RESEARCH ARTICLE

Birds, feather-degrading bacteria and preen glands: the antimicrobial activity of preen gland secretions from turkeys (*Meleagris gallopavo*) is amplified by keratinase

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One sentence summary: Keratinase activates the antimicrobial potential of preen gland secretions.

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ABSTRACT

The function of uropygial glands (preen glands) has been subject to controversial debates. In this study, we evaluated the antimicrobial potential of preen gland secretions of turkeys (*Meleagris gallopavo*) against 18 microbial strains by means of diffusion tests, broth microdilutions, checkerboard assays and time-kill curves. Furthermore, we tested the hypothesis that lipids exert direct antimicrobial effects on pathogens. Moreover, we checked for mutualistic relationships between the preen gland bacterium *Corynebacterium uropygiale* with its hosts. We found that preen gland secretions significantly inhibited the growth of a broad spectrum of bacteria and fungi, particularly when combined with keratinase. Combinations effectively killed multidrug resistant microorganisms in a strongly synergistic manner. Since feather-degrading microorganisms (FDM) express keratinase and thereby disrupt the integrity of the plumage, our data suggests that preen gland secretions of turkeys are specifically activated in the presence of FDM, and specifically eliminate FDM from feathers. However, antimicrobial effects did not originate from lipids, but were mediated by highly polar compounds which might be antimicrobial peptides (AMPs). Finally, *C. uropygiale* is apparently not involved in the antimicrobial activity of preen gland secretions of turkeys. In conclusion, our results suggest that turkeys can antagonize FDM by amplifying the antimicrobial properties of their preen gland secretions.

Keywords: uropygial gland; drug interaction; symbiosis; checkerboard microdilution; AMP hypothesis; lipid hypothesis

INTRODUCTION

Birds are exposed to numerous threats. Many birds live in surroundings where potentially detrimental bacteria, as well as harmful fungi are highly abundant (Walther 2003; Haribal et al. 2005). However, birds have developed effective defense strategies whose mechanisms have not, to date, been fully resolved (Moreno-Rueda 2017). For example, maintenance behaviors such...
as preening, scratching, bathing, dusting, sunning and shaking could have been arisen in response to the pronounced parasite pressure (Walther 2003). Taking into account that preening consumes between 5 to 30% of a bird’s daily time budget (Haribal et al. 2005) and significantly increases the metabolic rate (Croll and McLaren 1993), the preen gland and its secretion are likely to play a critical role regarding bird fitness. Among other things, preen glands and preening have been associated with antimicrobial defense in birds (e.g. Baxter and Trotter 1969; Pugh and Evans 1970; Bandyopadhyay and Bhattacharyya 1996; Jacob, Eigener and Hoppe 1997; Law-Brown 2001; Soini et al. 2007; Soler et al. 2008).

Preen gland and preen gland secretion

The preen gland, also called uropygial gland or oil gland, is a holocrine, mostly bilobate integumentary sebaceous gland located dorsally between the fourth caudal vertebrate and the pygostyle. It produces a waxy secretion (preen gland secretion, preen oil or preen wax) which is channeled through the papilla to body surface (Jacob and Ziswiler 1982; Salibian and Montalti 2009) and mainly consists of long-chained esters of fatty acids and fatty alcohols (Jacob and Ziswiler 1982; Shawkey, Pillai and Hill 2003; Thomas et al. 2010).

During preening, preen oil is applied to the feathers with the beak by spreading the secretion all over the plumage (Haribal et al. 2005). There has been much controversy about the functional importance of preen gland secretions. While there is consensus that they contribute to the suppleness and water repellent properties of the feather coat (Moreno-Rueda 2017), other functions such as thermal insulation (Bakken et al. 2006), endocrine regulation (Bhattacharyya and Chowdhury 1978), olfactory conspicuousness, kin recognition (Amo et al. 2012; Whittaker et al. 2009), predator avoidance (Reneerkens, Piersma and Sinninghe Damsté 2002) and intraspecific signaling (Leclaire et al. 2014) need more support. Also in terms of defense against microbes, the roles of the preen gland and preen gland secretions are not fully understood (Elder 1954; Haribal et al. 2005; Salibian and Montalti 2009; Moreno-Rueda 2017).

Antimicrobial activity of preen gland secretions

Information on the potential antimicrobial activity of preen gland secretions is still rare and inconclusive (Shawkey, Pillai and Hill 2003; Moreno-Rueda 2017). While the secretions of several bird species have shown to exhibit antimicrobial activity, preen gland secretions of other or even the same species (e.g. chicken) were inactive (Bandyopadhyay and Bhattacharyya 1996; Wellman-Labadie et al. 2010). Some authors suggested that preen gland secretions prevent structural damage of feathers by inhibiting the growth of feather-degrading microorganisms (FDM) (e.g. Moyer, Rock and Clayton 2003; Shawkey, Pillai and Hill 2003; Reneerkens et al. 2008). However, this hypothesis has neither been confirmed, nor been rejected and corresponding results are inconsistent (Moreno-Rueda 2017; Verea et al. 2017). Furthermore, the active components in preen gland secretions have mostly not been identified.

A mutualistic relationship between birds and bacteria has been proposed for Enterococcus faecalis and Enterococcus phoeniculicola in the preen glands of hoopes (Upupa epops) and green woodhoopoes (Phoeniculus purpureus), respectively, where bacteria produce antimicrobial metabolites of known structure and thereby confer antimicrobial activity to the preen gland secretions of the host species (Law-Brown 2001; Soler et al. 2008). However, similar bacteria have not been reported from other birds. Besides, lipids of preen gland secretion may directly reduce microbial growth (Baxter and Trotter 1969; Pugh and Evans 1970; Bandyopadhyay and Bhattacharyya 1996; Jacob, Eigener and Hoppe 1997; Soini et al. 2007).

The present work is a follow-up study of antimicrobial screenings of preen gland secretions of different bird species including turkeys (Meleagris gallopavo) (M.S. Braun and M. Wink, unpublished). Turkey preen gland secretions inhibited bacterial proliferation and thus are promising candidates for further investigation. Turkey secretions differ from the preen oils of most other species in that their wax esters exclusively consist of fatty acids esterified with fatty 2,3-diol (upropygole). Since none of the diol-free preen gland secretions of the 25 species tested in our preliminary screenings exerted consistent antimicrobial effects, we hypothesized that these 2,3-diol-containing diester waxes are responsible for the antimicrobial actions of the preen gland secretions of turkeys (‘lipid hypothesis’).

