Prevalence of *Bacillus licheniformis* on Northern Saw-Whet Owls (*Aegolius acadicus*)

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Abstract

Birds are hosts to a variety of bacteria and fungi including some that are keratinolytic. These feather degrading bacteria, including *Bacillus licheniformis*, are capable of degrading β-keratin which is a major component of feathers. While there is little evidence to indicate that feather degrading bacteria are capable of degrading feathers on live birds, there is ample evidence to indicate that many bird species experience altered feather coloration. There is significant variation in the reported prevalence of *B. licheniformis* on birds with percentages ranging from 6.7% to 99%. This study sought to provide further support for an overall prevalence of *B. licheniformis* on Northern Saw-Whet Owls. 14 possible *B. licheniformis* colonies were isolated resulting in a prevalence of 85.7%.

Introduction

Birds are hosts to a variety of organisms on their feathers including bacteria, lice (Kent & Burtt, 2016), and fungi (Clayton, 1999). A small proportion of these bacteria and fungi are keratinolytic, and thus capable of degrading β-keratin which is a major component of feathers (Burtt & Ichida, 1999). Feather degrading bacteria are a group of keratinolytic bacteria found on bird feathers and are mainly from the genus *Bacillus.* Bacillus species are Gram positive, rod-shaped, endospore formers and are the most common feather degrading bacterial species on bird feathers (Kent & Burtt, 2016). Of these, *Bacillus licheniformis* is one of the most studied. *B. licheniformis* is a common halotolerant and thermophilic soil bacterium (Burtt & Ichida, 1999). Due to this, birds are likely contaminated when they are in contact with soil (Burtt & Ichida, 1999). This is consistent with the discovery that *B. licheniformis* is more prevalent on the venter feathers and on birds that are ground foragers (Burtt & Ichida, 1999). However, because *B. licheniformis* is an endospore former, it is also possible that birds are also contaminated via airborne spores that are spread by the wind (Burtt & Ichida, 1999). A later study found that ground foraging, fly-catching, aerial foraging, and foliage gleaning birds all had similar prevalence levels of *B. licheniformis* (Kent & Burtt, 2016). Moreover, genetic studies have shown that birds can be contaminated with multiple strains at the same time indicating that birds are likely contaminated through random environmental contact (Whitaker et al., 2005).

The prevalence of feather degrading bacteria has been found to vary seasonally: they are most common in the late fall and least prevalent in the spring (Burtt & Ichida, 1999). Some variation has also been found between species which may be the result of behavioral differences such as foraging style or variation in the microbiome and microhabitat of species (Kent & Burtt, 2016). However, Kent & Burtt, 2016 found that the prevalence of *B. licheniformis* did not vary

as much between foraging guilds as Burtt & Ichida, 1999 reported. Prevalence has also been found to differ between males and females of the same species such as in Great Tits (Saag et al., 2011). It is unknown exactly why this occurred but it could be due to behavioral differences between the males and females during the breeding season (Saag et al., 2011). Furthermore, there is even variation on a single feather as bacteria tend to be more common on the distal than the proximal ends of feathers (Muza et al., 2000). Ultimately, the most significant factor associated with detection is sample size (Burtt & Ichida, 1999) indicating that seasonal or species-specific behavior likely does not have a large influence on the detection of feather degrading bacteria. Therefore, it is highly probable that feather degrading bacteria, specifically *Bacillus licheniformis*, will be isolated if enough individuals from a species are sampled.

In addition to feather degrading bacteria, there are at least thirteen known culturable species of keratinolytic fungi (Burtt & Ichida, 1999). Seven of these have been found on feathers, two were isolated only from nests, and four have never been isolated on birds but are known to be keratinolytic.

Having high quality feathers are essential to a bird's health as they are necessary for bird flight, thermoregulation, communication, and more (Gunderson, 2008). Understanding the impact of feather degrading bacteria on feathers is important because a feather is composed of over 90% β-keratin by mass and several studies have confirmed the ability of feather degrading bacteria to degrade feathers, specifically to degrade β-keratin *in vivo* (Burtt & Ichida 1999, Gunderson et al. 2008, Saranathan & Burtt 2007). Feathers consist of several main structures. The calamus is the part of the feather that is embedded in the skin and this portion extends into the rachis which is the center part of the feather (Foth, 2020). Barbs are small projections off of the rachis that make up the majority of most feathers (Foth, 2020). They are connected by

smaller, hooked projections called barbules (Foth, 2020). While all portions of a feather contain β-keratin, barbs and barbules are the components that are examined the most for the effects of feather degrading bacteria as these bacteria are known to thin and break barbs and barbules (Shawkey et al., 2007). Additionally, these components create the color of the feather (Foth, 2020) which is also impacted by feather degrading bacteria, and are the outermost portion so they would be the first portion of a feather exposed to feather degrading bacteria.

