predilection sites when compared to control skin, opposite to the finding in AMP expression. The reduction in infection and possibly inflammation by the topical antimicrobial therapy (T_1) reduced the severity of the lesions of the skin, concomitant to reduction of levels of cytokine expression in most of the AD samples, both from predilection and non-predilection sites of AD. In addition, expression of IL-17 in skin of AD dogs slightly increased 4 weeks after treatment (T_2) compared to control skin, which coincides with an increase in IL-22. The increased levels of Th1 cytokine (IFN-γ), pro-inflammatory (TNF-α), Th2 cytokines (IL-4, IL-13), Th17 cytokines (IL-17, IL-22) and regulatory cytokine (IL-10) in lesional atopic skin without treatment are consistent with several reports describing the differences in expression of cytokines in the skin in both spontaneous (human and canine) and experimental (canine) atopic dermatitis[15-21]. In general, AMP production may be stimulated by increased levels of IL-17 and IL-22[21,58], but in contrast, partially inhibited by Th2 cytokines[22,23,59]. Although AMP mRNA levels increased in predilection sites of atopic skin compared to control, the actual biological activity in the tissue was not determined in this study. Further investigations of AMP activities should be considered.

Taken together, the variation in mRNA expression level of both AMP and AD-related cytokines, both in AD and control samples is likely to be dependent not only on skin location but may also be a result of treatment and concomitant disease activity. The alterations in local expression of AMP and cytokines may predispose skin to be AD predilection site. Most likely the treatment helps reduce skin microbes and (indirectly) inflammation, which alleviates the severity of the lesions, resulting in reduction of expression of AMP and AD-related cytokines in AD skin immediately after Malaseb® treatment as compared to before and some weeks after therapy withdrawal. After treatment withdrawal, both AMP and cytokine expressions tended to increase in the AD skin.

Only a limited number of studies have been executed pertaining the effect of AMP alone or in combination with (pro-)inflammatory cytokines on canine skin in canine

atopic dermatitis. Moreover, the complicated pathogenesis, of AD skin, due to different types of immune responsiveness and changes in skin barrier integrity involving connections between microbiota and cells producing AMP and cytokines, and resulting in clinical signs are not well-established yet and need further study.

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References

1. Lancto, C. A., Torres, S. M., Hendrickson, J. A., Martins, K. V. & Rutherford, M. S. Altered expression of antimicrobial peptide genes in the skin of dogs with atopic dermatitis and other inflammatory skin conditions. *Vet. Dermatol.* **24**, 414-21, e90 (2013).
2. Leonard, B. C., Affolter, V. K. & Bevins, C. L. Antimicrobial peptides: agents of border protection for companion animals. *Vet. Dermatol.* **23**, 177-e36 (2012).
3. Bals, R. Epithelial antimicrobial peptides in host defense against infection. *Respir. Res.* **1**, 141-150 (2000).
4. Zanetti, M. Cathelicidins, multifunctional peptides of the innate immunity. *J. Leukoc. Biol.* **75**, 39-48 (2004).
5. Okumura, K. *et al*. C-terminal domain of human CAP18 antimicrobial peptide induces apoptosis in oral squamous cell carcinoma SAS-H1 cells. *Cancer Lett.* **212**, 185-194 (2004).
6. van Damme, C. M., Willemse, T., van Dijk, A., Haagsman, H. P. & Veldhuizen, E. J. Altered cutaneous expression of beta-defensins in dogs with atopic dermatitis. *Mol. Immunol.* **46**, 2449-2455 (2009).
7. Wingate, K. V., Torres, S. M., Silverstein, K. A., Hendrickson, J. A. & Rutherford, M. S. Expression of endogenous antimicrobial peptides in normal canine skin. *Vet. Dermatol.* **20**, 19-26 (2009).
8. Sang, Y. *et al*. Canine cathelicidin (K9CATH): gene cloning, expression, and biochemical activity of a novel pro-myeloid antimicrobial peptide. *Dev. Comp. Immunol.* **31**, 1278-1296 (2007).
9. Gallo, R. L. & Nakatsuji, T. Microbial symbiosis with the innate immune defense system of the skin. *J. Invest. Dermatol.* **131**, 1974-1980 (2011).
10. Gedon, N. K. Y. & Mueller, R. S. Atopic dermatitis in cats and dogs: a difficult disease for animals and owners. *Clinical and translational allergy* **8**, 41; 41-41 (2018).
11. Oyoshi, M. K., He, R., Kumar, L., Yoon, J. & Geha, R. S. Cellular and molecular mechanisms in atopic dermatitis. *Adv. Immunol.* **102**, 135-226 (2009).
12. Novak, N. & Leung, D. Y. M. in *Pediatric Allergy: Principles and Practice* (eds Leung, D. Y. M. & Sampson, H.) 552-63 (W.B.Saunders, Edinburgh, 2010).
13. Bitschar, K., Wolz, C., Krismer, B., Peschel, A. & Schittek, B. Keratinocytes as sensors and central players in the immune defense against Staphylococcus aureus in the skin. *J. Dermatol. Sci.* **87**, 215-220 (2017).
14. Di Cesare, A., Di Meglio, P. & Nestle, F. O. A role for Th17 cells in the immunopathogenesis of atopic dermatitis? *J. Invest. Dermatol.* **128**, 2569-2571 (2008).
15. Schlotter, Y. M., Rutten, V. P., Riemers, F. M., Knol, E. F. & Willemse, T. Lesional skin in atopic dogs shows a mixed Type-1 and Type-2 immune responsiveness. *Vet. Immunol. Immunopathol.* **143**, 20-26 (2011).
16. Pucheu-Haston, C. M. *et al*. A canine model of cutaneous late-phase reactions: prednisolone inhibition of cellular and cytokine responses. *Immunology* **117**, 177-187 (2006).
17. Pucheu-Haston, C. M. *et al*. Review: Lymphocytes, cytokines, chemokines and the T-helper 1-T-helper 2 balance in canine atopic dermatitis. *Vet. Dermatol.* **26**, 124-e32 (2015).
18. Olivry, T., Dean, G. A., Tompkins, M. B., Dow, J. L. & Moore, P. F. Toward a canine model of atopic dermatitis: amplification of cytokine-gene transcripts in the skin of atopic dogs. *Exp. Dermatol.* **8**, 204-211 (1999).
19. Marsella, R., Olivry, T. & Maeda, S. Cellular and cytokine kinetics after epicutaneous allergen challenge (atopy patch testing) with house dust mites in high-IgE beagles. *Vet. Dermatol.* **17**, 111-120 (2006).
20. Nuttall, T. J., Knight, P. A., McAleese, S. M., Lamb, J. R. & Hill, P. B. Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis. *Clin. Exp. Allergy* **32**, 789-795 (2002).
21. Nograles, K. E. *et al*. IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. *J. Allergy Clin. Immunol.* **123**, 1244-52.e2 (2009).
22. Kisich, K. O., Carspecken, C. W., Fieve, S., Boguniewicz, M. & Leung, D. Y. Defective killing of Staphylococcus aureus in atopic dermatitis is associated with reduced mobilization of human beta-defensin-3. *J. Allergy Clin. Immunol.* **122**, 62-68 (2008).
23. Nomura, I. *et al*. Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J. Immunol.* **171**, 3262-3269 (2003).
24. Howell, M. D. *et al*. Interleukin-10 downregulates anti-microbial peptide expression in atopic dermatitis. *J. Invest. Dermatol.* **125**, 738-745 (2005).
25. Eyerich, K. *et al*. IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response. *J. Allergy Clin. Immunol.* **123**, 59-66.e4 (2009).
26. Wolk, K. *et al*. IL-22 increases the innate immunity of tissues. *Immunity* **21**, 241-254 (2004).
27. Gittler, J. K. *et al*. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *J. Allergy Clin. Immunol.* **130**, 1344-1354 (2012).

28. Fazakerley, J. *et al*. Staphylococcal colonization of mucosal and lesional skin sites in atopic and healthy dogs. *Vet. Dermatol.* **20**, 179-184 (2009).
29. Bradley, C. W. *et al*. Longitudinal Evaluation of the Skin Microbiome and Association with Microenvironment and Treatment in Canine Atopic Dermatitis. *J. Invest. Dermatol.* **136**, 1182-1190 (2016).
30. Santoro, D. *et al*. Review: Pathogenesis of canine atopic dermatitis: skin barrier and host-micro-organism interaction. *Vet. Dermatol.* **26**, 84-e25 (2015).
31. Young, R., Buckley, L., McEwan, N. & Nuttall, T. Comparative in vitro efficacy of antimicrobial shampoos: a pilot study. *Vet. Dermatol.* **23**, 36-40, e8 (2012).
32. Bond, R., Rose, J. F., Ellis, J. W. & Lloyd, D. H. Comparison of two shampoos for treatment of Malassezia pachydermatis-associated seborrhoeic dermatitis in basset hounds. *J. Small Anim. Pract.* **36**, 99-104 (1995).
33. Mueller, R. S., Bergvall, K., Bensignor, E. & Bond, R. A review of topical therapy for skin infections with bacteria and yeast. *Vet. Dermatol.* **23**, 330-41, e62 (2012).
34. Favrot, C., Steffan, J., Seewald, W. & Picco, F. A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. *Vet. Dermatol.* **21**, 23-31 (2010).
35. Bizikova, P. *et al*. Review: Clinical and histological manifestations of canine atopic dermatitis. *Vet. Dermatol.* **26**, 79-e24 (2015).
36. Hensel, P., Santoro, D., Favrot, C., Hill, P. & Griffin, C. Canine atopic dermatitis: detailed guidelines for diagnosis and allergen identification. *BMC Vet. Res.* **11**, 196-015-0515-5 (2015).
37. Olivry, T., Marsella, R., Iwasaki, T., Mueller, R. & International Task Force On Canine Atopic Dermatitis. Validation of CADESI-03, a severity scale for clinical trials enrolling dogs with atopic dermatitis. *Vet. Dermatol.* **18**, 78-86 (2007).
38. Olivry, T. *et al*. Determination of CADESI-03 thresholds for increasing severity levels of canine atopic dermatitis. *Vet. Dermatol.* **19**, 115-119 (2008).
39. Brinkhof, B., Spee, B., Rothuizen, J. & Penning, L. C. Development and evaluation of canine reference genes for accurate quantification of gene expression. *Anal. Biochem.* **356**, 36-43 (2006).
40. Schlotter, Y. M. *et al*. A GeNorm algorithm-based selection of reference genes for quantitative real-time PCR in skin biopsies of healthy dogs and dogs with atopic dermatitis. *Vet. Immunol. Immunopathol.* **129**, 115-118 (2009).
41. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45 (2001).
42. Veenhof, E. Z. *et al*. Characterisation of T cell phenotypes, cytokines and transcription factors in the skin of dogs with cutaneous adverse food reactions. *Vet. J.* **187**, 320-324 (2011).
43. Veenhof, E. Z., Rutten, V. P., van Noort, R., Knol, E. F. & Willemse, T. Evaluation of T-cell activation in the duodenum of dogs with cutaneous food hypersensitivity. *Am. J. Vet. Res.* **71**, 441-446 (2010).
44. Santoro, D., Bunick, D., Graves, T. K. & Segre, M. Evaluation of canine antimicrobial peptides in infected and noninfected chronic atopic skin. *Vet. Dermatol.* **24**, 39-e10 (2013).
45. Grice, E. A. & Segre, J. A. The skin microbiome. *Nat. Rev. Microbiol.* **9**, 244-253 (2011).
46. Miller, W. H., Griffin, C. E., Campbell, K. L. & Muller, G. H. in *Muller and Kirk's Small Animal Dermatology7: Muller and Kirk's Small Animal Dermatology* (Elsevier/Mosby, 2013).
47. Rodrigues Hoffmann, A. *et al*. The skin microbiome in healthy and allergic dogs. *PLoS One* **9**, e83197 (2014).
48. Meason-Smith, C. *et al*. What is living on your dog's skin? Characterization of the canine cutaneous mycobiota and fungal dysbiosis in canine allergic dermatitis. *FEMS Microbiol. Ecol.* **91**, 10.1093/femsec/fiv139. Epub 2015 Nov 4 (2015).
49. Harder, J. *et al*. Enhanced expression and secretion of antimicrobial peptides in atopic dermatitis and after superficial skin injury. *J. Invest. Dermatol.* **130**, 1355-1364 (2010).
50. Gambichler, T. *et al*. Differential mRNA expression of antimicrobial peptides and proteins in atopic dermatitis as compared to psoriasis vulgaris and healthy skin. *Int. Arch. Allergy Immunol.* **147**, 17-24 (2008).
51. Santoro, D., Marsella, R., Bunick, D., Graves, T. K. & Campbell, K. L. Expression and distribution of canine antimicrobial peptides in the skin of healthy and atopic beagles. *Vet. Immunol. Immunopathol.* **144**, 382-388 (2011).
52. Olivry, T. *et al*. Treatment of canine atopic dermatitis: 2010 clinical practice guidelines from the International Task Force on Canine Atopic Dermatitis. *Vet. Dermatol.* **21**, 233-248 (2010).
53. Mullin, J., Carter, S., Williams, N., McEwan, N. & Nuttall, T. Transcription of canine toll-like receptor 2, beta-defensin 1 and beta-defensin 103 in infected atopic skin, non-infected atopic skin, healthy skin and the CPEK cell line. *Vet. Microbiol.* **162**, 700-706 (2013).
54. Leonard, B. C. *et al*. Activity, expression and genetic variation of canine beta-defensin 103: a multifunctional antimicrobial peptide in the skin of domestic dogs. *J. Innate Immun.* **4**, 248-259 (2012).

55. Chermprapai, S., Broere, F., Schlotter, Y. M., Veldhuizen, E. J. A. & Rutten, V. P. M. G. A canine keratinocyte cell line expresses antimicrobial peptide and cytokine genes upon stimulation with bacteria, microbial ligands and recombinant cytokines. *Veterinary Immunology and Immunopathology* **206**, 35-40 (2018).
56. Goldman, M. J. *et al*. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* **88**, 553-560 (1997).
57. Braff, M. H., Bardan, A., Nizet, V. & Gallo, R. L. Cutaneous defense mechanisms by antimicrobial peptides. *J. Invest. Dermatol.* **125**, 9-13 (2005).
58. Eyerich, K. *et al*. IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response. *J. Allergy Clin. Immunol.* **123**, 59-66.e4 (2009).
59. Ong, P. Y. *et al*. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* **347**, 1151-1160 (2002).