In this study, we quantified the antimicrobial activity of preen gland secretions of turkeys against microorganisms of seven different genera of fungi, Gram-positive and Gram-negative bacteria, including multidrug resistant clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA) from human patients. We further simulated the interactions between preen gland secretions and FDM in drug interaction assays by adding keratinase, an enzyme released by FDM. In order to test the lipid hypothesis, we extracted the hydrophobic components of preen gland secretions of turkeys, chemically confirmed the presence of wax diesters and tested the extract for its antimicrobial potential against a set of microbial indicator strains. Apart from this, we checked for a possible mutualistic relationship between turkeys and Corynebacterium upropygale, an actinobacterium which has recently been discovered in the preen gland of healthy turkeys (Braun et al. 2016).

MATERIALS AND METHODS

Microorganisms and culture conditions

Seven different genera, which have been found to be associated with birds, were utilized as indicator strains in our antimicrobial tests. Namely, we used the Gram-positives Escherichia coli XL1-Blue MRF’ (Stratagene, San Diego, CA, USA) (Berrang, Buhr and Cason 2000) and Pseudomonas montelii (feather isolate) (Shawkey, Pillai and Hill 2003), the Gram-positives Kocuria rhizophila (feather isolate) (Shawkey, Pillai and Hill 2003), Staphylococcus auricularis ATCC 33753, Staphylococcus aureus ATCC 25923 (Shawkey, Pillai and Hill 2003), Bacillus megaterium ATCC 14580 (Shawkey, Pillai and Hill 2003), the yeast Candida lactiscondens (ATCC 60137) (Kuttin, Beemer and Meroz 1976) and the filamentous fungus Aspergillus niger (soil isolate) (Hubalek 1974). All microorganisms, except for the isolates from environment, were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Furthermore, MRSA NCTC 10442 and nine clinical isolates of MRSA from patients hospitalized in 2016 at the Heidelberg University Hospital were provided by the Department of Infectious Diseases, Medical Microbiology and Hygiene, Heidelberg University, and were used in checkerboard assays (see Supplementary Table S1 for antibiograms). Bacteria were maintained on Columbia Agar
supplemented with 5% sheep blood, while fungi were grown on Sabouraud Dextrose Agar (SDA) at their optimum temperatures.

**Sampling and preparation of preen glands**

Preen glands were excised from freshly slaughtered animals in turkey farms and stored at -40°C until further processing. The samples were acquired at different times from two local breeders in Southern Germany (GeflügelSpezialitäten Annerose Ziegler GbA, Bammental and Putenhof Ullrich, Helmstadt-Bargen) (Table 1).

Half-frozen preen glands were superficially disinfected with 70% ethanol and the secretions were removed without contamination by the surrounding tissue using sterile scalpels and forceps. The method of Braun et al. (2016) was followed to recover C. uropygiale from the preen gland secretions from turkeys from each location and the Heidelberg Zoo, Heidelberg, Germany. The isolates described in Braun et al. (2016) were used for the mutualism test series (see 'Tests for mutualism'). The remaining preen gland secretions were used for chemical analyses and antimicrobial susceptibility tests. All samples were processed under the same conditions.

**Selection of representative samples**

Batches mentioned in Table 1 were screened for their antimicrobial activity alone and with the addition of keratinase in well diffusion tests against S. auricularis and E. coli (see 'Well diffusion tests'). Based on the zone of inhibition, batches were categorized as highly active and weakly active (Table 1). As keratinase we used proteinase K which is a serine protease with a broad spectrum of substrates that has been originally isolated from Engyodontium album (formerly known as Tritirachium album). The name of this protease originates from its ability to digest keratin (Betzel, Pal and Saenger 1988) and it thereby can be used as a model protease simulating the action of keratinases being secreted by FDM on feathers.

Active batches showing strong interaction with proteinase K originated from both breeders (Ziegler 1 and Ullrich 1) (Table 1). The batch ‘Ullrich 1’ contained secretions of male and female birds and both exerted strong antimicrobial effects on the bacterial indicator strains. The level of activity was identical for both sexes and breeders. Vice versa, the remaining batches (Ziegler 2 and Ullrich 1), originating from the same two breeders and containing secretions of both sexes, exhibited only very weak antimicrobial activities (Table 1). Hence, for the subsequent lipid analyses and the antimicrobial experiments (diffusion tests, checkerboard microdilution and time-kill curves) against a larger number of microbes and for chemical analyses, the preen gland secretions of the batches ‘Ziegler 1’ (hereinafter referred to as highly active sample) were merged, as were the preen gland secretions of the batch ‘Ziegler 2’ (referred to as weakly active sample), and taken as representative samples.

**Fractionation of preen gland secretions and chemical analysis**

In order to test if lipids of avian preen oils inhibit microbial growth, the lipophilic fraction was extracted from the representative samples. Since diester waxes were identified as candidates responsible for the antimicrobial activities of turkey preen gland secretions, we also confirmed the presence of these waxes by analyzing aliquots of the extracts by gas liquid chromatography (GLC) and GLC/mass spectrometry (MS). The remaining substance was used for the antimicrobial tests.

**Chemical extractions of preen gland secretions**

Lipid extractions of aliquots of the representative samples were based on the method of Bligh and Dyer (1959) which has been widely used for preen oils before (Haashtil et al. 1964; Wertz et al. 1985; Jacob, Eigener and Hoppe 1997; Jacob et al. 2014). Briefly, tissue-free aliquots of the samples were extracted with chloroform/methanol. Water was added and the organic phase was dried over anhydrous sodium sulfate. Aliquots of the extracts were used for the detection of diester waxes (see paragraph below) and in antimicrobial tests. Besides, the polar fraction of turkeys’ preen gland secretions was obtained by extracting the samples three times with water. The extracts were freeze-dried, weighed and subjected to antimicrobial tests.

**Isolation of wax esters and derivatization**

Lipid extracts of the representative samples were applied on preparative thin-layer chromatography (TLC) plates (Kieselgel 60G F254, Merck, Darmstadt, Germany) and developed in chloroform. Plates were evaluated under UV (254 nm) and the band at Rf 0.52 was scraped off and eluted in chloroform (Jacob and Grimmer 1970). Solvent was removed using a rotary evaporator (JKA RV8, GmbH & Co. KG, Staufen, Germany) and fractions of the isolated compounds were subjected to GLC and transesterification in methanol hydrochloric acid. For transesterification, 0.2 mL toluol, 1.5 mL methanol and 0.3 mL 8% hydrochloric acid were added and heated overnight at 45°C in stoppered glass tubes (Ichihara and Fukubayashi 2010). One volume of water was added and fatty acid methyl esters (FAMEs) were extracted three times with cyclohexane. The extract was dried over anhydrous sodium sulfate, filtered and dried in a rotary evaporator. Silylation was performed by the addition of N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA, Sigma Aldrich Corp., Steinheim, Germany) and pyridine for 20 min at 60°C (Rijpstra et al. 2007). The samples were subsequently used for GLC and GLC/MS analysis.