Currently, there is little evidence that feather degrading bacteria degrade feathers on live birds. One study attempted to determine the effects of feather degrading bacteria in seminatural conditions using captive Northern Cardinals and European Starlings (Cristol et al., 2005). The researchers determined that feather degrading bacteria had no effect on feather degradation but the conditions under which the study was conducted were unfavorable for feather degrading bacteria (Gunderson 2008). Another study found a positive correlation between tail feather wear and *B. licheniformis,* but it is important to note that tail feathers are exposed to many possible sources of degradation so the researchers were unable to definitively claim that the observed degradation was due to *B. licheniformis* (Kent & Burtt, 2016). Thus, it is possible that feather degrading bacteria only degrade loose feathers that are on the soil (Whitaker et al., 2005). However, more research needs to be done before this can be firmly concluded.

There is evidence to indicate that feather degrading bacteria may impact feather coloration (Gunderson et al., 2009; Shawkey et al., 2007; Shawkey et al., 2009; Leclaire et al., 2014) which would imply degradation of feather structure as the structure of the feather impacts the coloration. This is significant because coloration is essential for communication, crypsis, and more (Gunderson et al., 2009). Feather degrading bacteria have been shown to impact color on Eastern Bluebirds (Gunderson et al., 2009; Shawkey et al., 2007), Feral Pigeons (Leclaire et al.,

2014) and House Finches (Shawkey et al., 2009), but a study on Great Tits found that feather degrading bacteria did not affect coloration (Jacob et al., 2014). The impact of feather degrading bacteria on feather color may be specific to species or to pigments and coloration. Furthermore, it is possible that feather degrading bacteria degrade the feather structure enough to alter the coloration of feathers but not enough to produce a noticeable degradation impact on the feather when just observed with the naked eye.

Bacterial and fungal load have also been connected with other reductions in fitness such as increased predation risk (Møller et al. 2012, Rubiaee et al. 2017). Microorganisms on the feathers could impact flight by degrading feathers (Møller et al., 2012) or by disrupting the normal airflow over the surface of the feathers (Clayton, 1999). Both of these ultimately impact a bird's ability to outmaneuver, and thus escape from, a predatory bird (Rubiaee et al., 2017). Furthermore, even slight damage to the feathers can significantly increase the risk of predation (Møller et al., 2012). Feather degrading bacteria could have further impacts on small owls, such as Northern Saw-Whet Owls, the birds sampled in this study, which rely on the ability to fly silently to hunt and avoid predators. An owl's ability to fly silently is due to the structure of their feathers (Wagner et al., 2017). Specifically, the outermost primary wing feather is serrated which allows for quieter flight (Roulin et al., 2013). Thus, if feather degrading bacteria impact even the microscopic structure of an owl's feather, its ability to fly silently could be compromised resulting in that owl becoming extremely disadvantaged in both hunting and escaping predators.

There are several ways in which a bird can limit the number of microorganisms on its feathers. Some are innate such as the manner in which keratin is tightly folded in a feather (Gunderson, 2008), molting (Burtt & Ichida, 1999), and the fact that melanized feathers are more resistant to degradation by feather degrading bacteria (Gunderson et al., 2008). Others are

behaviors a bird can perform that reduce bacterial and fungal load. For example, both sunbathing and dustbathing dry out feathers resulting in unfavorable environments for feather degrading bacteria (Gunderson, 2008). During these conditions, *B. licheniformis,* and other endospore forming feather degrading bacteria, will form spores and are unable to degrade feathers while in this state (Cristol et al., 2005). Sunbathing has also been shown to limit the growth of *B. licheniformis* (Saranathan & Burtt, 2007). Preening is another such behavior and preen oil secretions serve as both a physical and antimicrobial barrier for feather degrading bacteria (Jacob et al., 2014). In fact, when keratinases produced by feather degrading bacteria are present, the antimicrobial effect of preen oil can become even stronger (Braun et al., 2014). Preening is a normal and essential function for birds and they can spend anywhere from 5 to 30% of their daily activity preening (Haribal et al., 2005). Removing the uropygial gland, an external gland that produces secretions used during preening (Jacob et al., 2014), drastically alters the skin microbiome (Gunderson, 2008) and results in brittle, rough feathers (Haribal et al., 2005). Moreover, experimentally lowering the bacterial load results in decreased time spent preening (Leclaire et al., 2014) and increasing the bacterial load can lead to an increase in the amount of time spent preening as well as changes to the preen oil composition and volume of the uropygial gland (Jacob et al., 2014).