Chapter 6

A canine keratinocyte cell line expresses antimicrobial peptide and cytokine genes upon stimulation with bacteria, microbial ligands and recombinant cytokines

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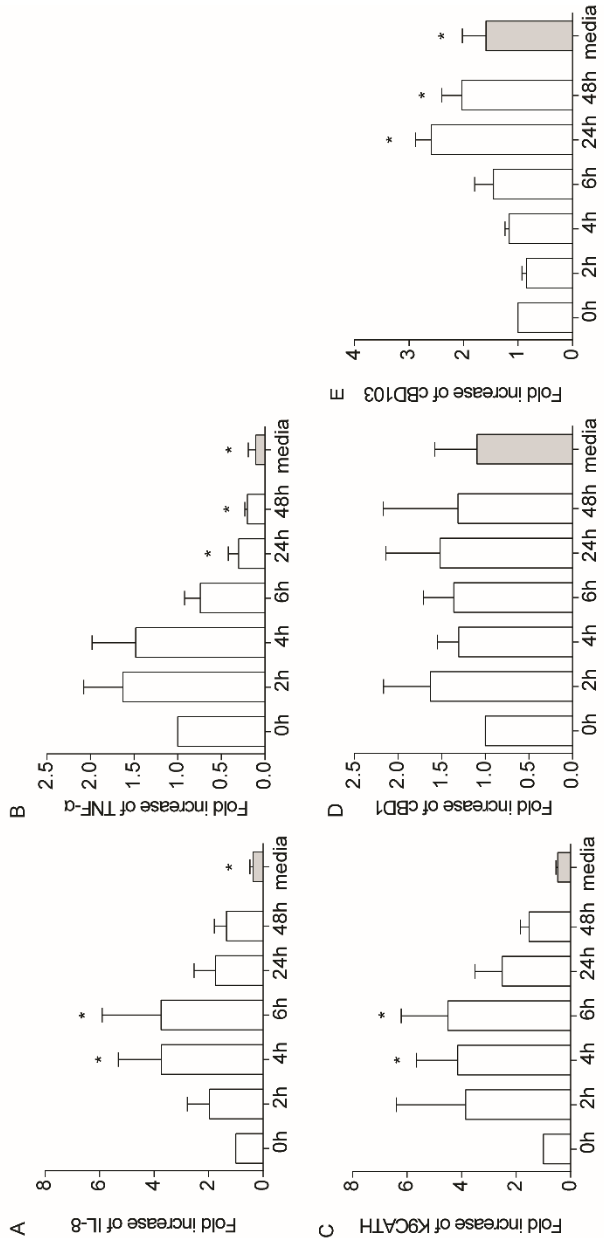
Graphical abstract



Abstract

Keratinocytes (KC) are the main cellular components of the stratum corneum that constitutes a solid physical skin barrier representing the first line of defense against pathogens. Moreover, KC are potent producers of inflammatory mediators and antimicrobial peptides (AMP) when activated through their pattern recognition receptors. In atopic dermatitis (AD) the protective skin barrier may be compromised due to barrier disruption, secondary infection and accelerated secretion of inflammatory cytokines which may also affect AMP expression in the skin. In the present study, we addressed the responses of a canine KC cell line upon exposure to *Staphylococcus pseudintermedius,* typically found on canine atopic skin during secondary infections, and stimulation by individual AD-associated ligands and cytokines. All stimuli induced a significant increase in expression of the pro-inflammatory cytokine genes tumor necrosis factor (TNF)-α and interleukin (IL)-8, but with different kinetics. Limited effects were observed on AMP gene expression except for K9CATH which was significantly upregulated upon bacterial infection but with none of the individual AD-associated ligands. Interestingly, K9CATH possessed antimicrobial activity towards *Staphylococcus pseudintermedius,* indicating that K9CATH expression is a specific defense reaction towards bacterial infection and not part of a general pro-inflammatory profile of KC.

Keywords: keratinocyte; antimicrobial peptide; β-defensin; cathelicidin; pattern recognition receptor; canine.

1. Introduction

In the stratum corneum of the skin, the major constituents, keratinocytes (KC) embedded in their lipid matrix, establish a solid physical barrier representing the first line of defense against pathogens[1,2]. Moreover KC are potent producers of inflammatory mediators and antimicrobial peptides (AMP) when activated through their pattern recognition receptors (PRRs) as observed in both human[3] and canine[4] studies, thereby contributing to local immune homeostasis. Canine skin homeostasis may be disturbed by changes in the microbiome as well as the micro-environment that contribute to alterations of innate and adaptive immune responsiveness[2]. One of the most common skin disorders in dog is atopic dermatitis (AD). Of the multitude of microbiota colonizing the canine skin, those most frequently involved in exacerbation of AD due to secondary infection are the Gram-positive *Staphylococcus pseudintermedius* and the yeast *Malassezia pachydermatis*; however, Gram-negative bacteria may contribute as well[5].

In acute disease AD is characterized by a Th2-type cytokine response, whereby IL-4 and IL-13 dominate the microenvironment of keratinocytes[6]. In contrast, in the chronic phase of AD the Th1-type cytokine IFN-γ is most prominent[7]. In addition to release of typical Th1 and Th2-related cytokines, the expression of IL-17 is increased in acute lesions of human AD skin[8]. Alterations in Th1, Th2 and Th17-type cytokine responses may have differential impact on both expression of AMP and pro-inflammatory cytokines which may render the skin more susceptible to infection[8,9].

Two families of AMP, cathelicidins and β-defensins, are important in the first line of defense against pathogens[10]. A broad antimicrobial spectrum[10], as well as immunomodulatory activity has been ascribed to these peptides[11], that are produced by both immune cells (often neutrophils) and epithelial tissues[4,12]. The β-defensins, cBD1 and cBD103, and the only known cathelicidin K9CATH are found in canine atopic skin and their expression is higher relative to that in healthy skin[13,14], which is comparable to what is reported for their homologues in human AD[15]. In contrast, gene expression levels of cBD102, cBD107 and cBD108 were found to be low in atopic dog skin[16]. In view of the putative clinical impact of bactericidal and immune-modulatory properties of AMP[4], better understanding of AMP as well as cytokine expression by, and their impact on canine KC may contribute to the identification of therapeutic targets for modulation of defense mechanisms in the canine skin.

The aim of this study was to determine the effects of viable *S. pseudintermedius*, synthetic microbial ligands, and AD-related cytokines on expression of AMP and pro-inflammatory cytokine genes of a canine keratinocyte cell line, thus, mimicking interactions occurring in the skin of dogs with (allergic) dermatitis. In addition, the antimicrobial activity of K9CATH to viable *S. pseudintermedius* was assessed.

2. Materials and methods

2.1 MSCEK cell line

The canine keratinocyte cell line (MSCEK) was cordially provided by Koji Nishifuji, Tokyo University, Japan[17]. The cell line is derived from a skin biopsy of a healthy dog and passaged over 90 times in culture. Cells were grown in William's E Medium (Life Technologies, Carlsbad, USA) supplemented with 10% of Nu-Serum IV Growth medium supplement (Corning, New York, USA); 5 ng/ml of Epidermal Growth Factor (EGF; Sigma, New Jersey, USA); 100 pM of Cholera Toxin (Sigma, New Jersey, USA); Penicillin-streptomycin (100 units/mL of penicillin and 100 µg/mL of streptomycin; Life Technologies, Carlsbad, USA). MSCEK were seeded in 12 well-plates, (coated with type 1 collagen; Corning, New York, USA) 1 x 10^5 cells/well in a volume of 1 ml, grown at 37 ^{o}C in a humidified atmosphere at 5% CO_2. The culture medium was refreshed every 48 h until cells were confluent, prior to use.

2.2 Staphylococcus pseudintermedius preparation

An overnight culture of methicillin-susceptible *S. pseudintermedius*, a clinical isolate from dog skin with bacterial infection (provided by the Veterinary Microbiological Diagnostic Center, VMDC, Faculty of Veterinary Medicine, Utrecht University, The Netherlands), was prepared by picking a colony from the selective agar and dispersing it into Miller Hilton broth (MHB) for selection of a mid-logarithmic phase bacteria. OD measurement at 620 nm was performed to determine the bacterial density using the formula: OD value 0.1 ≈ 1 x 10^8 CFU/ml and subsequently diluted to the desired density for each experiment.

2.3 Co-culture of S. pseudintermedius and MSCEK

Freshly-prepared 10^4, 10^6 and 10^8 CFU of *S. pseudintermedius* were added to a >90% confluent MSCEK cell monolayer, in 12 well-plates. To optimize the incubation time, bacteria and cells were co-cultured for 1 and 4 h at 37 ^{o}C. After incubation wells were washed with PBS to remove bacteria not involved with keratinocytes. To assess the total number of MSCEK associated bacteria (adhesion + invasion), cells were lysed with 1% Triton-X100 in PBS for 5 min to release all associated bacteria. The resultant bacterial suspension was diluted 10-10000 fold and plated out on Tryptone Soy Agar (TSA; Oxoid LTD, Hampshire, UK).

To assess invasion, in separate wells, after co-culture and washing as described above, cells were incubated with gentamicin (25 µg/ml) for 1 h at 37 ^{o}C to kill extracellular bacteria. Cells were washed with PBS, 1% Triton-x100 in PBS was added, and lysate was plated to determine the number of invaded bacteria.

2.4 Stimulation of MSCEK cells with S. pseudintermedius

Confluent MSCEK cultures were incubated with 10^8 CFU/ml *S. pseudintermedius* for 4 h at 37 ^oC or culture medium alone as negative control. After killing extracellular bacteria with gentamicin (25 µg/ml) and washing, cells were lysed at time points 0, 2, 4, 6, 24 and 48 h for qPCR analysis of AMP and pro-inflammatory cytokine gene activity. All stimulation conditions were repeated in three independent experiments.

2.5 Stimulation of MSCEK cells with selected synthetic microbial ligands and recombinant cytokines

MSECKS were stimulated with the synthetic microbial ligands Pam3CSK4 (10 µg/ml), LTA (10 µg/ml), TBD (20 µg/ml) (Invivogen, Carlsbad, CA) and LPS (50 µg/ml) (Sigma, New Jersey, USA). Alternatively MSCEKS were stimulated with the recombinant canine cytokines TNF-α, IFN-γ and IL-17 (20 ng/ml) and IL-4 and IL-13 at 50 ng/ml (R&D systems, Minnesota, USA). Cell cultures without stimulants (medium only) were used as a negative control. After 7, 24 and 48 h incubation cells were harvested for qPCR analysis of AMP and pro-inflammatory cytokine gene expression.

2.6 Expression of AMP and pro-inflammatory cytokine genes

RNA was isolated using the FavorPrep™ Blood/Cultured Cell Total RNA Mini Kit (FAVORGEN-Europe, Vienna, Austria) according to the manufacturer's recommendation. Five hundred nanogram of RNA per sample was used for cDNA synthesis by the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, USA) according to the manufacturer's instruction. RT-qPCR was performed with 1 µl cDNA (diluted 1:10) as a template using IQ™ SYBR® Green (Bio-Rad Laboratories Inc. licensed by Molecular Probes, Eugene, USA) in a MyiQ™ single-color real-time PCR detection system (Bio-Rad laboratories, Richmond, USA). Total reaction volume was 25 µl with primer concentration of 300 nM for the AMP (cBD1, cBD103 and K9CATH) and 400 nM for the reference gene RPS19, and the pro-inflammatory cytokine genes IL-8 and TNF-α. The primer pairs were used as either previously described or custom designed by the program of NCBI using Primer3 and BLAST (Table S1). The PCR conditions consisted of 5 min of initial denaturation at 95 ^oC, followed by 40 cycles of 20 s at 95 ^oC, 30 s of T_m at the specific temperatures for each primer pair and 30 s elongation at 72 ^oC (in case the $T_m \geq 58$ ^oC, the elongation step was skipped)[18]. Relative mRNA expression levels were determined by the MyiQ software and calculated by the Pfaffl method normalized to the reference gene RPS19[19].

2.7 Antimicrobial activity of K9CATH

The K9CATH was synthesized using Fmoc solid-phase synthesis as described previously[20]. The peptide was purified to >95% by reverse phase high-performance liquid

chromatography prior to biological testing. Peptide was dissolved in water. Different concentrations of K9CATH (0-40 µM) were added to 2 x 10^6 CFU/ml of *S. pseudintermedius* in a polypropylene 96-well plate and incubated for 3 h at 37 °C in 50% MHB medium. Subsequently, bacteria were diluted 10-1000 fold and 100 µl from each dilution was plated out on TSA plates. After 24-hour incubation at 37 °C, colonies on the plates were counted.

2.8 Statistical analyses

To compare the differences in relative expression of mRNA cBD1, cBD103, K9CATH, TNF-α and IL-8 by the MSCEK cells stimulated by *S. pseudintermedius* 10^8 CFU/ml at indicated time-points to the baseline (0 h) (**Figure 2**), a general linear model was used and corrected by Dunnett's Post Hoc test at the significance level $P<0.05$. Non-parametric Kruskal-Wallis corrected by Dunn's multiple comparisons testing was performed for the comparison between any contrasts of multiple groups (**Figures 3-4**). Statistical analysis was performed by GraphPad Prism 8.0 and SPSS.