**Gas liquid chromatography (GLC)**

Gas liquid chromatography of the wax esters was conducted in order to quantify the preen gland components and was performed on a Shimadzu GC-2010 Plus gas chromatograph equipped with a flame ionization detector (Nakagyo-ku, Kyôto, Japan) and a ZB-5 capillary column (30 m × 0.25 mm ID × 0.25 μm) (Phenomenex, Aschaffenburg, Germany). Helium was used as carrier gas, the injector temperature was set at 250°C and detector temperature at 320°C. Oven temperature was programmed according to Table 3. GCsolution v. 2.41.00 SU1 (Nakagyo-ku, Kyôto, Japan) was used for data acquisition and data analysis including semi-quantification based on peak areas.

**Gas liquid chromatography/mass spectrometry (GLC/MS)**

The presence of wax esters was confirmed by means of the fragmentation patterns of the transesterified and silylated lipid derivatization products. GLC/MS was conducted using a HP 5890 Series II gas chromatograph (Hewlett Packard Inc., Böblingen, Germany) equipped with a ZB-5 column (30 m × 0.25 mm ID, film thickness 0.25 μm) (Phenomenex, Aschaffenburg, Germany). Oven temperature was programmed as mentioned before in Table 3. The columns head pressure was 100 kPa. Helium served as carrier gas and injector temperature was set at 250°C in split
Antimicrobial activity of preen gland secretions and their extracts

Well diffusion tests

Kirby–Bauer diffusion tests using the highly active and weakly active samples as well as extracts thereof were conducted with minor modifications according to Hudzicki (2009). Vegetative cells or spores were harvested, adjusted to 0.5 McFarland units and spread on Müller–Hinton Agar (MHA) and SDA plates for bacteria and fungi, respectively. 6 mm wells were punched out and each well was filled with 4 μL preen gland secretion or lipid extract stabilized in 2% Cremophor RH 40 (Caesar & Loretz, Hilden, Germany). Water extracts were tested at a concentration of 0.25 mg per well. Plates were incubated at 37°C for 24 h, except for C. lactiscondensi, which was kept at 24°C for 48 h. Antimicrobial activity was assessed by means of zones of inhibition following incubation. The effect of keratinase was tested by adding 12.5 U proteinase K (Sigma Aldrich Corp., Steinheim, Germany) to the respective wells. Ampicillin (AppliChem, Darmstadt, Germany), ciprofloxacin (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and nystatin (Sigma Aldrich Corp., Steinheim, Germany) were used as positive controls. 2% Cremophor RH 40 in combination with proteinase K served as a negative control. All tests were run in triplicate. Highly active samples were subjected to broth microdilution, checkerboard and time-kill assays.

Broth microdilutions

Highly active representative samples were stabilized as mentioned before and tested against Staphylococcus auricularis, S. aureus and ten strains of MRSA by means of broth microdilution according to the method of CLSI (2012). Briefly, 96-well plates were loaded with Müller–Hinton Broth (MHB) and preen gland secretion, and two-fold dilution series were conducted in MHB. Bacteria were added at a cell density of approximately 5 × 10^5 cfu/mL and incubated at 35°C for 18 h. The minimum inhibitory concentration (MIC) was taken as the minimum sample concentration without any signs of visible growth. Cremophor RH 40 was used as a negative control, while vancomycin (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) served as a positive control. All tests were conducted in duplicate per plate and performed three times.

Checkerboard assays

In order to quantify interactions between preen gland secretions and keratinase, S. aureus, S. auricularis, MRSA NCTC 10442 and clinical isolates of MRSA were further investigated using checkerboard microdilutions following a slightly modified method of Verma (2007). The maximum concentrations of preen gland secretion and proteinase K were 2048 μg/mL and 64 U/mL, respectively. All tests were carried out three times. Bacterial growth was quantified using a Tecan NanoQuant infinite M200 Pro plate reader (Tecan Group AG, Männedorf, Switzerland), normalized and plotted in 3D response surface graphs using OriginPro 2017 (OriginLab Corp., Northampton, MA, USA). Drug Interactions were assessed based on two competing zero-interaction theories, namely Loewe additivity and Bliss independence. Besides, drug reduction indices (DRIs) were calculated. Criteria used for assessing drug interactions are denoted in Table 2.

Time-kill assays

Time-kill curves were the fourth approach in our antimicrobial susceptibility repertoire. They were used to characterize the synergistic interactions between preen gland secretions and keratinase seen in well diffusion tests and checkerboard microdilutions. Additionally, they served to eradicate the shortcomings of microdilutions and checkerboard assays in a way that time-kill curves allow the continuous monitoring of bacteriostatic and bactericidal drug kinetics. The analyses were performed with MRSA NCTC 10442 according to Verma (2007). Individual drugs as well as combinations thereof were prepared as follows: (1) high concentration of preen gland secretion (2048 μg/mL), (2) high concentration of keratinase (64 U/mL), (3) high concentration of preen gland secretion and low concentration of keratinase (2048 μg/mL and 8 U/mL), (4) low concentration of preen gland secretion and high concentration of keratinase (512 μg/mL and 64 U/mL) and (5) high concentration of preen gland secretion and high concentration of keratinase (2048 μg/mL and 64 U/mL). The samples were incubated in glass tubes under agitation at 35°C. The indicator strain was adjusted to approximately 5 × 10^5 cfu/mL. 20 μL of bacterial suspension were withdrawn at the beginning of the incubation, after 1 h, 2 h, 3 h, 6 h, 12 h, 24 h and 30 h, diluted in MHB, streaked on agar plates and incubated until visible growth was obtained. Colonies were counted and the number of viable cells was plotted on a logarithmic scale. All tests were carried out three times.

Tests for mutualism

Microorganisms and cultivation

The lipophilic strains Iso10T (DSM 46817T) and C4 of Corynebacterium ureygialae were maintained on Luria–Bertani agar (LB) supplemented with 0.3% Tween-80 (LBT). The mutualistic bacteria Enterococcus faecalis MRR-10 from the preen glands of hoopes

Table 1. Overview of samples from turkeys used in this study and corresponding antimicrobial activities.