Several studies have been conducted to determine the overall prevalence of *B. licheniformis* on birds but there is a wide variety in the results ranging from 6.7% (Burtt & Ichida, 1999) to 99% (Gunderson et al., 2009). Other studies have examined the relationship of the feather degrading bacterial load with other variables such as feather degradation (Kent & Burtt, 2016) or color (Gunderson et al., 2009; Leclaire et al., 2014; Ruiz-De-Castañeda et al., 2012; Shawkey et al., 2007; Shawkey et al., 2009). Thus, to continue adding to the current knowledge of feather

degrading bacteria and *B. licheniformis*, this study will examine the presence and abundance of *B. licheniformis* on Northern Saw-Whet Owls (*Aegolius acadicus*), whether *B. licheniformis* occurs more often on the dorsum, wing, or tail, of the owls sampled, and whether the dorsum, wing, or tail has the greatest bacterial load and diversity of bacterial morphotypes. Northern Saw-Whet Owls are small, migratory owls that are dark brown with white spotting and streaking and a white or buff facial disk and throat (Wails et al., 2018). Northern Saw-Whet Owls were chosen as the study species because they were already being banded at the location of this study as part of Project OwlNet, which monitors migration, and they have already been examined once for presence of *B. licheniformis* (Whitaker et al., 2005). Therefore, the results of this study will build off of the previous study. In addition to that, the majority of feather degrading bacterial research has been conducted on songbirds from Order *Passeriformes* so this study will continue to add to the current understanding of feather degrading bacteria by examining feathers from a bird belonging to Order *Strigiformes.*

An overall prevalence around 59% is expected as this is the value reported by another study on the same species also completed in Virginia (Whitaker et al., 2005). It is also expected that the largest number of *B. licheniformis* colonies will be found on the tail as this is the portion of the owl most in contact with the soil and other surfaces, and that the lowest number of *B. licheniformis* colonies will be found on the wings as the wing feathers are frequently preened for flight. Likewise, the most diversity and highest bacterial load are also expected to occur on the tail and the lowest diversity and smallest bacterial load are expected to occur on the wings.

Methods

In November 2020, Northern Saw-whet Owls were captured using mist nets and an audio lure in a forested area owned by Liberty University in Lynchburg, VA. Samples were then taken

from the dorsal, wing, and tail feathers. A plate with tryptic soy agar (TSA) was gently pressed on the owls' dorsum. For the tail feather samples, both sides of the tail feathers were pressed onto a TSA plate. Feathers on one wing were sampled by running a sterile swab throughout the feathers and a TSA plate was immediately inoculated using that swab. TSA was chosen because it is a general medium that supports growth of many microorganisms (Kent & Burtt, 2016). The sampling was done by the same person each time and was the first part of the processing procedure. To limit potential contamination, all people who handled the owls prior to sampling wore disposable gloves sprayed with ethanol. The mist nets used to capture the owls are a potential source of contamination but this is unlikely to significantly alter the results as the owls sampled did not enter the nets at the exact same spots. Moreover, the nets are exposed to natural means of sanitation such as sunlight which is known to inhibit the growth of *B. licheniformis (*Saranathan & Burtt, 2007). After the samples were taken, the owls were weighed, their wingspans were measured, they were banded with U.S. Fish and Wildlife bands, and then released. A total of 21 samples were collected from 7 owls. The TSA plates were incubated at room temperature for 2 to 3 days to allow for bacterial growth. After this, the plates were refrigerated until further analysis could be done.