3. Results and discussion

As a first step towards understanding the response of KC towards *S. pseudintermedius,* regularly involved in secondary infection in AD, interaction of these bacteria with MSCEK cells was investigated. Adhesion and invasion of *S. pseudintermedius* were assessed at three different bacterial densities during 1 or 4 h of incubation. Both adhesion and invasion of bacteria were time- and dose-dependent as shown by increasing bacterial counts (**Figures 1A-B**). Nonetheless, the number of invaded (intracellular) bacteria after incubation of MSCEK with *S. pseudintermedius* for 1 h was low, i.e. under or just above the detection limit of 2 Log CFU, at all bacterial densities. Longer incubation of MSCEK cells

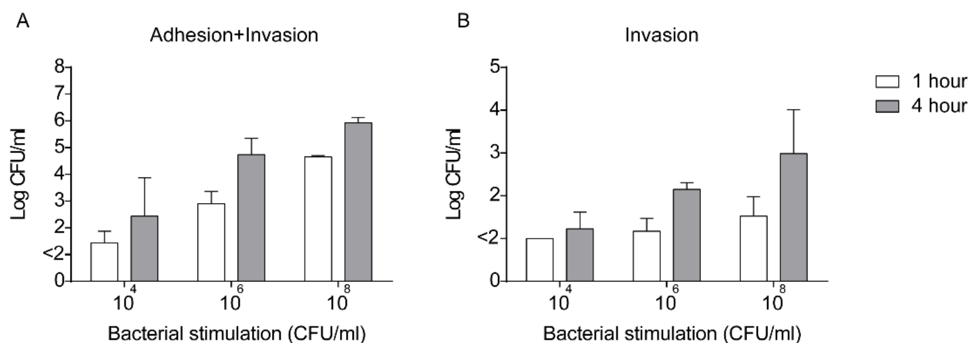


Figure 1. Adhesion and invasion assay of *S. pseudintermedius* to the MSCEK cell line. Three different densities (10^4, 10^6 and 10^8 CFU/ml) of freshly-prepared *S. pseudintermedius* were added to the MSECK cells and incubated for 1 and 4 h at 37 °C. A) To assess all bacteria associated with MSCEK (adhesion + invasion). B) To assess bacterial invasion.

with *S. pseudintermedius* enhanced the bacterial invasion at 10^8 CFU/ml, which was still less than 1% of the total number of associated bacteria (adhesion + invasion), indicating that MSCEK cells are relatively well equipped to prevent invasion.

Next, the response of MSCEK cells upon interaction with *S. pseudintermedius* was studied. Stimulation with 10^8 CFU/ml for 4 h resulted in significantly increased expression of the gene of the pro-inflammatory cytokine IL-8 at 4 and 6 h after exposure (**Figure 2A**). Gene expression of TNF-α was not affected at these early time points (**Figure 2B**). The pro-inflammatory cytokine (TNF-α and IL-8) responses to *S. pseudintermedius* occurred in a time-dependent fashion (**Figures 2A-B**) in line with previous studies using human KC in co-culture with live or killed *S. aureus*[21-23]. In AD, increased expression of TNF-α and IL-8 in the skin may induce maturation of antigen presenting cells and neutrophil migration to sites of infection *in vivo*[24]. Interestingly expression of the K9CATH gene followed the same pattern of induction, peaking at 4 and 6 h after infection, with higher than 4 fold increase (**Figure 2C**) (of note: some samples of 0 h were below the detection limit, indicating that the shown fold increase is an underestimation). On the other hand the defensins cBD1 and cBD103 did not increase significantly (**Figures 2D-E**), showing that expression of AMP is differentially regulated for each (family of) AMP, as has been noted before, in humans[25] and dogs[26]. At later time-points cBD103 was significantly expressed as compared to the expression at 0 h, but this was partially an effect of culturing as cells incubated with control medium also

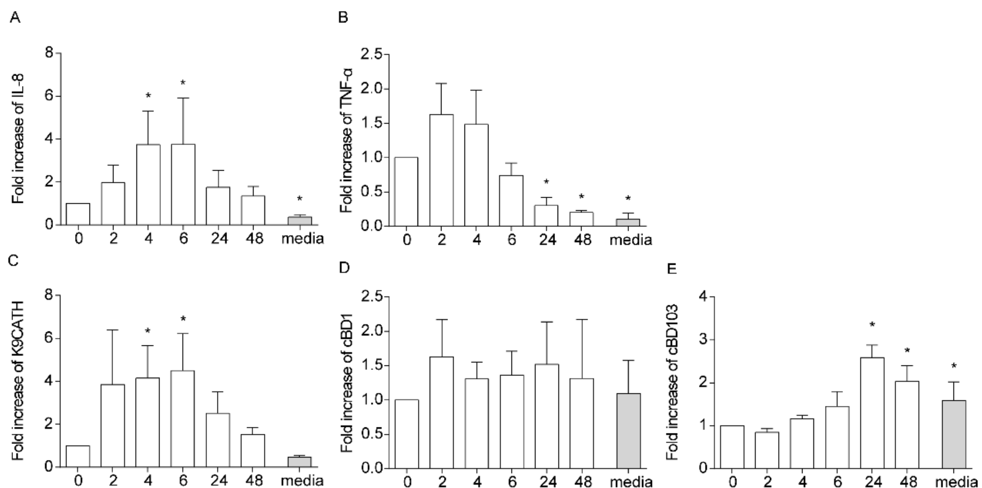


Figure 2. **MSCEK stimulated by live *S. pseudintermedius*.** Relative mRNA expression of IL-8 (A), TNF-α (B), K9CATH (C), cBD1 (D) and cBD103 (E) by MSCEK cells unstimulated (media) or following 0, 2, 4, 6, 24 and 48 h after an initial stimulation of 4-hours with *S. pseudintermedius*, 10^8 CFU/ml, at indicated hours assessed by qRT-PCR normalized to the reference gene RPS19. Fold increase calculated by the relative gene expression at the indicated time points compared to the relative expression at 0 h. Data are expressed as mean +/- SEM fold increase of two to three independent experiments in triplicate. Significance level at * $P<0.05$.

showed increased expression. Differences in constitutive versus induced expression level were observed between AMP[4]. For example, hBD-1 is constitutively expressed in epithelial cells but not upregulated during inflammation due to lacking of NF-kB transcription factor binding sites in the promotor region. Whereas, the promotor region of hBD-2 and hBD-3 (the orthologue of cBD103) consist of functional NF-kB binding sites exhibiting inducible expression in human skin upon inflammation or infection[4]. The constitutive expression may be important for an innate defense mechanism and the induced expression may help limit the severity of clinical symptoms. Although the observed effects on gene expression of AMP and cytokines in this study are relatively mild, exposure to *S. pseudintermedius* caused a clear pro-inflammatory state of MSCEKs.

Since bacteria consist of a complex mixture of potential stimulatory compounds, the effects observed with viable bacteria were related to MSCEK stimulation with single ligands, hence via individual PRR in order to obtain an insight in the complexity of bacterial immune stimulation. Incubation of MSCEK cells with LPS, Pam3CSK4 and TBD (ligands for TLR4, TLR2/1 and MINCLE, respectively) had significant effects on pro-inflammatory cytokines, especially TNF-α showing more than a 20 fold increase at 7 h (**Figure 3A**).

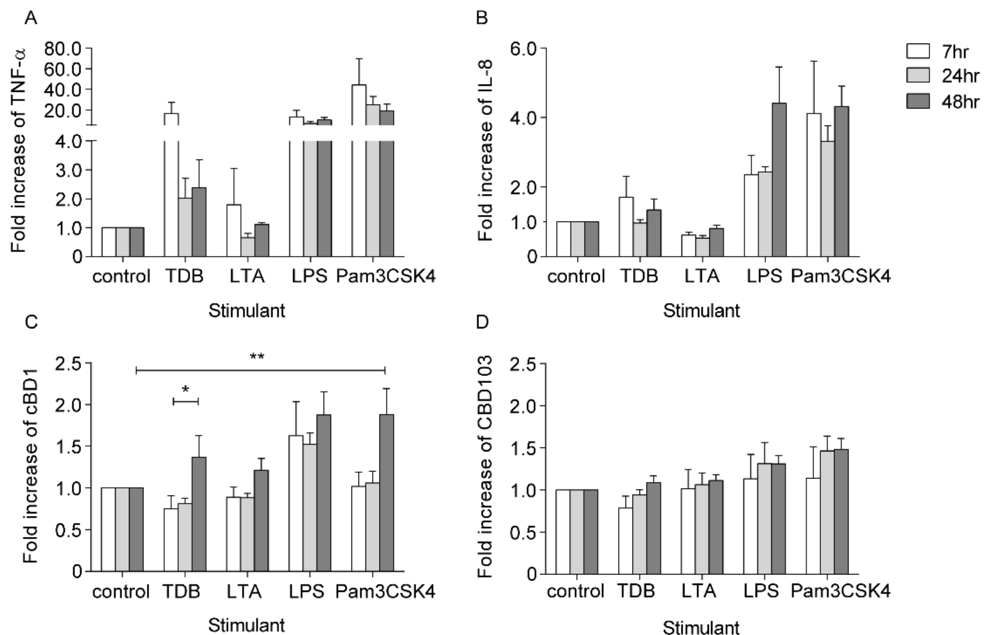


Figure 3. MSCEK stimulated by selected synthetic microbial ligands. Relative TNF-α (A), IL-8 (B), cBD1 (C) and cBD103 (D) mRNA expression by MSCEK cells stimulated for 7, 24 and 48 h with selected PRR ligands: synthetic TDB (20 µg/ml), LTA (10 µg/ml), LPS (50 µg/ml) and Pam3CSK4 (10 µg/ml) as assessed by qRT-PCR normalized to the reference gene RPS19. Fold increase was calculated by the relative gene expression of stimulated cells compared to non-stimulated cells (control) at the same time-points. Data are expressed as mean fold increase +/- SEM of at least three independent experiments. Significant differences between groups (P <0.05, P <0.01 denoted by *, **, respectively) were tested by non-parametric Kruskal-Wallis and Dunn's multiple comparison.

IL-8 mRNA levels were upregulated up to 5 fold compared to the control, although mainly after long incubation times (48 h, **Figure 3B**). On the contrary, LTA, a TLR2 agonist and major component of the Gram positive bacterial cell wall, had no effect on pro-inflammatory cytokine production (**Figures 3A-B**). With respect to AMP, small increases in cBD1 expression were observed after long incubation times with LTA (**Figure 3C**), but there was no effect on cBD103 (**Figure 3D**) or K9CATH where gene expression often remained under the detection limit of the assay for all stimulations (data not shown). The observed induction of pro-inflammatory cytokines by *in vitro* stimulated KC is comparable to that described earlier in human[22,27] and canine studies[28]. Clearly, the kinetics and extents of induction using individual ligands were different, mostly less intense, from that after whole bacterial stimulation, indicating that a multi factor based stimulation happens in the latter situation that cannot be simply reproduced by single ligands. Besides the stimulatory impact of bacterial ligands, the inflammatory state of the skin in AD itself is likely to have an effect on cytokine and AMP expression of keratinocytes. Therefore, we also analyzed the effect of AD-related cytokines involved in this inflammatory state on cBD1, cBD103, TNF-α and IL-8 gene expression.

Incubation of MSCEK with recombinant cytokines did not lead to statistically significant difference for any of the tested AMP genes (**Figures 4A-B**). Again gene expression of K9CATH was below the detection level for most samples (data not shown). Contrary to AMP gene expression after stimulation with AD related cytokines, TNF-α was able to induce a 15-fold higher expression of IL-8, as compared to the control at 48 h (**Figure 4C**). The presence of IFN-γ significantly decreased the expression of IL-8 at 24 h (**Figure 4C**). The presence of Th-2 associated cytokines IL-4, IL-13, or pro-inflammatory cytokine IL-17 had no significant effect on either AMP or pro-inflammatory cytokine mRNA levels.

Despite the differences in kinetics and extent of induction, the main difference between stimulation with ligands/cytokines and viable *S. pseudintermedius* was the induction of the K9CATH that was significantly increased upon stimulation with the whole viable bacteria. This prompted us to also test the antimicrobial activity of this peptide against the bacterium. The minimum bactericidal concentration (MBC) of K9CATH was 30 µM but significant killing activity was also observed in the 10-20 µM range (> 2log reduction in number of viable bacteria, data not shown). In another study a minimal inhibitory concentration (MIC) of >30 µM of K9CATH against *S. aureus* was reported[12]. Other AMP such as of human (LL-37), mouse (CRAMP) and pig (PR-39)[12,29,30] have similarly high MBC or MIC, which could indicate that *Staphylococci* are relatively resistant to (some) AMP compared to other bacteria. These results contrast, lower MIC values previously determined for K9CATH against other Gram positive and Gram negative bacteria[12]. Overall, it is an interesting observation that *S. pseudintermedius* induces an antimicrobial compound (K9CATH) that is not directly induced by individual PRR ligands or components of the pro-inflammatory immune response *in vitro*, thought to be important on an individual basis.

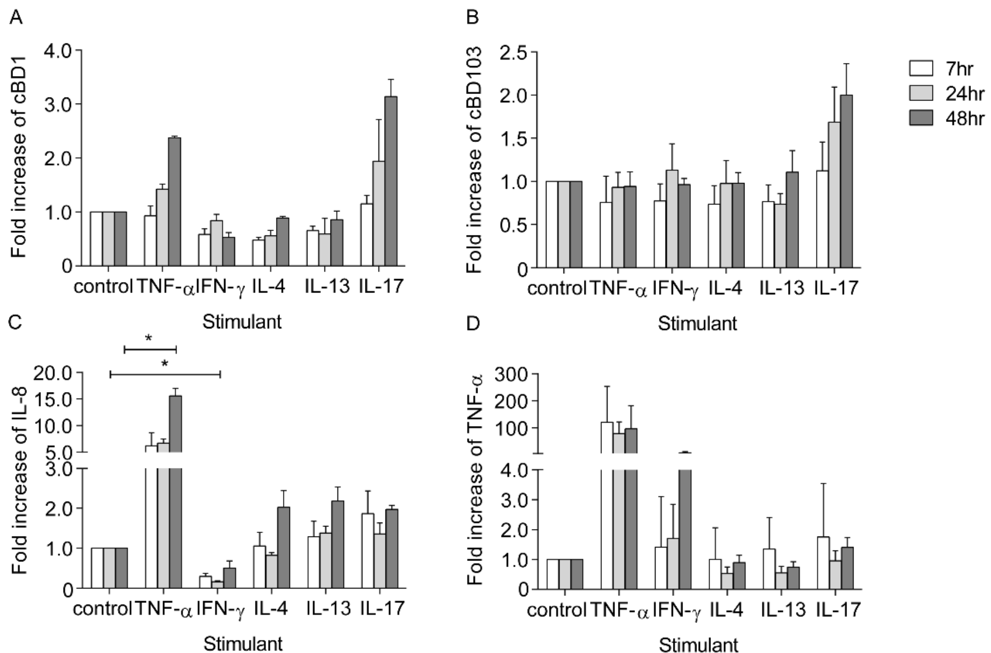


Figure 4. MSCEK stimulated by selected recombinant (canine) cytokines. Relative cBD1 (A), cBD103 (B), IL-8 (C) and TNF-α (D) mRNA expression by MSCEK cells stimulated for 7, 24 and 48 h with selected recombinant canine cytokines: TNF-α (20 ng/ml), IFN-γ (20 ng/ml), IL-4 (50 ng/ml), IL-13 (50 ng/ml) and IL-17 (20 ng/ml) as assessed by qRT-PCR normalized to the reference gene RPS19. Fold increase was calculated by the relative gene expression of stimulated cells compared to non-stimulated cells (control) at the same time-points. Data are expressed as mean fold increase +/- SEM of at least three independent experiments. Significant differences between groups ($P < 0.05$ denoted by *) were tested by non-parametric Kruskal-Wallis and Dunn's multiple comparison.