<table>
<thead>
<tr>
<th>Batch</th>
<th>No. of preen glands</th>
<th>Time of sampling</th>
<th>Antimicrobial activity</th>
<th>Sex of birds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ziegler 1*</td>
<td>30</td>
<td>January 2013</td>
<td>++</td>
<td>♂</td>
</tr>
<tr>
<td>Ziegler 2*</td>
<td>30</td>
<td>February 2016</td>
<td>+</td>
<td>♂</td>
</tr>
<tr>
<td>Ullrich 1</td>
<td>20</td>
<td>September 2015</td>
<td>++</td>
<td>♂ + ♀</td>
</tr>
<tr>
<td>Ullrich 2</td>
<td>20</td>
<td>July 2017</td>
<td>+</td>
<td>♂ + ♀</td>
</tr>
</tbody>
</table>

*zones of inhibition in well diffusion tests after addition of keratinase <10 mm.

**zones of inhibition in well diffusion tests after addition of keratinase ≥15 mm.

*used as representative batches in in-depth antimicrobial and chemical characterizations.
Protease expression was determined to test if protease activity of bacteria indicated strain. The cultures were transferred to 35°C for 4 days. The cultures were subsequently overlaid with the indicator strain C. auricularis ATCC 33753 in liquefied semi-solid MHA. After solidification at room temperature, the cultures were transferred to 35°C and incubated overnight at ambient atmosphere.

Overlay diffusion tests
Overlay diffusion tests were conducted to check for the production of antimicrobial metabolites by C. uropygiale. Two strains of C. uropygiale were grown at 35°C on MHA plus 0.3% Tween-80 (MHTA) and on preen gland secretion agar (1.5% (w/v) preen gland secretion, 1% gum arabic, 1.5% bacto agar, autoclaved for 15 min at 121°C) for 4 days. The cultures were subsequently overlaid with the indicator strain S. auricularis ATCC 33753 in liquefied semi-solid MHA. After solidification at room temperature, the cultures were transferred to 35°C and incubated overnight at ambient atmosphere.

Test for synergistic interactions between C. uropygiale and keratinase
In order to test if C. uropygiale produces metabolites that can be activated by keratinase, cell material of Iso10 and C4 was taken from agar plates, suspended in MHB, tested alone and in combination with proteinase K in well diffusion tests according to the method described above. S. auricularis ATCC 33753 was used as indicator strain.

Protease activity of bacteria
Protease expression was determined to test if C. uropygiale produces proteases, which could interact with preen gland secretions of turkeys. Two independent assays were carried out, namely the agar plate method and the azocasein test, and are described in the following paragraphs.

Agar plate assays
Protease expression of the preen gland bacteria from turkeys was assessed on Skim Milk Agar (SMA 10% (w/v) skim milk powder, 1.5% bacto agar, pH 7.0, autoclaved for 5 min). SMA was spot-inoculated with C. uropygiale strains Iso10 and C4 and incubated for 5 days at 35°C. Protease activities were measured as the diameters of the halos around bacterial growth subsequent to incubation.

Azocasein tests
Azocasein is an azo-dye and substrate for proteases. Proteolytic activity results in the formation of azopeptides with high UV absorption, that can be used to quantify protease activity. Azocasein tests were conducted with modifications according to Bergmeyer (1970).

Briefly, 1 mg/mL azocasein (Sigma Aldrich Corp., Steinheim, Germany) was dissolved in 0.1 M potassium phosphate buffer (pH 7.0). It was incubated with bacterial suspensions of C. uropygiale and E. faecalis MRR-10 for 1 h at 37°C. Trichloroacetic acid was added to stop the reaction. After centrifugation, 1 M potassium hydroxide was added to the supernatant and the absorbance of the released azo dye was measured at 436 nm. Blanks were processed in parallel under the same conditions, but azocasein and trichloroacetic acid were added simultaneously and centrifuged without further incubation. One unit of protease activity was defined as the amount of bacterial suspension required to produce an increase in absorbance of 0.01 under the described conditions.

RESULTS

Analytics
Gas chromatograms of the undervatized wax esters showed a pattern typical for diesters in preen gland secretions (Fig. 1). Peaks of both representative samples show identical retention times. When analyzing the transesterified and silylated samples both retention times and peak areas matched. As expected, the peaks in the chromatogram of the undervatized samples gave rise to a complex pattern of FAMEs and long-chained diols (Fig. 1). Diagnostic ions in the fragmentation pattern of GLC/MS analyses demonstrated that preen oil diesters in turkey secretions consist of a homologous series of fatty acids ranging from C10 to C31 and a homologous series long-chained C18 to C24 2,3-alkanediols. Main FAME was C16 (~10% of the total peak area) and the most abundant diol was a C22 compound (~15% of the peak area) (Table 4). Representative mass spectra of FAMEs and trimethylsilyl ethers are illustrated in Fig. 2.

Antimicrobial activity of preen gland secretions
Well diffusion tests: antimicrobial activity against bacteria and fungi
The investigation of the spectrum of antimicrobial activities of preen gland secretions showed that the highly active sample exerted inhibitory effects on all the microorganisms tested. This was particularly true when keratinase was added. As expected, the sample classified as weakly active in previous tests, either completely lacked antimicrobial properties or was only slightly active (Table 5).

The lipid extracts of the highly active and weakly active samples were unable to inhibit microbial proliferation when applied alone. Supplementation with proteinase K did not influence the antimicrobial potency of these samples. Thus, the activity of preen gland secretions could not be attributed to lipids, but it
could be recovered in the water phase. When combined with proteinase K, the antimicrobial potential of the water extract of the weakly active sample slightly increased, whereas the activity of the highly active sample was drastically augmented upon keratinase supplementation (Table 5; Fig. S1, Supporting Information).