The total number of bacterial and fungal colonies and morphotypes were counted and characterized for each plate. Morphotypes were determined by morphological characteristics such as color, height, margin, and texture. Bacterial colonies that were likely to be *B. licheniformis* were identified visually as *B. licheniformis* is characterized by wrinkled, cone-shaped colonies (Kent & Burtt, 2016). To avoid underestimating the true occurrence of *B. licheniformis,* all colonies that were cream or white in color, slightly opaque, and flat were isolated on plates consisting of R2A media. All of the following tests were done using these

plates as the source. If fungal growth occurred on these plates, a new R2A plate was immediately inoculated.

B. licheniformis is a highly heat tolerant and salt tolerant bacterium. To test for heat tolerance, a new set of R2A plates were inoculated for every bacterium isolated and incubated at 50ºC for seven days. Plates were checked daily for growth and if no growth occurred within seven days the bacterium was determined to not be *B. licheniformis.* Next, R2A plates were made with a 7% NaCl concentration and a plate was inoculated for each bacterium that grew under the 50ºC conditions. These new plates were incubated at room temperature and checked daily for growth for seven days. Again, if no growth occurred within seven days the bacterium was determined to not be *B. licheniformis.* Tests for oxygen requirements were then done by inoculating thioglycolate broth. These were incubated at room temperature and checked daily for growth for seven days. If no growth occurred, a new tube of thioglycolate broth was inoculated for that bacterial colony. Gram and Endospore stains were then conducted on all bacterium that grew in 50ºC, 7% NaCl R2A, and were facultative anaerobes. All colonies that were gram positive with nonswollen central elliptical spores were considered likely to be *B. licheniformis.*

Results

A total of 1,189 bacterial colonies and 166 bacterial morphotypes were counted. There were 360 colonies and 77 morphotypes from the tail samples, 508 colonies and 39 morphotypes from the dorsal samples, and 321 colonies and 50 morphotypes from the wing samples (Figure 1, Figure 2). There was a statistically significant difference between the number of colonies found on the three areas (x^2=282.2, d.f.=12, P=2.5x10^{-53}) but no significant difference for the number of morphotypes between the three areas (x^2=20.0, d.f.=12, P=0.066). The plates with the highest number of bacterial morphotypes had less than 60 colonies, and all the plates with more than 100

colonies had less than 10 morphotypes (Figure 3). However, there was no significant relationship between the number of bacterial colonies and morphotypes found on the same plate (Y_i=8.86 + -0.009X_i where Y_i is the number of bacterial morphotypes and X_i is the number of bacterial colonies on that plate, n=19, F=0.32, d.f.=19, P=0.58)

The tail plate from owl 2123 and four other isolated colonies were lost due to fungal growth.

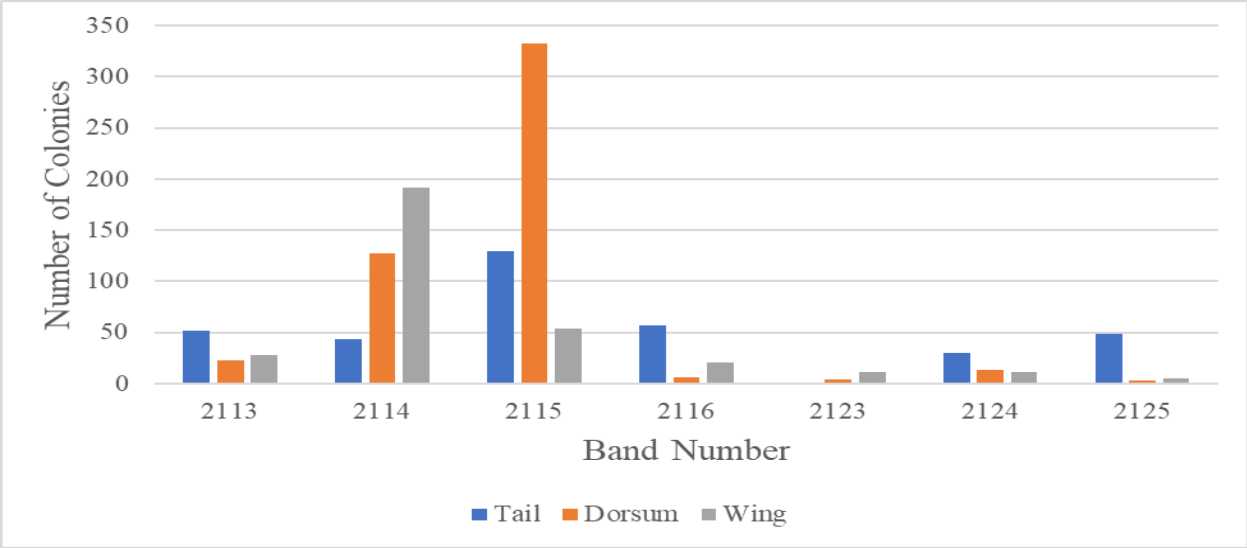

Figure 1. Graph of the number of bacterial colonies that grew on the initial sample plates by the owls' band number and location sampled.