The alterations in the AMP and inflammatory cytokine expressions upon stimulation of the MSCEK cell with microbial components and analogs are comparable to those of the widely used canine keratinocyte progenitor cell line (CPEK)[28,31], or canine primary KC from healthy donors[32]. Moreover, the increased expression of pro-inflammatory cytokines and AMP by the MSCEK in co-culture with *S. pseudintermedius* are in agreement with the finding in human KC co-cultured with *S. aureus*[21-23,33,34]. Even though one should be careful extrapolating the research done in cell lines to the *in vivo* situation, these first results with the MSCEK cell line indicate that it can be an easy and useful new tool to study the KC role in immune reactivity in the canine skin. Since a good understanding of the complex immune response in atopic dermatitis is currently lacking, results of the present study potentially extended with those of similar studies with canine keratinocytes may unveil new therapeutic targets for AD and other (chronic) inflammatory disorders.

Declarations of interest

None.

Acknowledgements

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References

1. Chermprapai, S. *et al*. Altered lipid properties of the stratum corneum in Canine Atopic Dermatitis. *Biochim. Biophys. Acta* **1860**, 526-533 (2018).
2. Santoro, D. *et al*. Review: Pathogenesis of canine atopic dermatitis: skin barrier and host-micro-organism interaction. *Vet. Dermatol.* **26**, 84-e25 (2015).
3. Bitschar, K., Wolz, C., Krismer, B., Peschel, A. & Schittek, B. Keratinocytes as sensors and central players in the immune defense against Staphylococcus aureus in the skin. *J. Dermatol. Sci.* **87**, 215-220 (2017).
4. Leonard, B. C., Affolter, V. K. & Bevins, C. L. Antimicrobial peptides: agents of border protection for companion animals. *Vet. Dermatol.* **23**, 177-e36 (2012).
5. Miller, W. H., Griffin, C. E., Campbell, K. L. & Muller, G. H. in *Muller and Kirk's Small Animal Dermatology7: Muller and Kirk's Small Animal Dermatology* (Elsevier/Mosby, 2013).
6. Jassies-van der Lee, A., Rutten, V. P., Bruijn, J., Willemse, T. & Broere, F. CD4+ and CD8+ skin-associated T lymphocytes in canine atopic dermatitis produce interleukin-13, interleukin-22 and interferon-gamma and contain a CD25+ FoxP3+ subset. *Vet. Dermatol.* **25**, 456-e72 (2014).
7. Schlotter, Y. M., Rutten, V. P., Riemers, F. M., Knol, E. F. & Willemse, T. Lesional skin in atopic dogs shows a mixed Type-1 and Type-2 immune responsiveness. *Vet. Immunol. Immunopathol.* **143**, 20-26 (2011).
8. Eyerich, K. *et al*. IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response. *J. Allergy Clin. Immunol.* **123**, 59-66.e4 (2009).
9. Gittler, J. K. *et al*. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *J. Allergy Clin. Immunol.* **130**, 1344-1354 (2012).
10. Gallo, R. L. & Hooper, L. V. Epithelial antimicrobial defence of the skin and intestine. *Nat. Rev. Immunol.* **12**, 503-516 (2012).
11. Jenssen, H., Hamill, P. & Hancock, R. E. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* **19**, 491-511 (2006).
12. Sang, Y. *et al*. Canine cathelicidin (K9CATH): gene cloning, expression, and biochemical activity of a novel pro-myeloid antimicrobial peptide. *Dev. Comp. Immunol.* **31**, 1278-1296 (2007).
13. Santoro, D., Marsella, R., Bunick, D., Graves, T. K. & Campbell, K. L. Expression and distribution of canine antimicrobial peptides in the skin of healthy and atopic beagles. *Vet. Immunol. Immunopathol.* **144**, 382-388 (2011).
14. Santoro, D., Bunick, D., Graves, T. K. & Segre, M. Evaluation of canine antimicrobial peptides in infected and noninfected chronic atopic skin. *Vet. Dermatol.* **24**, 39-e10 (2013).
15. Harder, J. *et al*. Enhanced expression and secretion of antimicrobial peptides in atopic dermatitis and after superficial skin injury. *J. Invest. Dermatol.* **130**, 1355-1364 (2010).
16. van Damme, C. M., Willemse, T., van Dijk, A., Haagsman, H. P. & Veldhuizen, E. J. Altered cutaneous expression of beta-defensins in dogs with atopic dermatitis. *Mol. Immunol.* **46**, 2449-2455 (2009).
17. Ide, K. *et al*. Expression analysis of desmosomal components of the novel canine epidermal keratinocyte cell line (MSCEK). *J. Vet. Med. Sci.* **72**, 1479-1482 (2010).
18. Brinkhof, B., Spee, B., Rothuizen, J. & Penning, L. C. Development and evaluation of canine reference genes for accurate quantification of gene expression. *Anal. Biochem.* **356**, 36-43 (2006).
19. Schlotter, Y. M. *et al*. A GeNorm algorithm-based selection of reference genes for quantitative real-time PCR in skin biopsies of healthy dogs and dogs with atopic dermatitis. *Vet. Immunol. Immunopathol.* **129**, 115-118 (2009).
20. Bikker, F. J. *et al*. Evaluation of the antibacterial spectrum of drosocin analogues. *Chem. Biol. Drug Des.* **68**, 148-153 (2006).
21. Wiegand, C., Abel, M., Ruth, P. & Hipler, U. C. HaCaT keratinocytes in co-culture with Staphylococcus aureus can be protected from bacterial damage by polihexanide. *Wound Repair Regen.* **17**, 730-738 (2009).
22. Sasaki, T. *et al*. Effects of staphylococci on cytokine production from human keratinocytes. *Br. J. Dermatol.* **148**, 46-50 (2003).
23. Secor, P. R. *et al*. Staphylococcus aureus Biofilm and Planktonic cultures differentially impact gene expression, mapk phosphorylation, and cytokine production in human keratinocytes. *BMC Microbiol.* **11**, 143-2180-11-143 (2011).
24. Koga, C., Kabashima, K., Shiraishi, N., Kobayashi, M. & Tokura, Y. Possible pathogenic role of Th17 cells for atopic dermatitis. *J. Invest. Dermatol.* **128**, 2625-2630 (2008).
25. Midorikawa, K. *et al*. Staphylococcus aureus susceptibility to innate antimicrobial peptides, beta-defensins and CAP18, expressed by human keratinocytes. *Infect. Immun.* **71**, 3730-3739 (2003).
26. Santoro, D. & Maddox, C. W. Canine antimicrobial peptides are effective against resistant bacteria and yeasts. *Vet. Dermatol.* **25**, 35-e12 (2014).

27. Niebuhr, M., Heratizadeh, A., Wichmann, K., Satzger, I. & Werfel, T. Intrinsic alterations of pro-inflammatory mediators in unstimulated and TLR-2 stimulated keratinocytes from atopic dermatitis patients. *Exp. Dermatol.* **20**, 468-472 (2011).
28. Hendricks, A. *et al*. Ciclosporin modulates the responses of canine progenitor epidermal keratinocytes (CPEK) to toll-like receptor agonists. *Vet. Immunol. Immunopathol.* **147**, 91-96 (2012).
29. Veldhuizen, E. J. *et al*. Antimicrobial and immunomodulatory activities of PR-39 derived peptides. *PLoS One* **9**, e95939 (2014).
30. Gallo, R. L. *et al*. Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. *J. Biol. Chem.* **272**, 13088-13093 (1997).
31. Mullin, J., Carter, S., Williams, N., McEwan, N. & Nuttall, T. Transcription of canine toll-like receptor 2, beta-defensin 1 and beta-defensin 103 in infected atopic skin, non-infected atopic skin, healthy skin and the CPEK cell line. *Vet. Microbiol.* **162**, 700-706 (2013).
32. Santoro, D., Ahrens, K., Marsella, R. & Segre, M. Evaluation of antimicrobial peptides and cytokine production in primary keratinocyte cell culture from healthy and atopic beagles. *Exp. Dermatol.* **24**, 317-319 (2015).
33. Menzies, B. E. & Kenoyer, A. Staphylococcus aureus infection of epidermal keratinocytes promotes expression of innate antimicrobial peptides. *Infect. Immun.* **73**, 5241-5244 (2005).
34. Wanke, I. *et al*. Skin commensals amplify the innate immune response to pathogens by activation of distinct signaling pathways. *J. Invest. Dermatol.* **131**, 382-390 (2011).
35. Asahina, R., Kamishina, H., Kamishina, H. & Maeda, S. Gene transcription of pro-inflammatory cytokines and chemokines induced by IL-17A in canine keratinocytes. *Vet. Dermatol.* **26**, 426-31, e100 (2015).
36. Veenhof, E. Z., Rutten, V. P., van Noort, R., Knol, E. F. & Willemse, T. Evaluation of T-cell activation in the duodenum of dogs with cutaneous food hypersensitivity. *Am. J. Vet. Res.* **71**, 441-446 (2010).
37. Veenhof, E. Z. *et al*. Characterisation of T cell phenotypes, cytokines and transcription factors in the skin of dogs with cutaneous adverse food reactions. *Vet. J.* **187**, 320-324 (2011).

Supplemental material

Table S1. Primer sequences

Gene	Forward primer (5’-3’)	Reverse primer (5’-3’)	T_m
RPS19[18]	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG	61
cBD1[16]	AGCCTCTCTCCCTTCTCTGG	GCCATCGATCCTGGTAAAGA	57
cBD103[16]	GCCGCTGCTTACTTGTACCT	CCTCATGACCAACAGGCTTC	57
K9CATH[a]	TGTGTCCCAAGACGACACAG	ATGAGCTCTTTCAGCCGGTC	56
IL-8[35]	CTTCCAAGCTGGCTGTTGCTC	TGGGCCACTGTCAATCACTCTC	60
TNF-α[36,37]	CCCCGGGCTCCAGAAGGTG	GCAGCAGGCAGAAGAGTGTGGTG	64

[a] see https://www.ncbi.nlm.nih.gov/tools/primer-blast/. Accession number: NM_001003359.1

Chapter 7

General discussion

General discussion

Atopic dermatitis (AD) is a genetically predisposed inflammatory and pruritic allergic skin disease associated with immunoglobulin E (IgE) antibodies most commonly directed against environmental allergens[1]. Canine AD shares several characteristics with human AD[2-5], and is one of the most common skin diseases of dogs with a history of pruritus and recurrent skin or ear infection[6]. The exact pathogenesis of AD remains unclear for both human and canine AD and various hypotheses have been postulated[7-9].

Two major disease models are covered by the inside-out and the outside-in hypothesis. The inside-out hypothesis focuses on an immunological imbalance as primary cause of the disease, i.e. an initial over-activation of Th2-dominated immune responsiveness to harmless environmental allergens that subsequently causes skin barrier dysfunction[9]. The second model, concerns the outside-in hypothesis, which states that AD primarily develops as result of intrinsic defects of the skin barrier, facilitating penetration of otherwise harmless environmental allergens, that give rise to an inappropriate activation of both innate and adaptive immunity[9]. The two hypotheses for the immune-pathogenesis cannot be completely separated, clearly, loss of integrity of the skin barrier and activation of the immune system are closely linked. Due to disturbance of the homeostasis in the skin, altered keratinocyte and leukocyte activation result in changes in both antimicrobial peptide (AMP)[10] as well as cytokines production[10-15]. Consequently, the skin microbiome may alter and secondary infections can occur which further deteriorates the situation[16,17].

Some areas of the skin are prone to be affected by the disease (predilection sites i.e. axilla and inguinal region) while other locations (non-predilection site i.e. lateral thorax) are less involved[18]. It is currently unclear whether differences in skin composition at these specific locations contribute to being predilection site. The severity of the lesions may vary depending on the stage of disease (acute or chronic), the extent of concurrent secondary infection and self-traumatic injury[19,20].

Topical antimicrobial therapy reducing the microbial load on the skin and in addition the severity of clinical signs and pruritus is a major component of therapeutic approach of canine AD[21-24]. The reduction in infection and inflammation by the topical antimicrobial therapy may also alter AMP and AD-related cytokines expression in AD skin.

Major aspects of the immune-pathogenesis of canine AD described in this thesis were

i) Integrity of the skin as determined by the presence of lipids in the stratum corneum (SC), the major component of the skin barrier produced by keratinocytes (KC).

ii) Constitution of the skin microbiome (bacteria and fungi) on canine AD skin as well as healthy skin.

iii) Expression of antimicrobial peptides (AMP) and (pro-)inflammatory cytokines in canine AD and healthy skin *in vivo*.
iv) The *in vitro* interaction of a canine KC cell line (MSCEK) with specific purified microbial ligands and intact bacteria (genus *Staphylococcus*), shown to be abundantly present on AD skin, as well as with AMP and (pro-)inflammatory cytokines to study the relationship amongst the main findings under ii) and iii)

Canine skin barrier (Stratum corneum lipid)

Little is known about lipid composition and structure in the skin of dogs. Since lipid properties are considered to be important for the skin barrier[25], their composition and organization in the stratum corneum (SC) in AD and control dogs were examined (**Chapter 3**[26]). To the authors' knowledge this is the first report on the SC lamellar lipid organization analyses by small angle X-ray diffraction (SAXD) and Fourier transform infrared spectroscopy (FTIR) of canine skin. In addition, the lipid composition of several skin regions was analyzed by High performance thin layer chromatography (HPTLC) and Mass spectrometry (MS) to relate lipid compositions to those of the lipid organization.

The lipids in dog SC assembled in long and short periodicity phases (LPP and SPP, respectively) and LPP was less prominently present in atopic skin compared to the control, similar to the findings in human AD[27,28]. The reduction in LPP formation is associated with increased permeability of the skin barrier[29] that may contribute to development of AD. In atopic dog skin the conformational disordering of lipid organization was more prominent than in healthy skin, consistent with findings in human AD that coincided with a decrease in skin barrier function[27,30,31]. We showed that the lamellar organization of canine skin shared many characteristics observed in human SC.