**Broth microdilutions and checkerboard assays**

Broth microdilutions demonstrated that the MICs of the single substances proteinase K and preen gland secretions were above 64 U/mL and 2048 μg/mL, respectively. Thus, for the analysis of the checkerboard assays, these off-scale MICs were converted into the next highest twofold concentrations (Meletiadis et al. 2005; Li et al. 2008).
Table 4. Overview of fatty acid and diol moieties of preen gland secretion wax esters after derivatization, their diagnostic ions and peak areas. Values in brackets correspond to the percentages of total lipids.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Compound</th>
<th>RT (min)</th>
<th>Highly active sample</th>
<th>Weakly active sample</th>
<th>Diagnostic ions (m/z)</th>
<th>M*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C10 FAME</td>
<td>13.7</td>
<td>1.3 (0.9)</td>
<td>2.2 (1.6)</td>
<td>74^1, 87, 101, 115, 129, [M-29]^+, [M-31]^+, [M-43]^+</td>
<td>186</td>
</tr>
<tr>
<td>2</td>
<td>C11 FAME</td>
<td>16.2</td>
<td>2.0 (1.5)</td>
<td>2.3 (1.6)</td>
<td>74^1, 87, 101, 115, 129, [M-29]^+, [M-31]^+, [M-43]^+</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>C12 FAME</td>
<td>18.9</td>
<td>4.3 (3.1)</td>
<td>4.1 (2.9)</td>
<td>74^1, 87, 101, 115, 129, [M-29]^+, [M-31]^+, [M-43]^+</td>
<td>214</td>
</tr>
<tr>
<td>4</td>
<td>C13 FAME</td>
<td>21.6</td>
<td>1.9 (1.4)</td>
<td>1.8 (1.3)</td>
<td>74^1, 87, 101, 115, 129, [M-29]^+, [M-31]^+, [M-43]^+</td>
<td>228</td>
</tr>
<tr>
<td>6</td>
<td>C15 FAME</td>
<td>27.0</td>
<td>1.6 (1.2)</td>
<td>1.3 (0.9)</td>
<td>74^1, 87, 101, 115, 129, [M-29]^+, [M-31]^+, [M-43]^+</td>
<td>256</td>
</tr>
<tr>
<td>7</td>
<td>C16 FAME</td>
<td>29.7</td>
<td>4.6 (3.4)</td>
<td>4.7 (3.3)</td>
<td>74^1, 87, 101, 115, 129, [M-29]^+, [M-31]^+, [M-43]^+</td>
<td>270</td>
</tr>
<tr>
<td>8</td>
<td>Unidentified</td>
<td>30.6</td>
<td>1.6 (1.2)</td>
<td>0.9 (0.6)</td>
<td>75, 103, 111, 299</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>C18 FAME</td>
<td>33.0</td>
<td>2.1 (1.5)</td>
<td>1.5 (1.1)</td>
<td>75^1, 75^2, 117, 132^1, 145, [M-15]^+</td>
<td>328</td>
</tr>
<tr>
<td>12</td>
<td>Unidentified</td>
<td>35.4</td>
<td>12 (9.9)</td>
<td>9.9 (6.6)</td>
<td>75^1, 75^2, 103, 111, 327</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>C21 FAME</td>
<td>37.3</td>
<td>0.6 (0.4)</td>
<td>0.6 (0.4)</td>
<td>75^1, 75^2, 117, 132^1, 145, [M-15]^+</td>
<td>356</td>
</tr>
<tr>
<td>15</td>
<td>C22 diol TMS</td>
<td>38.4</td>
<td>2.0 (1.5)</td>
<td>1.7 (1.2)</td>
<td>75^1, 75^2, 117, 147^3, 149^3, [M-117]^+, [M-15]^+</td>
<td>430</td>
</tr>
<tr>
<td>16</td>
<td>C23 FAME</td>
<td>39.2</td>
<td>1.6 (1.2)</td>
<td>1.9 (1.3)</td>
<td>74^1, 87, 101, 115, 129, [M-29]^+, [M-31]^+, [M-43]^+, [M-45]^+</td>
<td>326</td>
</tr>
<tr>
<td>17</td>
<td>C24 FAME</td>
<td>40.5</td>
<td>1.0 (0.7)</td>
<td>0.9 (0.6)</td>
<td>75^1, 75^2, 117, 147^3, 149^3, [M-117]^+, [M-15]^+</td>
<td>444</td>
</tr>
<tr>
<td>18</td>
<td>C25 FAME</td>
<td>40.8</td>
<td>0.2 (0.1)</td>
<td>0.1 (0.1)</td>
<td>74^1, 87, 101, 115, 129, [M-29]^+, [M-31]^+, [M-43]^+, [M-45]^+</td>
<td>340</td>
</tr>
<tr>
<td>19</td>
<td>C26 diol TMS</td>
<td>42.6</td>
<td>8.7 (6.4)</td>
<td>8.7 (6.2)</td>
<td>75^1, 75^2, 117, 147^3, 149^3, [M-117]^+, [M-15]^+</td>
<td>458</td>
</tr>
<tr>
<td>20</td>
<td>C27 diol TMS</td>
<td>44.6</td>
<td>10.6 (7.7)</td>
<td>11.3 (8)</td>
<td>75^1, 75^2, 117, 147^3, 149^3, [M-117]^+, [M-15]^+</td>
<td>472</td>
</tr>
<tr>
<td>21</td>
<td>C28 diol TMS</td>
<td>46.6</td>
<td>15.2 (11.1)</td>
<td>17 (12.1)</td>
<td>75^1, 75^2, 117, 147^3, 149^3, [M-117]^+, [M-15]^+</td>
<td>486</td>
</tr>
<tr>
<td>22</td>
<td>C29 diol TMS</td>
<td>48.3</td>
<td>5.7 (4.2)</td>
<td>6.8 (4.8)</td>
<td>75^1, 75^2, 117, 147^3, 149^3, [M-117]^+, [M-15]^+</td>
<td>500</td>
</tr>
<tr>
<td>23</td>
<td>C30 diol TMS</td>
<td>50.0</td>
<td>0.5 (0.4)</td>
<td>0.5 (0.4)</td>
<td>75^1, 75^2, 117, 147^3, 149^3, [M-117]^+, [M-15]^+</td>
<td>514</td>
</tr>
</tbody>
</table>

1McLafferty rearrangement ion.
2Characteristic for silyl ethers (Sawaya 1972).
3Expected for molecules with polysilyl ether functions (Sawaya 1972).
FAME: fatty acid methyl ester.
TMS: trimethylsilyl derivative.

Strong synergistic interactions were found for all strains tested. All FICI values calculated for Loewe additivity based analysis were far below the cutoff for synergism and isobolograms showed concave isoboles well below the line of additivity (Fig. 3). Besides, the results based on Bliss independence theory further corroborated these results as interaction surfaces suggested synergistic interactions for all eleven strains used in checkerboard assays (SSI was always >200%, Table 6). Antagonism occurred for a few combinations of preen gland secretion and keratinase, but in most cases, was not significant. Correspondingly, the absolute values of SSI of these combinations were <100 for all strains and can be thus considered weak (Fig. S2, Supporting Information).

Response surface plots illustrated the synergistic effects of keratinase and preen gland secretions. While keratinase and preen gland secretion alone were of little toxicity, the combination of both agents significantly inhibited bacterial growth over a wide range of concentrations (Fig. 4). It reduced the concentrations of the single substances needed to completely inhibit growth by at least two to eightfold (see DRI in Table 6).