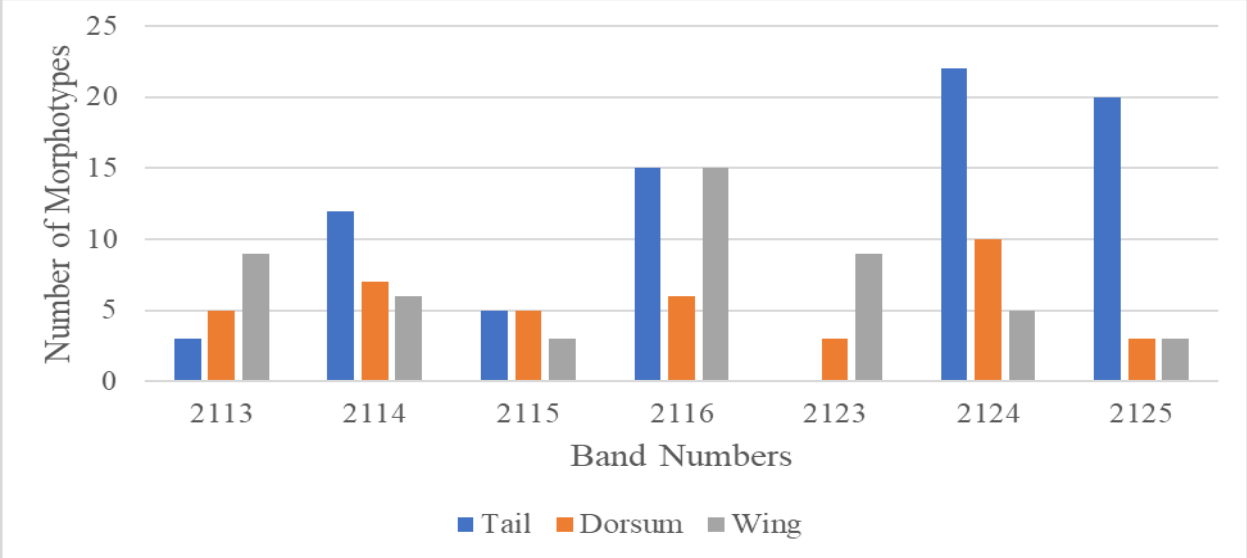

Figure 2. Graph of the number of bacterial morphotypes counted and characterized from the initial sample plates by the owls' band number and location sampled.