The altered lipid organization observed in lesional skin of AD dogs compared to healthy control skin may be associated with the changes in the lipid composition that was observed. Significant differences in lipid composition between lesional AD and healthy skin of dogs were a decreased level of FFA and a decreased ratio of CER[NS] C44/C34, in lesional skin**.** Studies in lipid model systems indicated that a reduction in the chain length of the CERs or an increase of short chain CER[NS] C34 as well as a reduction in FFA level can cause a reduced lamellar distance[29,32,33] promoting hexagonal lateral packing rather than orthorhombic packing, resulting in an increased lamellar disordered organization[34,35]. Furthermore, a decreasing nonlinear relationship between the carbon chain length of CER[NS/NdS] C44/C34 and AD severity score (CADESI-03) was observed in this study indicating that the high abundance of shorter chain CERs correlates with the increased severity of AD in dog skin, similar to the findings previously reported in human[27,28]. This confirmed the correlation between lipid composition and disease severity.

In human AD, the altered lipid organization and composition observed in SC of lesional skin correlated with decreased barrier function (increased trans epidermal water loss), increased disease severity (SCORAD) and decreased natural moisturizing factor[27,30]. Although the barrier function of SC in dogs was not investigated in the present studies the alterations of SC lipid organization and composition observed in lesional skin of AD dogs were in agreement with findings described in human AD previously[27,30,31]. Hence, the observed changes in lipid properties in SC of AD dogs possibly indicate impaired skin barrier function and skin prone to develop AD and enhanced severity of AD. Alterations in SC lipid may be the primary cause of AD.

Canine skin microbiome

Another aspect important in canine AD pathogenesis is the high recurrence of bacterial and fungal dermatitis. Commensal microbiota are likely to, amongst others, prevent the overgrowth of pathogenic microbes and play a role in maintenance of homeostasis of the skin[16,17,36], changes in the microbiome may impair the skin barrier and/or local immune responsiveness, hence trigger AD progression[16,17,37]. Bacterial, *Staphylococcus* and yeast, *Malassezia* infections are the most common complicating pathogens diagnosed by culture[38-40]. In the present study, **Chapter 4**[41], the molecular sequencing analyses by the Illumina platform provided the skin bacterial and fungal compositions, 16S and ITS profiles respectively. Multiple different skin locations were sampled, either defined as predilection or lesional sites (axilla, inguinal region, periocular) and non-predilection or non-lesional site (trunk) in healthy and AD dogs. In addition, these sites were also sampled over time to address changes in microbiome due to topical antimicrobial treatment (Malaseb®). This high-throughput analysis revealed a strong between dog variability (individual) effect on the 16S and ITS profiles, but the opposite effect was described in human study[42]. A trend of reduced diversity in AD compared to control dog skin was observed for the 16S and ITS profiles, similar to the findings reported in human AD[43] and other canine studies[44-46]. Topical antimicrobial treatment altered the microbiome composition of both 16S and ITS profiles. In addition, this study also demonstrated a strong breed effect on the ITS profile, which however was less than the individual effect. This suggests that inter-dog variation in skin microbiome composition is more pronounced than intra-dog variation. Also in humans, ethnic differences are reflected in variability in microbiome composition[47-49].

Whereas human skin microbiome analyses, revealed that the microbiome composition was dependent on body sites[50,51], significant differences were not found in the microbiome composition among skin sites in dogs in this study. Another study on fungal composition of canine skin that classified more skin types also did not find significant differences between skin sites[46]. Human skin sites and their respective microbiome, may be grouped based on microenvironment (such as the number of

sebaceous glands, the temperature and humidity) into three types: dry, sebaceous and moist area[50,51]. Two studies in canine skin classified skin types into three groups: haired skin, mucosal sites and mucocutaneous junctions[44,52]. Comparison between human and canine skin microbiomes is complicated due to different classifications of skin types. All sample sites in the present study can be classified as haired skin. However, the sample sites were selected based on the validated predilection sites related to AD as described earlier[18] and we aim to emphasize the relationship between the actually affected skin area and its skin microbiome constitution in relation to AD. Using haired skin is prerequisite for our objectives and we removed the haircoat as much as possible before swabbing the skin samples.

The genera *Staphylococcus* and *Unidentified#01* (a known classifiable fungal taxon but without currently assigned nomenclature) are the most highly abundant in bacterial and fungal profiles, on AD dog skin, and during flare-up, but the relative abundance of these microbes was not significantly different compared to control skin. The absence of significant differences can be due to the limitation of the experiment (low sample size). Nevertheless, finding *Staphylococcus* as the predominant colonizer of AD skin in this study is consistent with many other studies in canine[44,45] feline[53] and human skin[43,50,54].

The genus *Malassezia* is one of the most dominant yeasts (fungi) on canine[46], and human skin[51]. Based on ITS sequences, the *"Unidentified"* genera abundantly present on the AD skin in our study do not belong to the phylum Basidiomycota, hence cannot be *Malassezia*. Unfortunately, due to the limitation of the sequencing database, no further specific information about the observation of the "*Unidentified*" genera is available. Only a using and improved sequence database could further elucidate their identity. In contrast to reports in literature that classify *Malassezia pachydermatis* as the most common yeast complication in canine AD[55], our study showed very low abundancy by the sequencing and the culture method using the selective agar confirmed the presence of *Malassezia* in our samples. This contradictory finding may be due to small sample size of our study, housing condition of dogs enrolled in this study, environmental humidity, lack of predisposing factors of individuals such as the presence of skin folds, cutaneous pH levels, previous medication, etc.[56]. This should be confirmed by larger studies.

Interestingly, only the relative abundance of the genus *Blumeria* was significant affected by both AD and topical antimicrobial treatment. Its presence was significantly higher on AD as compared to control skin and reduced by the topical treatment effect. The relationship between the genus *Blumeria* on the skin and AD, has never been reported in human or canine health before, hence further studies should be considered.

Canine skin AMP and AD-related (pro-)inflammatory cytokines expression

Apart from the skin barrier and the skin microbiome addressed in Chapters 3 and 4, keratinocytes and immunocompetent cells in the skin, crucial in skin homeostasis, are involved in the immune-pathogenesis of AD. Expression of AMP and AD-related (pro-)inflammatory cytokines were also studied (**Chapter 5**) in skin biopsies taken simultaneously to sampling for the "Microbiome study" described in Chapter 4**.** Due to this, alterations in microbiome composition could be related to changes in expression of AMP and (pro-)inflammatory cytokines in healthy and AD skin as well as during treatment.

The expression of AMP (cBD1 and cBD103) was higher prior to treatments and during disease flare in the non-predilection site (thorax) of AD dogs compared to healthy dog skin. Whereas the expression of (pro-)inflammatory cytokines in the predilection sites of AD skin before treatment and during flare was higher than that in the non-predilection site, opposite to the finding in AMP expression. The increased expression levels of Th2 cytokines (IL-4, IL-13), Th1 cytokine (IFN-γ), pro-inflammatory cytokine (TNF-α), Th17 cytokines (IL-17, IL-22) and the regulatory cytokine (IL-10) in predilection sites of AD skin without treatment compared to controls are consistent with several studies published previously on both spontaneous (human and canine) and experimental (canine) atopic dermatitis[5,57-62]. The topical antimicrobial therapy may reduce microbial load and possibly inflammation resulting in subsequent decrease in the severity of the lesion on the skin and the expression levels of both AMP and AD-related cytokines in most of both predilection and non-predilection sites of AD samples immediately after therapy.

Apart from differences induced by treatment, differential expression of both AMP and cytokines was also observed between different skin sites in both AD and healthy skin samples, potentially influenced by anatomical differences of the skin but severity of clinical disease at different locations studied cannot be excluded. A single AD dog with highest CADESI score pertaining acute lesions at inguinal region and the thorax is responsible for a relatively higher averaged expression of TNF-α, IL-4, IL-13, IL-17 and IL-22 in AD skin after treatment withdrawal.

Both expression of AMP and (pro-)inflammatory cytokines in predilection sites of AD skin tended to be increased during exacerbation (flare), that may have been caused by the overgrowth of certain microbes of which the genera i.e. *Staphylococcus* (16S) and *Unidentified#01* (ITS) were prominent as described in Chapter 4[26].

Only a limited number of studies has been executed on the role of AMP alone or the combined role of AMP and (pro-)inflammatory cytokines in canine skin health and disease, like in canine AD. Further studies on AMP and (pro-)inflammatory activity in canine skin (including AD) are needed to unravel the correlation between skin barrier function, immune responsiveness and pathogenesis of disease.

A canine keratinocyte cell line (MSCEK) expresses antimicrobial peptide and cytokine genes upon stimulation with bacteria, microbial ligands and recombinant cytokines

Differential activation of a canine keratinocyte cell line (MSCEK) as a result of interactions between different Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) expressed on its surface and the type of microbial components acting as ligands for the receptors, differentially influences expression of canine AMP (cBD1 and cBD103) as well as (pro-)inflammatory cytokines (TNF-α and IL-8) by **Chapter 6**[26]. The expression of AMP and (pro-)inflammatory cytokines as a response to stimulation by selected microbial ligands occur in a time dependent fashion, (pro-)inflammatory cytokines being produced early on after stimulation. This may suggest that the host immune response may act quickly to the triggers (invaders) causing acute inflammation and thereby in second instance disturb constitutive AMP production.

Although increased expression of the pro-inflammatory cytokines (TNF-α, IL-8), the Th2 cytokines (IL-4, IL-13) and the Th17 cytokines (IL-17, IL-22) was observed in lesional atopic skin as described in Chapter 5, no significant differences in the expression of AMP (cBD1 and cBD103) were found in the canine KC cell line (MSCEK) when stimulated by those recombinant (canine) cytokines as compared to the controls (**Chapter 6**). In the literature, all these cytokines, separately, differentially regulated the AMP expression in the AD skin. Whereas IL-17 and IL-22 might stimulate AMP production, AMP production can be partially inhibited by Th2 cytokines[10,11,63]. This suggests that extrapolating the research findings from cell line experiments to the *in vivo* situation should be done with caution, especially under the complicated conditions as AD.

Based on the findings of overgrowth genera during abstention of treatment of AD skin as described in Chapter 4 and previous reports, we selected *S. pseudintermedius* to study the interaction between this skin microbe, AMP, cytokines and a KC cell line *in vitro* in Chapter 6. The production of pro-inflammatory cytokines (TNF-α and IL-8) showed to be significantly increased in a time-dependent fashion. Whereas, the cBD1 and cBD103 expression did not increase significantly. K9CATH expression was extremely low under normal conditions (unstimulated KC) or when stimulated by individual synthetic microbial ligands, but significantly increased in KC stimulated by live *S. pseudintermedius*, likely to be the most important of the staphylococcal genus in AD (**Chapter 4**). This is potentially due to the more complex interactions of whole *S. pseudintermedius* with KC, as compared to that of the individual ligands, and might also indicate an antibacterial role of the peptide. Analysis of the antimicrobial activity of K9CATH against *S. pseudintermedius* showed killing with a minimum bactericidal concentration (MBC) of 30 µM (**Chapter 6**)**,** relatively high compared to that found for other Gram positive including *S. aureus* and Gram negative bacteria[64]. Other AMP such as of human (LL-37), mouse (CRAMP) and pig (PR-39)[64-68] have similarly high MBC or minimal inhibitory concentrations (MIC), which could indicate that *Staphylococcus* are relatively resistant to AMP compared to other

bacteria. The diminished production of local AMP caused by disrupted skin barrier in context of AD plus the relatively high resistance of the bacteria may be the reason for their high ability to infect skin and aggravate the severity of AD[63, 69, 70].

The *in vitro* study of expression or activity of a limited number of AMP is most likely not representative for the situation *in vivo* where multiple antibacterial compounds will be present, potentially augmenting each other's antimicrobial activities[71-74]. In that respect, it would be interesting to test combinations of AMP for example K9CATH and cBD1 against *S. pseudintermedius* to determine synergism between these molecules. On top of that, abundant numbers and diverse types of microorganisms are inhabiting the skin as also described in Chapter 4, and express multiple types of microbial ligands, interact with multiple PRRs constituting a more complex immune responsiveness.

Next to direct antimicrobial activity also immunomodulatory properties of AMP might contribute to skin homeostasis. Cathelicidins expressed in some other species (i.e. human LL-37 or porcine PR-39) have shown to act as chemo-attractor of T-cells and phagocytes[75], and aid in induction of pro-inflammatory macrophages[76]; all functions that help indirectly to eliminate the pathogenic microorganisms should be further investigated.

Our study demonstrated that *S. pseudintermedius* can stimulate AMP and (pro-) inflammatory cytokine productions by canine keratinocytes cell line (MSCEK) *in vitro* and that may mimic the increased expression of AMP and AD-related cytokines on the AD predilection skin as described in **Chapter 5.** Not only the imbalance of immune response but also the trigger by skin microbes can alter AMP mRNA expression in the skin.

Alterations in SC lipid both organization and composition may be correlated with skin barrier dysfunction that allow allergens and/or microorganisms penetrating the skin and consequently develop AD. By that, loss of skin homeostasis may alter the microbiome composition, innate (AMP) and adaptive immunity responsiveness (cytokines), individually or even the relationship between those. Although, there are several possible mechanisms related to canine AD, the findings described in this thesis contribute to knowledge in this field.