Time-kill curves
Time-kill kinetics of MRSA NCTC 10442 showed that preen gland secretion and keratinase alone exerted only slight antimicrobial activity when compared to the growth control. However, when applied in combination, cell counts collapsed and indicated considerable synergistic interactions. Preen gland secretion supplemented with keratinase quickly pushed multidrug resistant bacteria under the limit of detection (LOD, 50 cfu/mL). However, only under high dose conditions (2048 μg/mL preen gland secretion plus 64 μg/mL proteinase K) the number of cells remained below the LOD (Fig. 5).

Tests for mutualism
Overlay diffusion tests
In overlay diffusion tests investigating possible antibacterial effects of the turkey isolates, S. auricularis flourished in the presence of C. uropygiale strains Iso10 and C4 (Fig. S3, Supporting Information). Under the conditions used in these tests, there is no evidence for the production of antimicrobial metabolites and mutualism. Only the positive control E. faecalis MRR-10 from hoopoe preen glands inhibited the indicator strain.

Test for synergistic interactions between C. uropygiale and keratinase
Zones of inhibition could be observed for none of the samples. There is no evidence that C. uropygiale produces antimicrobial components in need of activation by keratinase.

Protease expression
Tests for protease activity of C. uropygiale on SMA yielded negative results, i.e. no halos could be detected after incubation.
Table 5. Antimicrobial activity of preen gland secretions of turkeys and their extracts against a set of microorganisms. Values are given as means ± standard deviations. Wax: complete preen gland secretion, KER: keratinase, CRH: Cremophor RH 40 (stabilizer), NI: no inhibition, ND: not done.

<table>
<thead>
<tr>
<th>Zone of inhibition (mm)</th>
<th>E. coli XL1- Blue MRF'</th>
<th>P. montelli feather isolate</th>
<th>K. rhizophila feather isolate</th>
<th>S. auricularis ATCC 33753</th>
<th>MRSA NCTC 10442</th>
<th>B. megaterium ATCC 14581</th>
<th>C. lactiscondensi ATCC 60137</th>
<th>A. niger soil isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highly active sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wax</td>
<td>10.2±0.6</td>
<td>10.5±0.5</td>
<td>8.3±1.2</td>
<td>10.2±0.8</td>
<td>8.6±0.6</td>
<td>8.0±1.0</td>
<td>7.1±0.3</td>
<td>6.7±0.6</td>
</tr>
<tr>
<td>Wax + KER</td>
<td>15±0.3</td>
<td>20±1</td>
<td>14.7±1.5</td>
<td>22.3±1.5</td>
<td>24.3±1.2</td>
<td>14.0±1.0</td>
<td>9.0±1.0</td>
<td>8.0±1.0</td>
</tr>
<tr>
<td>Lipid extract</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Lipid extract + KER</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Water extract'</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>12±3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Water extract + KER*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>25±4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Weakly active sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Wax</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>7.3±0.6</td>
<td>6.3±0.6</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Wax + KER</td>
<td>7.8±1.0</td>
<td>7.3±0.6</td>
<td>7.7±0.06</td>
<td>8.6±0.6</td>
<td>8.3±0.6</td>
<td>7.3±0.6</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Lipids</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Lipid + KER</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Water extract'</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>8.2±1.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Water extract + KER*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>10.2±0.8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>CRH + KER</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>20.8±3.2</td>
<td>NI</td>
<td>17.3±0.6</td>
<td>18.5±1.5</td>
<td>15.0±2.0</td>
<td>22.3±1.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>ND</td>
<td>25.3±2.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Nystatin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>18.1±1.3</td>
<td>15.3±0.6</td>
<td></td>
</tr>
</tbody>
</table>

*The concentration of the water extract was 16× lower than the concentrations of the complete preen gland secretion and the lipid extract.

Figure 3. Normalized isobologram of representative staphylococcal strains illustrating interactions between preen gland secretion and keratinase.
### Table 6. Results of the broth microdilutions and checkerboard assays showing the interactions between preen gland secretion (Wax) and keratinase (KER) based on Loewe additivity and Bliss independence theories. MIC: minimum inhibitory concentration, Vanco: vancomycin (positive control), FIC: fractional inhibitory concentration, FICI: fractional inhibitory concentration index, $\sum$SSI: sum of statistically significant interactions (in brackets: number of statistically significant synergistic interactions), Int.: interpretation, SYN: synergism, SSYN: strong synergism. DRI: dose reduction index.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>MIC</th>
<th>MIC alone</th>
<th>MIC in combination</th>
<th>Loewe additivity</th>
<th>Bliss independence</th>
<th>DRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. auricularis</td>
<td>ATCC 33753</td>
<td>0.5</td>
<td>≥2048</td>
<td>64</td>
<td>265</td>
<td>0.5</td>
<td>0.0625</td>
</tr>
<tr>
<td>S. aureus</td>
<td>ATCC 25923</td>
<td>0.5</td>
<td>≥2048</td>
<td>64</td>
<td>1024</td>
<td>8</td>
<td>0.25</td>
</tr>
<tr>
<td>MRSA</td>
<td>NCTC 10442</td>
<td>1</td>
<td>≥2048</td>
<td>64</td>
<td>512</td>
<td>8</td>
<td>0.125</td>
</tr>
<tr>
<td>MRSA</td>
<td>KL602821</td>
<td>≤0.5</td>
<td>≥2048</td>
<td>64</td>
<td>512</td>
<td>8</td>
<td>0.125</td>
</tr>
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<td>MRSA</td>
<td>BL518716</td>
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<td>≥2048</td>
<td>64</td>
<td>512</td>
<td>8</td>
<td>0.125</td>
</tr>
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<td>≥2048</td>
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<td>512</td>
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<td>512</td>
<td>8</td>
<td>0.125</td>
</tr>
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<td>512</td>
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<td>BL602098</td>
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<td>≥2048</td>
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<td>512</td>
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<td>0.125</td>
</tr>
<tr>
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<td>≥2048</td>
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<td>512</td>
<td>8</td>
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</tr>
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<td>≥2048</td>
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<td>≥2048</td>
<td>64</td>
<td>512</td>
<td>8</td>
<td>0.125</td>
</tr>
</tbody>
</table>

1. For the calculations of the FIC and FICI values, the off-scale MICs of the single drugs were converted to the next highest twofold concentrations (Meletiadis et al. 2005; Li et al. 2008).
2. Synergistic interaction if $\text{FICI} \leq 0.5$.
3. Strong synergistic effect if $\sum \text{SSI} > 200\%$.

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**Figure 4.** 3D response surface plots depicting the combined effects of preen gland secretion and keratinase on the experimental growth of four representative strains of the study.
Hence, *C. uropygiale* does not produce keratinase or other proteases under the conditions used in this test (Fig. S4, Supporting Information).

Spectrophotometric tests using azocasein as a substrate confirmed these findings. While protease activity could be detected in *E. faecalis* from hoopoes, both turkey isolates failed to express proteases in vitro (Table S2, Supporting Information).

**DISCUSSION**

In this work, we investigated the antimicrobial activity of preen gland secretions of turkeys against a set of bacteria, molds and yeasts. We conducted drug combination assays with keratinase to simulate the presence of FDM. Furthermore, we determined the effect of combinations on clinical isolates of multidrug resistant *Staphylococcus aureus*. In addition, we tested the hypothesis that the antimicrobial activity of turkey preen gland secretions originates from their lipophilic fraction and shed light on the chemical nature of the active agents. Finally, possible mutualistic relationships between turkeys and two strains of the preen-gland inhabiting bacteria *Corynebacterium uropygiale* were assessed.

**Antimicrobial activity**

**Tests of native preen gland secretions**

Antimicrobial activity of preen gland secretions of birds has been frequently proposed, but, with a few exceptions for single species (mainly hoopoes), no general conclusion can be drawn based on the results of the available studies (Moreno-Rueda 2017). However, evidence comes from Shawkey, Pillai and Hill (2003) who investigated antimicrobial effects of preen gland secretion of house finches (*Haemorhous mexicanus*) against feather-degrading and non-feather-degrading plumage isolates in diffusion tests. As a result, preen gland secretion preferably inhibited keratinolytic bacteria (Shawkey, Pillai and Hill 2003). As the amount of substance applied by Shawkey, Pillai and Hill (2003) is unknown, their data cannot be compared to the turkey results.

Recently, the preen gland secretion of house sparrows (*Passer domesticus*) was shown to inhibit *Staphylococcus epidermidis* (Magallanes et al. 2016). However, even at a concentration as high as 5 mg/mL, antimicrobial activity was very weak (mean inhibition between 10 to 20% with standard deviations between 78 and 88%), and did not meet the criteria of an MIC. Accordingly, the antimicrobial potential of turkey secretions is far in excess of those reported for house sparrows.

In another study, the preen gland secretions of spectacled thrushes (*Turdus nudigenis*) favored the growth of some feather-degrading bacteria, in spite of retarding feather degradation in vitro (Verea et al. 2017).

Hoopoes and green woodhoopoes are special in that they harbor mutualistic bacteria in their preen glands that contribute to the broad-range antimicrobial activity of their preen gland secretions. Law-Brown (2001) as well as Martín-Vivaldi et al. (2010) provided striking evidence that injecting antibiotics into the preen glands of green woodhoopoes and hoopoes is followed by a significant alteration of the chemical composition of preen gland secretions and a loss in biological activity. Although the antimicrobial activity of native hoopoe preen gland secretions has not been quantified in tests comparable to our assays, the MICs of their single active agents (Martin-Vivaldi et al. 2010) have been found to be similar to those of turkey preen gland secretions. Chemically synthesized preen gland secretions of green woodhoopoes showed very high activity in diffusion

![Figure 5. Time-kill curves of the combined action of preen gland secretion and keratinase when compared to the single agents against MRSA NCTC 10442.](https://academic.oup.com/femsec/article-abstract/94/9/fiy117/5036518)
tests (Law-Brown 2001). However, the fact that synthesized secretions were used make comparisons with turkey preen gland secretions difficult.

Detection of diester waxes in turkey preen gland secretions and rejection of the lipid hypothesis

The divergent pattern of activity between the highly active and the weakly active samples raised the question about their molecular bases and whether or not the substantial discrepancies regarding their biological activity can be attributed to the amount or composition of diester waxes. Chemical analysis confirmed the presence of diester waxes in both highly active and weakly active preen gland secretions, the fragmentation patterns of which perfectly coincided with alkane-2,3-diols in Sawaya and Kolattukudy (1972) and FAMEs from the NIST library. Furthermore, our results were in line with Hansen, Tang and Edkins (1969) who reported on uropygial-containing esters as major component of the preen gland secretion of turkeys. All derivatization products mentioned in Hansen, Tang and Edkins (1969) were detected and the pattern of relative abundance coincided with values found in the literature (Hansen, Tang and Edkins 1969). However, it turned out that both the highly active and weakly active preen oil samples showed congruent peak patterns which were nearly identical in terms of qualitative and quantitative compositions. This was a hint that our lipid hypothesis has been based on a wrong assumption. Finally, the subsequent antimicrobial test series using the lipid extracts unequivocally rejected the lipid hypothesis and clearly demonstrated that preen gland lipids are by no means a main contributor to direct antimicrobial activities of turkey preen gland secretions.

Indirect antimicrobial effects

However, apart from direct antimicrobial effects, lipids in preen gland secretions could form a physical barrier preventing pathogens from accessing feather or skin keratin. The barrier hypothesis was introduced by Reneerkens et al. (2008), after the authors found that defatting feathers of red knots increases susceptibility to feather degradation by B. licheniformis which is independent of whether monoester or diester waxes predominated in preen gland secretions (Reneerkens et al. 2008). While preen gland secretions could indeed function as physical barrier, the effect observed by Reneerkens et al. (2008) could also be due to feather lipids produced by the skin. As reported by Jacob and Grimmer (1975b), plumage lipids contain compounds of non-preen gland origin, such as free fatty acids and free alcohols, which can exert antimicrobial effects (Menon and Menon 2000; Mukherjee et al. 2013).

Alternatively, esterase positive bacteria present on feathers or abiotic factors could theoretically hydrolyze ester waxes in preen gland secretions giving rise to antimicrobial free fatty acids and free fatty alcohols. This hypothesis needs further investigations, however.

Feather mites could also play a role in pathogen defense. Although generally known as parasites damaging feathers, they might serve functions beneficial to their hosts. By feeding on preen gland secretions, they could remove detrimental bacteria and fungi (Brown et al. 2006). Indeed, Zeman (1988) has shown that feather mites (Dermanyssus gallinae) preferentially feed on diester waxes originating from preen glands.

AMP hypothesis

After rejecting the lipid hypothesis, we were interested in the basic chemical properties of the active components of preen gland secretion of turkeys. We carried out water extraction and antimicrobial tests against one of the most sensitive of our indicator strains. While the lipid fractions were proven inactive, tests using water extracts consistently demonstrated that antimicrobially active components of preen gland secretions of turkeys are of polar nature. Given that this also applies to other bird species, this would explain the results of Shawkey, Pillai and Hill (2003) who reported on biological activity of preen gland secretions of house finches in diffusion tests. As preen wax is very viscous (as correctly stated by the authors), often claggy with the vast majority of compounds being highly lipophilic, it does not diffuse through the agar in diffusion tests easily, unless stabilized (M.S. Braun and M. Wink, unpublished). Since the authors did not make use of any stabilizer but still obtained very big zones of inhibition, this might be evidence for the antimicrobial agents being hydrophilic.