References

1. Halliwell, R. Revised nomenclature for veterinary allergy. *Vet. Immunol. Immunopathol.* **114**, 207-208 (2006).
2. Griffin, C. E. & DeBoer, D. J. The ACVD task force on canine atopic dermatitis (XIV): clinical manifestations of canine atopic dermatitis. *Vet. Immunol. Immunopathol.* **81**, 255-269 (2001).
3. Marsella, R. Fixing the skin barrier: past, present and future--man and dog compared. *Vet. Dermatol.* **24**, 73-6.e17-8 (2013).
4. Santoro, D. & Rodrigues Hoffmann, A. Canine and Human Atopic Dermatitis: Two Faces of the Same Host-Microbe Interaction. *J. Invest. Dermatol.* **136**, 1087-1089 (2016).
5. Pucheu-Haston, C. M. *et al*. Review: Lymphocytes, cytokines, chemokines and the T-helper 1-T-helper 2 balance in canine atopic dermatitis. *Vet. Dermatol.* **26**, 124-e32 (2015).
6. Olivry, T. *et al*. Treatment of canine atopic dermatitis: 2010 clinical practice guidelines from the International Task Force on Canine Atopic Dermatitis. *Vet. Dermatol.* **21**, 233-248 (2010).
7. Elias, P. M. & Steinhoff, M. "Outside-to-inside" (and now back to "outside") pathogenic mechanisms in atopic dermatitis. *J. Invest. Dermatol.* **128**, 1067-1070 (2008).
8. Novak, N. & Leung, D. Y. M. in *Pediatric Allergy: Principles and Practice* (eds Leung, D. Y. M. & Sampson, H.) 552-63 (W.B.Saunders, Edinburgh, 2010).
9. Gedon, N. K. Y. & Mueller, R. S. Atopic dermatitis in cats and dogs: a difficult disease for animals and owners. *Clinical and translational allergy* **8**, 41; 41-41 (2018).
10. Kisich, K. O., Carspecken, C. W., Fieve, S., Boguniewicz, M. & Leung, D. Y. Defective killing of Staphylococcus aureus in atopic dermatitis is associated with reduced mobilization of human beta-defensin-3. *J. Allergy Clin. Immunol.* **122**, 62-68 (2008).
11. Nomura, I. *et al*. Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J. Immunol.* **171**, 3262-3269 (2003).
12. Howell, M. D. *et al*. Interleukin-10 downregulates anti-microbial peptide expression in atopic dermatitis. *J. Invest. Dermatol.* **125**, 738-745 (2005).
13. Eyerich, K. *et al*. IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response. *J. Allergy Clin. Immunol.* **123**, 59-66.e4 (2009).
14. Wolk, K. *et al*. IL-22 increases the innate immunity of tissues. *Immunity* **21**, 241-254 (2004).
15. Gittler, J. K. *et al*. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *J. Allergy Clin. Immunol.* **130**, 1344-1354 (2012).
16. Miller, L. S. & Cho, J. S. Immunity against Staphylococcus aureus cutaneous infections. *Nat. Rev. Immunol.* **11**, 505-518 (2011).
17. Laborel-Preneron, E. *et al*. Effects of the Staphylococcus aureus and Staphylococcus epidermidis Secretomes Isolated from the Skin Microbiota of Atopic Children on CD4+ T Cell Activation. *PLoS One* **10**, e0141067 (2015).
18. Favrot, C., Steffan, J., Seewald, W. & Picco, F. A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. *Vet. Dermatol.* **21**, 23-31 (2010).
19. Olivry, T., Marsella, R., Iwasaki, T., Mueller, R. & International Task Force On Canine Atopic Dermatitis. Validation of CADESI-03, a severity scale for clinical trials enrolling dogs with atopic dermatitis. *Vet. Dermatol.* **18**, 78-86 (2007).
20. Hensel, P., Santoro, D., Favrot, C., Hill, P. & Griffin, C. Canine atopic dermatitis: detailed guidelines for diagnosis and allergen identification. *BMC Vet. Res.* **11**, 196-015-0515-5 (2015).
21. Olivry, T. *et al*. Treatment of canine atopic dermatitis: 2015 updated guidelines from the International Committee on Allergic Diseases of Animals (ICADA). *BMC Vet. Res.* **11**, 210-015-0514-6 (2015).
22. Hillier, A. *et al*. Guidelines for the diagnosis and antimicrobial therapy of canine superficial bacterial folliculitis (Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases). *Vet. Dermatol.* **25**, 163-75, e42-3 (2014).
23. Nam, E. H. *et al*. Evaluation of the effect of a 0.0584% hydrocortisone aceponate spray on clinical signs and skin barrier function in dogs with atopic dermatitis. *J. Vet. Sci.* **13**, 187-191 (2012).
24. Olivry, T., Mueller, R. S. & THE INTERNATIONAL TASK FORCE ON CANINE ATOPIC DERMATITIS. Evidence-based veterinary dermatology: a systematic review of the pharmacotherapy of canine atopic dermatitis. *Vet. Dermatol.* **14**, 121-146 (2003).
25. Harding, C. R. The stratum corneum: structure and function in health and disease. *Dermatol. Ther.* **17 Suppl 1**, 6-15 (2004).
26. Chermprapai, S. *et al*. Altered lipid properties of the stratum corneum in Canine Atopic Dermatitis. *Biochim. Biophys. Acta* **1860**, 526-533 (2018).

27. Janssens, M. *et al*. Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. *J. Lipid Res.* **53**, 2755-2766 (2012).
28. Janssens, M. *et al*. Lamellar lipid organization and ceramide composition in the stratum corneum of patients with atopic eczema. *J. Invest. Dermatol.* **131**, 2136-2138 (2011).
29. de Jager, M. *et al*. A novel in vitro percutaneous penetration model: evaluation of barrier properties with p-aminobenzoic acid and two of its derivatives. *Pharm. Res.* **23**, 951-960 (2006).
30. van Smeden, J. *et al*. The importance of free fatty acid chain length for the skin barrier function in atopic eczema patients. *Exp. Dermatol.* **23**, 45-52 (2014).
31. Pilgram, G. S. *et al*. Aberrant lipid organization in stratum corneum of patients with atopic dermatitis and lamellar ichthyosis. *J. Invest. Dermatol.* **117**, 710-717 (2001).
32. Thakoersing, V. S. *et al*. Nature versus nurture: does human skin maintain its stratum corneum lipid properties in vitro? *Exp. Dermatol.* **21**, 865-870 (2012).
33. Mojumdar, E. H., Kariman, Z., van Kerckhove, L., Gooris, G. S. & Bouwstra, J. A. The role of ceramide chain length distribution on the barrier properties of the skin lipid membranes. *Biochim. Biophys. Acta* **1838**, 2473-2483 (2014).
34. Uchiyama, M., Oguri, M., Mojumdar, E. H., Gooris, G. S. & Bouwstra, J. A. Free fatty acids chain length distribution affects the permeability of skin lipid model membranes. *Biochim. Biophys. Acta* **1858**, 2050-2059 (2016).
35. Mojumdar, E. H., Gooris, G. S. & Bouwstra, J. A. Phase behavior of skin lipid mixtures: the effect of cholesterol on lipid organization. *Soft Matter* **11**, 4326-4336 (2015).
36. Nakatsuji, T. *et al*. Antimicrobials from human skin commensal bacteria protect against Staphylococcus aureus and are deficient in atopic dermatitis. *Sci. Transl. Med.* **9**, 10.1126/scitranslmed.aah4680 (2017).
37. Cho, I. & Blaser, M. J. The human microbiome: at the interface of health and disease. *Nat. Rev. Genet.* **13**, 260-270 (2012).
38. Fazakerley, J. *et al*. Staphylococcal colonization of mucosal and lesional skin sites in atopic and healthy dogs. *Vet. Dermatol.* **20**, 179-184 (2009).
39. McEwan, N. A., Mellor, D. & Kalna, G. Adherence by Staphylococcus intermedius to canine corneocytes: a preliminary study comparing noninflamed and inflamed atopic canine skin. *Vet. Dermatol.* **17**, 151-154 (2006).
40. Tarpataki, N. Recent developments in canine atopic dermatitis: a review. *Acta Vet. Hung.* **54**, 473-484 (2006).
41. Chermprapai, S. *et al*. The bacterial and fungal microbiome of the skin of healthy dogs and dogs with atopic dermatitis and the impact of topical antimicrobial therapy, an exploratory study. *Veterinary Microbiology* **229**, 90-99 (2019).
42. Grice, E. A. & Segre, J. A. The skin microbiome. *Nat. Rev. Microbiol.* **9**, 244-253 (2011).
43. Kong, H. H. *et al*. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res.* **22**, 850-859 (2012).
44. Rodrigues Hoffmann, A. *et al*. The skin microbiome in healthy and allergic dogs. *PLoS One* **9**, e83197 (2014).
45. Bradley, C. W. *et al*. Longitudinal Evaluation of the Skin Microbiome and Association with Microenvironment and Treatment in Canine Atopic Dermatitis. *J. Invest. Dermatol.* **136**, 1182-1190 (2016).
46. Meason-Smith, C. *et al*. What is living on your dog's skin? Characterization of the canine cutaneous mycobiota and fungal dysbiosis in canine allergic dermatitis. *FEMS Microbiol. Ecol.* **91**, 10.1093/femsec/fiv139. Epub 2015 Nov 4 (2015).
47. Clemente, J. C. *et al*. The microbiome of uncontacted Amerindians. *Sci. Adv.* **1**, e1500183 (2015).
48. Rosenthal, M., Goldberg, D., Aiello, A., Larson, E. & Foxman, B. Skin microbiota: microbial community structure and its potential association with health and disease. *Infect. Genet. Evol.* **11**, 839-848 (2011).
49. Sultana, B., Cimiotti, J., Aiello, A. E., Sloan, D. & Larson, E. Effects of age and race on skin condition and bacterial counts on hands of neonatal ICU nurses. *Heart Lung* **32**, 283-289 (2003).
50. Grice, E. A. *et al*. Topographical and temporal diversity of the human skin microbiome. *Science* **324**, 1190-1192 (2009).
51. Findley, K. *et al*. Topographic diversity of fungal and bacterial communities in human skin. *Nature* **498**, 367-370 (2013).
52. Meason-Smith, C. *et al*. Characterization of the cutaneous mycobiota in healthy and allergic cats using next generation sequencing. *Vet. Dermatol.* (2016).
53. Older, C. E. *et al*. The feline skin microbiota: The bacteria inhabiting the skin of healthy and allergic cats. *PLoS One* **12**, e0178555 (2017).
54. Dekio, I. *et al*. Characterization of skin microbiota in patients with atopic dermatitis and in normal subjects using 16S rRNA gene-based comprehensive analysis. *J. Med. Microbiol.* **56**, 1675-1683 (2007).

55. Miller, W. H., Griffin, C. E., Campbell, K. L. & Muller, G. H. in *Muller and Kirk's Small Animal Dermatology7: Muller and Kirk's Small Animal Dermatology* (Elsevier/Mosby, 2013).
56. Bajwa, J. Canine Malassezia dermatitis. *The Canadian veterinary journal = La revue veterinaire canadienne* **58**, 1119-1121 (2017).
57. Schlotter, Y. M., Rutten, V. P., Riemers, F. M., Knol, E. F. & Willemse, T. Lesional skin in atopic dogs shows a mixed Type-1 and Type-2 immune responsiveness. *Vet. Immunol. Immunopathol.* **143**, 20-26 (2011).
58. Pucheu-Haston, C. M. *et al*. A canine model of cutaneous late-phase reactions: prednisolone inhibition of cellular and cytokine responses. *Immunology* **117**, 177-187 (2006).
59. Olivry, T., Dean, G. A., Tompkins, M. B., Dow, J. L. & Moore, P. F. Toward a canine model of atopic dermatitis: amplification of cytokine-gene transcripts in the skin of atopic dogs. *Exp. Dermatol.* **8**, 204-211 (1999).
60. Marsella, R., Olivry, T. & Maeda, S. Cellular and cytokine kinetics after epicutaneous allergen challenge (atopy patch testing) with house dust mites in high-IgE beagles. *Vet. Dermatol.* **17**, 111-120 (2006).
61. Nuttall, T. J., Knight, P. A., McAleese, S. M., Lamb, J. R. & Hill, P. B. Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis. *Clin. Exp. Allergy* **32**, 789-795 (2002).
62. Nograles, K. E. *et al*. IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. *J. Allergy Clin. Immunol.* **123**, 1244-52.e2 (2009).
63. Ong, P. Y. *et al*. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* **347**, 1151-1160 (2002).
64. Sang, Y. *et al*. Canine cathelicidin (K9CATH): gene cloning, expression, and biochemical activity of a novel pro-myeloid antimicrobial peptide. *Dev. Comp. Immunol.* **31**, 1278-1296 (2007).
65. Veldhuizen, E. J. *et al*. Antimicrobial and immunomodulatory activities of PR-39 derived peptides. *PLoS One* **9**, e95939 (2014).
66. Agerberth, B. *et al*. Amino acid sequence of PR-39. Isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides. *Eur. J. Biochem.* **202**, 849-854 (1991).
67. Gallo, R. L. *et al*. Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. *J. Biol. Chem.* **272**, 13088-13093 (1997).
68. Bals, R., Wang, X., Zasloff, M. & Wilson, J. M. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9541-9546 (1998).
69. Lloyd, D. H. Microbial diseases secondary to allergic skin disease - clinical significance and control. ID - 20103277386. *European Journal of Companion Animal Practice* **19**, 254-260 (2009).
70. Howell, M. D. The role of human beta defensins and cathelicidins in atopic dermatitis. *Curr. Opin. Allergy Clin. Immunol.* **7**, 413-417 (2007).
71. van Damme, C. M., Willemse, T., van Dijk, A., Haagsman, H. P. & Veldhuizen, E. J. Altered cutaneous expression of beta-defensins in dogs with atopic dermatitis. *Mol. Immunol.* **46**, 2449-2455 (2009).
72. Lancto, C. A., Torres, S. M., Hendrickson, J. A., Martins, K. V. & Rutherford, M. S. Altered expression of antimicrobial peptide genes in the skin of dogs with atopic dermatitis and other inflammatory skin conditions. *Vet. Dermatol.* **24**, 414-21, e90 (2013).
73. Santoro, D. & Maddox, C. W. Canine antimicrobial peptides are effective against resistant bacteria and yeasts. *Vet. Dermatol.* **25**, 35-e12 (2014).
74. Gallo, R. L. & Hooper, L. V. Epithelial antimicrobial defence of the skin and intestine. *Nat. Rev. Immunol.* **12**, 503-516 (2012).
75. Choi, K. Y. & Mookherjee, N. Multiple immune-modulatory functions of cathelicidin host defense peptides. *Front. Immunol.* **3**, 149 (2012).
76. van der Does, A. M. *et al*. LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature. *J. Immunol.* **185**, 1442-1449 (2010).