Hydrophilic compounds might be antimicrobial peptides (AMPs). The AMP hypothesis is supported by Martín-Platero et al. (2006) and Ruiz-Rodríguez et al. (2013) who showed that AMPs contribute to the antimicrobial capacity of the preen gland secretions of hoopies. Further evidence comes from van Dijk (2007) reporting on the inducible expression of a precursor of chicken myeloid AMP 27 (proCAMP27) in the preen glands of chickens. In consideration of human cathelicidin hCAP18, which is secreted in sweat and requires activation by stratum corneum tryptic enzyme (SCTE, kallikrein 5) or stratum corneum chymotryptic enzyme (SCCE, kallikrein 7) in skin (Yamasaki et al. 2006), Van Dijk (2007) suggests a similar process when proCAMP27 is applied to the skin of birds. Since putative AMPs in turkey secretions were shown to be activated by keratinase in our tests, we propose a second mechanism of enzymatic processing. When encountering weakly active antimicrobial precursors in preen gland secretions, keratinases expressed on feathers by FDM could interact in a similar manner as seen in our antimicrobial tests and rapidly clear keratinolytic microorganisms from the plumage. In this way, feather-degrading pathogens would be specifically eradicated, while non-detrimental microorganisms of the plumage would not be harmed. This interplay between FDM and birds would be point in case for a co-evolutionary arms race where FDM try to exploit their hosts, and birds develop counter-strategies to keep up with microbial parasites. Since CMAP27 is a potent antagonist of FDM (van Dijk 2007), this might also hold true for the chicken AMP. It would also agree with the inhibition pattern of house finch secretions which specifically target FDM (Shawkey, Pillai and Hill 2003).

The different antimicrobial potentials of the batches used in our study might be explained by diverging abundances of FDM on the plumage of the animals. The birds of ‘Ziegler 1’ and ‘Ullrich 1’ (highly active sample) could have been faced with a large number of FDM, triggering the production of high amounts of AMPs. On the contrary, FDM on the plumage of the birds of ‘Ziegler 2’ and ‘Ullrich 2’ (weakly active sample) could have been less abundant, leading to lower AMP levels in their preen gland secretions. Since domestic turkeys, on the farm, are kept in close vicinity to each other, bacteria can easily be transferred between the individuals, giving rise to similar bacterial burdens on the plumage and identical antimicrobial activities of the preen gland secretions of the individual birds of the same group (batch).
Symbiosis between C. uropygiale and turkeys

Bacteria were tested in overlay diffusion tests for the production of antimicrobial metabolites. Antimicrobial metabolites have been found to be synthesized by our positive control from hoopies, E. faecalis MRR-10 (Martín-Platero et al. 2006; Soler et al. 2008). There is some evidence that E. phoeniculicola, the preen gland bacterium of green woodhoopoes metabolizes constituents of preen gland secretion produced by their hosts to form antimicrobial agents (Law-Brown 2001). Hence, we included preen wax from turkeys into the formulation of the test medium. Although C. uropygiale exhibited luxuriant growth, there was no evidence for mutualism (Fig. S3, Supporting Information). Same conclusion was drawn from the protease expression and combination assays with proteinase K (Table S2, Fig. S4, Supporting Information).

In conclusion, C. uropygiale does not produce antimicrobial substances under the tested conditions and is likely not to modulate the activity of preen gland secretions of turkeys. Nevertheless, the bacteria might engage in a mutualistic relationship by occupying a niche which otherwise could be colonized by pathogenic microorganisms. Given the assumed important roles of the preen gland for birds (see introduction), C. uropygiale could be a decisive parameter concerning the fitness of turkeys.

There is some evidence, that C. uropygiale indeed plays an important role, because we recovered the same bacteria from turkeys at all three locations. The obligate lipophilism of this species is another sign for a close co-evolution between turkeys and coryneform bacteria.

Preen gland bacteria reported in the literature

Generally, the microbiome of preen gland secretions has not yet been thoroughly investigated and to date, only a few bird species have been reported to harbor bacteria in their preen glands. Evidence for mutualism comes from the hoopoee and Enterococcus spp. (Soler et al. 2010) as well as the green woodhoopoe and E. phoeniculicola (Law-Brown 2001). Furthermore, Burkholderia spp. and Pseudomonas spp. have been detected in the preen glands of dark-eyed juncos (Junco hyemalis) and could theoretically be able to produce antimicrobial metabolites (Whittaker and Theis 2016). Knowledge on other preen gland bacteria is restricted to some new species within Kocuria sp. colonizing the uropygial glands of great spotted woodpeckers (Dendrocopos major) (K. uropygialis and K. uropygioeca) (Braun et al. 2018a) and American barn owls (Braun and Wink, under review). Besides, novel coryneform bacteria have been found in the preen glands of Egyptian geese (Alopochen aegyptiacus) (Braun et al. 2018b). Possible mutualisms between the woodpecker, goose and owl isolates have not yet been studied but a close relationship between preen gland bacteria and their hosts is to be anticipated. In conclusion, the present lack of data regarding microbe-bird associations warrants further study.

CONCLUSIONS

The antimicrobial potential of preen gland secretions from birds is still controversially discussed. Evidence supporting either side is still rare (Shawkey, Pillai and Hill 2003; Moreno-Rueda 2017), sometimes rather descriptive and often contradictory (Moreno-Rueda 2017). With the present work, we contribute to the field by providing data on the antimicrobial activity of the preen gland secretions of turkeys. Our six key findings can be summarized as follows:

1. Preen gland secretion of turkeys inhibits a wide range of bacteria. When combined with keratinase, it acts in a strongly synergistic manner. Bactericidal effects are strong enough to fight multidrug resistant clinical isolates of MRSA.
2. Turkeys are likely to make use of keratinases expressed by FDM on the plumage. They thereby beat keratinolytic bacteria at their own game, while other microbes are not harmed to the same extent.
3. Lipophilic components including 2,3-diol-containing diester waxes do not mediate direct antimicrobial effects of preen gland secretions from turkeys but could do so after hydrolysis under field conditions.
4. Active components in turkey preen gland secretions are highly polar and might be of peptide nature.
5. We did not find evidence for C. uropygiale contributing to the antimicrobial potential of preen gland secretion in turkeys.
6. Current research is far from understanding the overall contribution of preen gland secretions to the fitness of birds. Further studies are needed to unravel the mechanisms of their antimicrobial activities and the functional importance of preen gland-associated microbiomes.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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