Chapter 8

Summaries in English, Dutch and Thai

English summary

Disturbances of the skin's gross-, microscopic- and molecular structure and consequent functional alterations amongst which those in immune responsiveness may pave the way for induction of skin diseases such as atopic dermatitis (AD). The pathogenesis of AD in dog skin is not fully understood. According to the "inside-out" hypothesis, the first of two describing the potential pathogenesis of AD, immunological abnormalities may lead to increased epidermal barrier permeability. The second hypothesis, the "outside-in" theory of pathogenesis of AD, proposes that an intrinsic skin barrier dysfunction may facilitate allergen penetration into the skin leading to hyperactivation of both innate and adaptive immunity. Due to loss of skin homeostasis in either of the two pathogenic routes the balance between skin commensals and opportunistic microbes may be disturbed. Consequent changes in the microbiome composition and the release of microbial components contribute to alterations in innate (e.g. antimicrobial peptides, AMP) and adaptive (e.g. cytokines) immune responsiveness. This may further aggravate skin barrier function, skin microbiome composition and subsequently lead to AD development or its increased severity.

In **Chapter 3**, we demonstrated that the skin of dogs affected by AD showed alterations in stratum corneum lipid properties, compared to that in normal skin as reflected by changes in the lamellar phases and decreased formation of LPP shown by SAXD, decreased conformational ordering uncovered by FTIR and reduced levels of FFA and the decreased ratio of CER[NS] C44/C34 by HPTLC and LC/MS. These changes, more pronounced in lesional skin of AD patients compared to non-lesional skin, indicate a role of skin barrier disturbance in pathogenesis. Loss of proper skin barrier function may have led to increased permeability of the skin and epidermal water loss and subsequently to facilitation of allergen penetration and/or secondary infection of the skin as a cause of the AD observed.

Next to the skin barrier also the skin microbiome may play a role in the pathogenesis of AD. In the exploratory study performed in the context of this thesis, the canine skin microbiome composition was shown to be dependent on individual variation and seemed to be less determined by skin topography. General, significant differences in the microbiome composition were not observed between AD and healthy dog skin. However, the microbial diversity (both in 16s and ITS profiles) tended to be lower in AD compared to healthy skin at every site and timepoint studied. The relative abundance of certain microbes was significantly different between AD and control skin, the relationship between those taxa and the skin integrity should be further investigated in a larger study. Antimicrobial topical treatment (Malaseb®) that is known to be able to decrease the microbial load and consequently reduce clinical severity of presenting signs was used in

this study and significantly changed the microbiome composition in course of time and at every site studied but irrespective of AD skin status (**Chapter 4**).

The expression levels of AMP (cBD1, cBD103, K9CATH) and AD-related Th2 cytokines (IL-4, IL-13), the Th1 cytokine (IFN-γ), the pro-inflammatory cytokine (TNF-α), Th17 cytokines (IL-17, IL-22), and a regulatory cytokine (IL-10) are likely to be related to skin location and severity of AD. In the context of the topical treatment (Malaseb®) described in Chapter 4, alterations in the expression of AMP and AD-related cytokines in AD and healthy dog skin as a result of the antimicrobial effect were studied. Disease severity tended to be influenced by topical treatment, that altered both AMP and cytokine expression. Increased expression of mRNA of AMP and cytokines in the skin biopsy of AD dogs described in this thesis, although consistent with previously reported findings, should be further investigated and confirmed by a larger study (**Chapter 5**).

The disrupted barrier caused by AD enables allergens and/or microorganisms to penetrate the skin and to alter the complex skin homeostasis including the interactions between keratinocytes (KC), skin microbiota and the production of AMP in the skin. Canine KC culture *in vitro* in **Chapter 6** uncovered alterations in AMP (cBD1, cBD103 and K9CATH) as well as pro-inflammatory cytokines (TNF-α and IL-8) when stimulated with live *S. pseudintermedius* or microbial components (Gram positive and negative) activating the cells through various TLR and other ligands. This mimics one of several possible causes of disturbance under the complicated conditions of AD in skin.

The findings described in this thesis indicate putative alterations occurring in or inherent to the AD skin. The altered lipid composition and organization in both lesional and non-lesional AD skin may support the “outside-in” hypothesis that states that abnormalities in skin barrier can be leading in AD pathogenesis. Next to the “outside-in” hypothesis, the “inside-out” hypothesis stating that the AD is immunologically driven initially is still being an alternative mechanism for the pathogenesis of AD. The microbiome composition may differ between AD and healthy dog skin, but the predilection sites of AD may not be predisposed by the microbiome composition. The microbiome of canine skin is likely to vary between individuals and to be influenced by the topical antimicrobial treatment. The microbiome and AD are likely to influence each other. The infected, inflammatory state of AD skin can induce both AMP and further cytokine expression, and differential expression levels can be due to location and topical treatment. Such distortions in the balance of the whole skin structure may lead to significant changes in skin barrier lipid, skin microbiome, AMP and inflammatory cytokines as shown in this thesis.

Overall, the contents of this thesis contribute to further understanding of the immunopathogenesis of atopic dermatitis in dogs, in view of new options for diagnosis and follow up of therapy.

Nederlandse samenvatting

Verstoring van de macroscopische -, microscopische - en moleculaire structuur van de huid, en de daarmee samenhangende functionele veranderingen, waaronder die in lokale immuunreactiviteit, kunnen aanleiding zijn tot het ontstaan van huidziekten zoals atopische dermatitis (AD). De pathogenese van AD in de huid van de hond is niet volledig duidelijk. De “inside-out” hypothese, de eerste van twee die de mogelijke pathogenese van AD beschrijven, stelt dat immunologische veranderingen kunnen leiden tot het toenemen van de permeabiliteit van de huidbarrière. De tweede, de “outside-in” hypothese, beschrijft de situatie waarin een verstoring van de huidbarrière het binnendringen van allergenen in de huid mogelijk maakt, met als gevolg hyperactivatie van zowel het aangeboren als het adaptieve immuunsysteem. Het resultaat bij beide hypothesen is het verlies van homeostase in de huid, en een verstoring van de de balans tussen commensale en opportunistische microben. De aangepaste samenstelling van het microbioom en het vrijkomen van microbiële componenten dragen bij aan verschuivingen in de aangeboren (o.a. antimicrobiële peptiden; AMP) en adaptieve (o.a. cytokines) immuunreactiviteit. Als gevolg daarvan kunnen huidbarrièrefunctie en samenstelling van het microbioom verder aangetast worden en kan AD ontstaan of verergeren.

In **Hoofstuk 3** tonen we aan dat in door AD aangedane huid van honden, lipiden in het stratum corneum andere eigenschappen hebben dan die in normale hondenhuid. We zien veranderingen in de lamellaire fasen en een afgenomen vorming van LPP gemeten door SAXD; een verminderde conformationele structurering aangetoond door FITR; lagere niveaus van FFA en een afgenomen ratio van CER[NS] C44/C34 gemeten door HPTLC en LC/MS. Deze veranderingen, duidelijker aanwezig in lesionale huid dan in huid zonder laesies, wijzen op betrokkenheid van een verstoorde huidbarrière in pathogenese van AD in de hond. Verlies van een functionele huidbarrière zal leiden tot een toegenomen permeabiliteit van de huid en verlies van water in de epidermis. Dit kan vervolgens leiden tot gemakkelijkere penetratie van allergeen in de huid en/of secondaire infectie van de huid, mogelijk geassocieerd met de ernst van AD.

Naast de huidbarrière speelt mogelijk ook het microbioom van de huid een rol in de pathogenese van AD. In de verkennende studie die uitgevoerd is voor deze thesis, bleek de samenstelling van het microbioom van hondenhuid vooral afhankelijk van individuele variatie en in mindere mate van de topografie van de huid. Algemene, significante verschillen in de samenstelling van het microbioom van AD- en gezonde hondenhuid werden niet waargenomen. Echter, de microbiële diversiteit (in zowel 16s als ITS profielen) bleek lager in AD huid vergeleken met gezonde huid op elke huid locatie en op elk tijdspunt waarop werd gemeten. Daarnaast was de relatieve vertegenwoordiging van bepaalde microben significant anders in AD dan in gezonde huid. De relatie tussen deze taxa en de integriteit van de huid zou moeten worden bestudeerd in een grotere

vervolgstudie. De lokale behandeling met antimicrobiële shampoo (Masaleb ®), bekend om zijn eigenschap het aantal microben van de huid te verlagen en daarmee de ernst van de klinische klachten te reduceren bij AD, zorgde in deze studie voor significante verandering in het microbioom in de tijd en op elke in de huid geteste locatie. Dit was onafhankelijk van de AD status van de huid (**Hoofdstuk 4**).

De expressie niveaus van AMP (cBD1, cBD103, K9CATH) en de AD-gerelateerde Th2 cytokines (IL-4, IL-13), pro-inflammatoire cytokine (IFN-γ, IL-17, IL-22) en het regulerende cytokine (IL-10) in de huid zijn waarschijnlijk gerelateerd aan de locatie en de ernst van de ziekte. In hoofdstuk 4 zijn de veranderingen in de expressie van AMP en AD-gerelateerde cytokines in AD- en gezonde hondenhuid, ten gevolge van het antimicrobiële effect van lokale behandeling (Masaleb®) beschreven. De ernst van de ziekte werd beïnvloed door de lokale behandeling, die zowel de AMP als de cytokine expressie veranderde. De waargenomen toename in expressie van mRNA van AMP en inflammatoire cytokines in de huidbiopten van honden met AD, hoewel consistent met eerdere beschreven bevindingen, zou verder moeten worden onderzocht en bevestigd in een grotere studie (**Hoofdstuk 5**).

De met AD geassocieerde verstoorde barrière stelt allergenen en/of micro-organismen in staat om de huid binnen te dringen en de complexe huidhomeostase te veranderen, inclusief de interacties tussen keratinocyten (KC), de huidmicrobiota en de productie van AMP en cytokines in de huid. *In vitro* kweek van honden KC in **Hoofdstuk 6** liet veranderingen zien in productie van AMP (cBD1, CBD103 en K9CATH) en in pro-inflammatoire cytokines (TNF-α en IL-8) na stimulatie van cellen met levende *S. pseudintermedius* of microbiële componenten en via verschillende TLR en andere receptoren. Reflectie van één van de verschillende mogelijke oorzaken van de verstoring van de huid in de ingewikkelde situatie van AD.

De bevindingen beschreven in deze thesis wijzen op veranderingen in of inherent aan de AD huid. De veranderde lipidensamenstelling en organisatie in zowel AD huid met en zonder laesies zou de ‘outside-in’ hypothese kunnen ondersteunen die stelt dat afwijkingen in de huidbarrière kan leiden tot AD pathogenese. Naast de ‘outside-in’ hypothese is de ‘inside-out’ hypothese, die veronderstelt dat de AD initieel immunologisch is gedreven, nog steeds een alternatief mechanisme voor de pathogenese van AD. Hoewel de samenstelling van het microbioom van de huid kan verschillen tussen AD- en gezonde hondenhuid wordt de voorkeur voor de locatie van AD in de huid mogelijk niet veroorzaakt door het microbioom. Het microbioom in hondenhuid varieert tussen individuen en wordt beïnvloed door de lokale antimicrobiële behandeling. Het microbioom en AD beïnvloeden elkaar. De geïnfecteerde, inflammatoire status van AD huid kan expressie van zowel AMP als van overige cytokines induceren, die kan verschillen in samenhang met locatie en lokale behandeling. Zulke verstoringen in de balans van de

gehele huidstructuur kunnen leiden tot significante veranderingen in de lipiden van de huidbarrière, het microbioom van de huid, AMP en inflammatoire cytokines zoals aangetoond in deze thesis.

Samenvattend, de inhoud van deze thesis draagt bij aan verder inzicht in de immuun-pathogenese van atopische dermatitis in honden, van belang voor diagnose en behandeling van AD.

บทสรุปภาษาไทย

การเปลี่ยนแปลงในด้านโครงสร้างและหน้าที่การทำงานของชั้นผิวหนังทั้งระดับกายภาคถึงระดับโมเลกุล ตลอดจนการตอบสนองทางภูมิคุ้มกันที่เปลี่ยนแปลงในชั้นผิวหนังเป็นปัจจัยโน้มนำในการก่อให้เกิดความผิดปกติที่ผิวหนัง เช่น โรคภูมิแพ้ผิวหนังต่อสิ่งแวดล้อม (Atopic dermatitis, AD) ในปัจจุบันพยาธิกำเนิดของ AD ในผิวหนังสุนัขยังไม่เป็นที่ทราบแน่ชัดและประกอบไปด้วย 2 สมมติฐานหลัก คือ สมมติฐาน “Inside-Out” ซึ่งอธิบายถึงการเกิดโรค AD จากความผิดปกติของระบบภูมิคุ้มกันที่นำไปสู่ความเสียหายของผิวหนังชั้นนอกในแง่ของการสูญเสียคุณสมบัติในการซึมผ่านเพิ่มขึ้น สมมติฐานที่สองคือทฤษฎี “Outside-In” โดยเกิดจากความผิดปกติของชั้นผิวหนังที่สูญเสียคุณสมบัติในการป้องกันสิ่งแปลกปลอม เป็นผลทำให้สารก่อภูมิแพ้แทรกซึมเข้าไปในผิวหนังได้ง่ายขึ้น และไปกระตุ้นการทำงานของระบบภูมิคุ้มกันทั้งแบบชนิดไม่จำเพาะและจำเพาะที่มากเกิน ทั้งนี้ การสูญเสียสภาวะสมดุลของผิวหนังจากทั้งสองสมมุติฐานจะไปมีผลเปลี่ยนแปลงสมดุลขององค์ประกอบทางจุลชีววิทยาและจุลินทรีย์ที่อยู่บนผิวหนัง ทำให้เกิดการเปลี่ยนแปลงในการสร้างสารภูมิคุ้มกันแบบไม่จำเพาะ (antimicrobial peptide, AMP) และแบบจำเพาะ (cytokines) ตามมา สิ่งเหล่านี้จะทวีความรุนแรงของภาวะการสูญเสียการทำหน้าที่ของผิวหนังในการป้องกันและซ้ำเติมภาวะเสียสมดุลให้แย่ยิ่งขึ้น และอาจจะมีผลสืบเนื่องในการพัฒนาหรือเพิ่มความรุนแรงของโรค AD

ในบทที่ 3 แสดงถึงการเปลี่ยนแปลงของไขมันในผิวหนังชั้น stratum corneum ของสุนัขที่มีโรคภูมิแพ้ผิวหนังต่อสิ่งแวดล้อม หรือ AD เมื่อเปรียบเทียบกับสุนัขปกติ จากการตรวจโดยวิธี SAXD พบการเปลี่ยนแปลงในความยาวของ lamellar และการก่อตัวที่ลดลงของ Long periodicity phase (LPP) จากการตรวจโดยวิธี FTIR พบโครงสร้างการเรียงตัวของไขมันที่มีความหนาแน่นที่น้อยลง และจากการตรวจโดยวิธี HPTLC และ LC/MS พบระดับ Free fatty acids (FFA) และอัตราส่วนของ CER[NS] C44/C34 ที่ลดลง การเปลี่ยนแปลงทั้งหมดนี้มีความสัมพันธ์กับการสูญเสียหน้าที่ของชั้นผิวหนังในการเป็นเกราะป้องกันของร่างกาย อีกทั้งยังพบความรุนแรงในบริเวณที่มีรอยโรคร่วมด้วยได้เด่นชัดกว่าในบริเวณที่ไม่มีรอยโรคของผิวหนังสุนัขตัวเดียวกัน ความผิดปกติเหล่านี้แสดงให้เห็นถึงบทบาทสำคัญที่อาจเป็นปัจจัยหนึ่งในขบวนการก่อให้เกิดโรค AD โดยการสูญเสียการทำงานในแง่การป้องกันของผิวหนังต่อร่างกายส่งผลให้เกิดการเพิ่มการซึมผ่านและการสูญเสียน้ำของผิวหนัง และเอื้ออำนวยให้สารก่อภูมิแพ้แทรกซึมผ่านผิวหนัง อีกทั้งยังเพิ่มโอกาสในการติดเชื้อแทรกซ้อน จึงนำเป็นสาเหตุโน้มนำของการเกิดโรคภูมิแพ้ในผิวหนังสุนัขได้

นอกจากคุณสมบัติของไขมันในชั้นผิวหนัง ชีวนิเวศจุลชีพ (microbiome) บนผิวหนังอาจเป็นอีกปัจจัยหนึ่งที่มีบทบาทสำคัญในขบวนการก่อโรคของ AD จากการศึกษาเชิงสำรวจในบทที่ 4 ของวิทยานิพนธ์ฉบับนี้ พบว่าองค์ประกอบของชีวนิเวศจุลชีพบนผิวหนังในสุนัขแต่ละตัวนั้นมีความแตกต่างกัน และมีความแตกต่างกันเพียงเล็กน้อยบนตำแหน่งต่างๆ ของร่างกาย เมื่อเปรียบเทียบระหว่างสุนัขที่มีสุขภาพดีและมีปัญหา AD ไม่พบความแตกต่างขององค์ประกอบทางชีวนิเวศจุลชีพบนผิวหนังอย่างมีนัยสำคัญ แต่พบความหลากหลายของจุลินทรีย์บนผิวหนัง (ทั้งแบคทีเรีย 16s และเชื้อรา ITS) ในสุนัขที่ทีปัญหา AD มีแนวโน้มต่ำกว่าสุนัขที่มีสุขภาพดี การรักษาด้วยแชมพูอาบน้ำที่มีส่วนผสมของยาต้านจุลชีพ Malaseb® ซึ่งเป็นที่รู้จักอย่างแพร่หลายในความสามารถลดปริมาณ

จุลินทรีย์และความรุนแรงของอาการทางคลินิก ถูกนำมาใช้ในการศึกษาครั้งนี้และออกฤทธิ์ส่งผลให้มีการเปลี่ยนแปลงขององค์ประกอบของจุลินทรีย์อย่างมีนัยสำคัญในทุกช่วงเวลาและตำแหน่งของร่างกายสุนัขที่ทำการศึกษาโดยไม่คำนึงถึงสภาวะของ AD นอกจากนี้ ความชุกชุมสัมพัทธ์ของจุลินทรีย์บางชนิดบนผิวหนังมีความแตกต่างอย่างมีนัยสำคัญระหว่างสุนัขที่มีปัญหา AD และสุนัขสุขภาพดี การศึกษาหาความสัมพันธ์ระหว่างจุลินทรีย์ชนิดนั้นๆ และความสมบูรณ์ของผิวหนังควรได้รับการพิจารณาศึกษาต่อในกลุ่มประชากรที่มีขนาดใหญ่ขึ้น

จากการศึกษาในบทที่ 5 พบว่าระดับการสร้างสารภูมิคุ้มกันที่เกี่ยวข้องกับภาวะการเกิด AD แบบชนิดไม่จำเพาะ AMP (cBD1, cBD103, K9CATH) และจำเพาะประเภท Th2 cytokine (IL-4, IL-13) ประเภท Th1 cytokine (IFN-γ) ประเภท Pro-inflammatory cytokine (TNF-α) ประเภท Th17 cytokine (IL-17, IL-22) และประเภท Regulatory cytokine (IL-10) มีแนวโน้มที่จะมีความสัมพันธ์กับตำแหน่งต่างๆ ของร่างกายและความรุนแรงของโรค AD ในสุนัข การนำแชมพูที่มียาต้านจุลชีพ Malaseb® ชนิดเดียวกับที่ใช้ในการศึกษาบทที่ 4 มาศึกษาเพื่อดูการเปลี่ยนแปลงของระดับการสร้างสารภูมิคุ้มกันที่เกี่ยวข้องกับภาวะการเกิด AD ทั้งแบบชนิดไม่จำเพาะ AMP และจำเพาะ cytokine จากชิ้นเนื้อที่เก็บตรวจจากผิวหนังสุนัขที่มีสุขภาพดีและมีปัญหา AD อันเป็นผลมาจากฤทธิ์ต้านจุลชีพ พบว่าการใช้แชมพูหรือที่เรียกว่าการรักษาแบบเฉพาะที่มีผลต่อการลดความรุนแรงของโรคและมีแนวโน้มที่จะเปลี่ยนแปลงระดับ mRNA ของการสร้างสารภูมิคุ้มกันที่เกี่ยวข้องกับภาวะการเกิด AD ทั้งแบบชนิดไม่จำเพาะ AMP และจำเพาะ cytokine ผลการศึกษาดังกล่าวมีความสอดคล้องกับผลการวิจัยอื่นที่มีรายงานก่อนหน้า อย่างไรก็ตามการศึกษาในกลุ่มประชากรที่มีขนาดใหญ่ขึ้นควรได้รับการพิจารณาศึกษาต่อเพื่อยืนยันผลการศึกษาในครั้งนี้

การสูญเสียความสามารถของผิวหนังในการป้องกันสิ่งแปลกปลอมจากภายนอกเนื่องด้วยภาวะ AD ทำให้เพิ่มโอกาสการแทรกผ่านของสารก่อภูมิแพ้และ / หรือจุลินทรีย์เข้าสู่ผิวหนัง และส่งผลรบกวนต่อสภาวะสมดุลของผิวหนังรวมถึงอันตรกิริยาระหว่างเซลล์ผิวหนัง keratinocytes (KC) จุลชีพประจำถิ่น และการสร้าง AMP ในผิวหนัง การเลี้ยงเซลล์ผิวหนังสุนัขในหลอดทดลอง ในบทที่ 6 พบว่า เมื่อเซลล์ผิวหนังถูกกระตุ้นด้วยแบคทีเรียชนิด *S. pseudintermedius* หรือองค์ประกอบแบคทีเรีย (ทั้งชนิดแกรมบวกและลบ) ผ่าน Toll-like receptor (TLR) และชีวเคมี ligand อื่น ๆ จะสังเกตพบการเปลี่ยนแปลงของการสร้างสารภูมิคุ้มกันที่เกี่ยวข้องกับภาวะการเกิด AD ทั้งแบบชนิดไม่จำเพาะ AMP (cBD1, cBD103, K9CATH) และจำเพาะประเภท Pro-inflammatory cytokine (TNF-α, IL-8) การทดลองในครั้งนี้จำลองสภาวะซึ่งเป็นหนึ่งในหลายสาเหตุของการเสียสมดุลของผิวหนังขณะที่มีปัญหา AD

ผลการศึกษาที่อธิบายไว้ในวิทยานิพนธ์ฉบับนี้แสดงถึงการเปลี่ยนแปลงที่เกิดขึ้นที่ผิวหนังซึ่งอาจเป็นปัจจัยโน้มนำหรือผลกระทบจากภาวะ AD โดยองค์ประกอบและการเรียงตัวของไขมันในชั้นผิวหนังที่เปลี่ยนแปลงไปทั้งในบริเวณที่มีรอยโรคและไม่มีรอยโรคของผิวหนังสุนัขที่มีปัญหา AD สอดคล้องกับสมมติฐานการเกิดโรคแบบ "Outside-In" ที่ชี้ให้เห็นว่าปัจจัยโน้มนำของการเกิด AD เป็นผลมาจากความผิดปกติของชั้นผิวหนังที่สูญเสียความสามารถในการป้องกันสิ่งแปลกปลอมจากภายนอกได้อย่างเหมาะสม แตกต่างจากสมมติฐาน "Inside-Out"

ที่ได้ระบุว่าความผิดปกติของการตอบสนองทางระบบภูมิคุ้มกันเป็นสาเหตุโน้มนำให้เกิด AD และความผิดปกติอื่นๆ ตามมา องค์ประกอบของชีวนิเวศจุลชีพอาจแตกต่างกันระหว่างผิวหนังสุนัขที่มีสุขภาพดีและมีปัญหา AD แต่ไม่มีอิทธิพลต่อการก่อให้เกิดความผิดปกติในตำแหน่งที่พบปัญหารอยโรคของ AD ได้บ่อย องค์ประกอบของชีวนิเวศจุลชีพบนผิวหนังสุนัขมีแนวโน้มที่จะแตกต่างกันไปตามแต่ละตัวและสามารถเปลี่ยนแปลงภายใต้อิทธิพลของการรักษาด้วยยาต้านจุลชีพแบบเฉพาะที่ และมีความเป็นไปได้ที่ชีวนิเวศจุลชีพและการเกิด AD มีอิทธิพลต่อกัน สภาวะการติดเชื้อหรืออักเสบของผิวหนังที่มีปัญหา AD จะส่งผลให้เกิดการกระตุ้นการสร้างสารภูมิคุ้มกันในผิวหนังทั้งแบบไม่จำเพาะ AMP และจำเพาะ cytokine โดยระดับของสารที่ตรวจพบอาจแตกต่างกันอันเนื่องมาจากความแตกต่างของตำแหน่งบนร่างกายและฤทธิ์ของการรักษาแบบเฉพาะที่ โดยสรุปคือ การสูญเสียสภาวะสมดุลทั้งในแง่ของโครงสร้างและหน้าที่ในชั้นผิวหนังนำไปสู่การเปลี่ยนแปลงอย่างมีนัยสำคัญของไขมันในชั้นผิวหนัง ชีวนิเวศจุลชีพที่ผิวหนัง สารภูมิคุ้มกันในผิวหนังทั้งแบบไม่จำเพาะ AMP และจำเพาะ cytokine ดังที่อธิบายไว้ในวิทยานิพนธ์ฉบับนี้

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Chapter 9

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Curriculum Vitae

List of publications

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Curriculum Vitae

Suttiwee Chermprapai was born on the 21st of April 1983 in Bangkok, Thailand. She graduated her Doctoral of Veterinary Medicine (DVM) with the first honor from Kasetsart University (KU), Thailand in 2007. After graduation, she worked as a veterinary practitioner at the Kasetsart University Veterinary Teaching Hospital (KUVTH), Bangkhen Campus. In 2008, she received a new position as a university lecturer in the Department of Companion Animal Clinical Sciences, the Faculty of Veterinary Medicine, KU, and simultaneously a chief of the Veterinary Dermatology unit of KUVTH. In 2012, she had an opportunity to get the scholarship from the employed faculty to pursue a PhD study at Utrecht University, the Netherlands. She started working in the Department of Infectious diseases and Immunology, and the Department of Clinical Sciences of Companion Animals in October 2012. After her thesis defense, she will return to work at the Faculty of Veterinary Medicine, Kasetsart University.

List of Publications

Chermprapai, S., Broere, F., Gooris, G., Schlotter, Y.M., Rutten, V.P.M.G., Bouwstra, J.A., 2018. Altered lipid properties of the stratum corneum in Canine Atopic Dermatitis. Biochim. Biophys. Acta 1860, 526-533.

Chermprapai, S., Broere, F., Schlotter, Y.M., Veldhuizen, E.J.A., Rutten, V.P.M.G., 2018. A canine keratinocyte cell line expresses antimicrobial peptide and cytokine genes upon stimulation with bacteria, microbial ligands and recombinant cytokines. Veterinary Immunology and Immunopathology 206, 35-40.

Chermprapai, S., Ederveen, T.H.A., Broere, F., Broens, E.M., Schlotter, Y.M., van Schalkwijk, S., Boekhorst, J., van Hijum, S.A.F.T., Rutten, V.P.M.G., 2019. The bacterial and fungal microbiome of the skin of healthy dogs and dogs with atopic dermatitis and the impact of topical antimicrobial therapy, an exploratory study. Veterinary Microbiology 229, 90-99.

Poster and Oral Presentation

Chermprapai, S., Broere, F., Schlotter, Y.M., Veldhuizen, E.J.A., Rutten, V.P.M.G., 2016. The canine epidermal keratinocyte cell line (MSCEK) express the Beta defensins cBD1 and cBD103 upon stimulation with selected bacterial ligands and recombinant cytokines. Proceedings of the Eighth World Congress of Veterinary Dermatology in Bordeaux, France.

Chermprapai, S., Broere, F., Schlotter, Y.M., Veldhuizen, E.J.A., Rutten, V.P.M.G., 2015. Expression of the Beta defensins cBD 1 and cBD103 by the canine epidermal keratinocyte cell line (MSCEK) stimulated with selected bacterial ligands and recombinant cytokines. Proceedings of the first International Symposium on Allergy meets Infection, Lübeck, Germany.

Kunakornsawat, S., Imsilp, K., **Chermprapai, S.**, Poapolathep, S., Netramai, S., 2012. Pegylated liposomal doxorubicin-induced palmar-plantar erythrodysthesia (hand-foot syndrome) in a nasal carcinoma dog. Proceeding of International Conference on Veterinary Sciences IICB, APHIS, FAO joint symposiums "ASIA WEB FOR WORLD FOOD SECURITY", Bangkok, Thailand.

Vipabusabakorn P., Intachat C., Chottikamporn J., Duangmontri K., Ngamcharoen J., Vichukit K., **Chermprapai S.**, Sirinarumitr T., Sirinarumitr K., 2018. Cryptococcal prostatitis in a dog. Proceeding of the 11th VPAT Regional Veterinary Congress 2018 (VRVC 2018), Nonthaburi, Thailand.