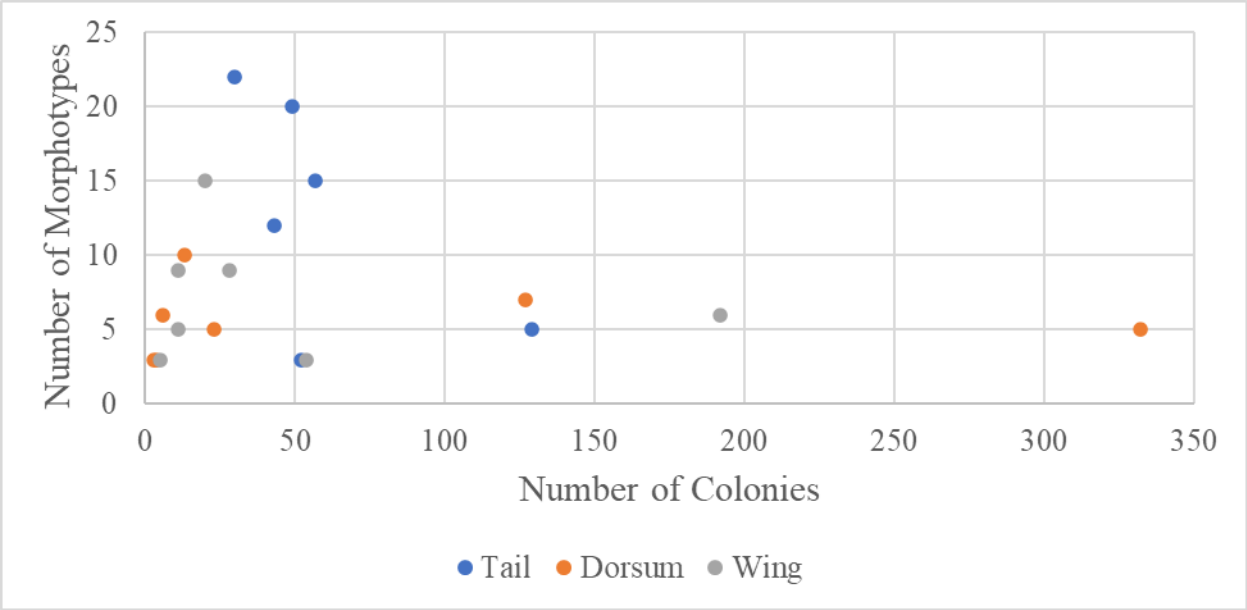

Figure 3. Graph of the number of bacterial morphotypes by the number of colonies for each initial sample plate.

There were 511 total fungal colonies with 327 of the colonies occurring on the tail, 87 on the dorsum, and 97 on the wing samples (Figure 4). Like the bacterial colonies, there was a statistically significant difference between the number of fungal colonies occurring in the three areas sampled (x^2=53.6, d.f.=12, P=3.2x10^{-7}). These colonies consisted of 120 morphotypes with 48 morphotypes on the tail samples, 35 on the dorsal samples, and 37 on the wing samples (Figure 5). There was no statistically significant difference between the number of fungal morphotypes across the three sampled areas (x^2=19.7, d.f.=12, P=0.07).

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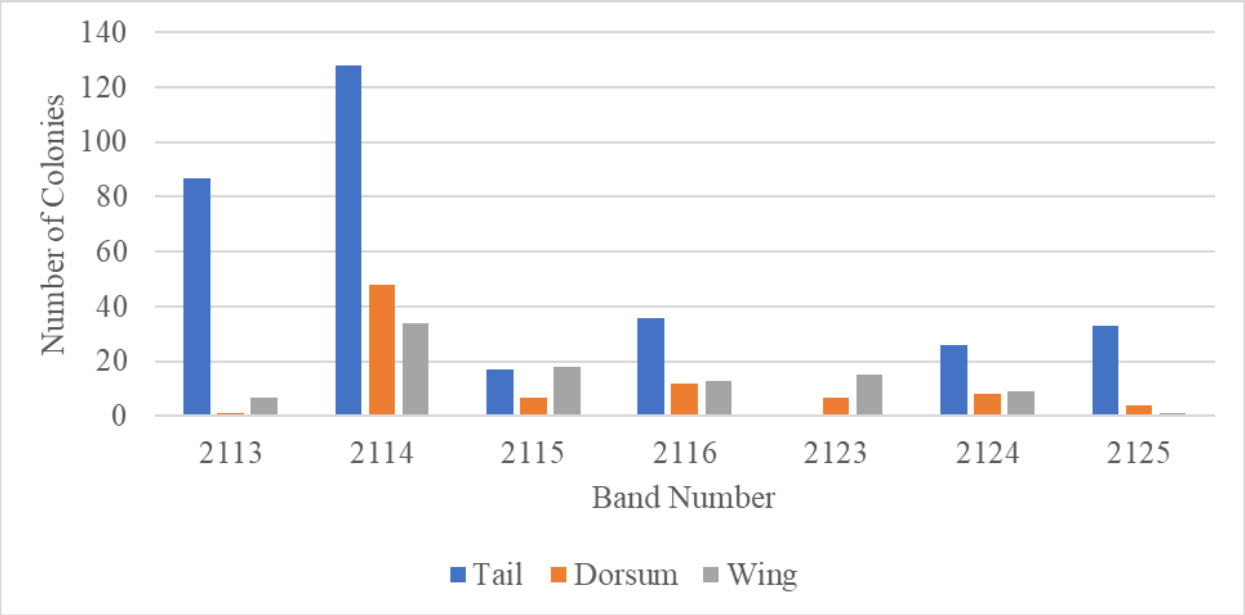

Figure 4. Graph of the number of fungal colonies that grew on the initial sample plates by the owls' band number and location sampled.

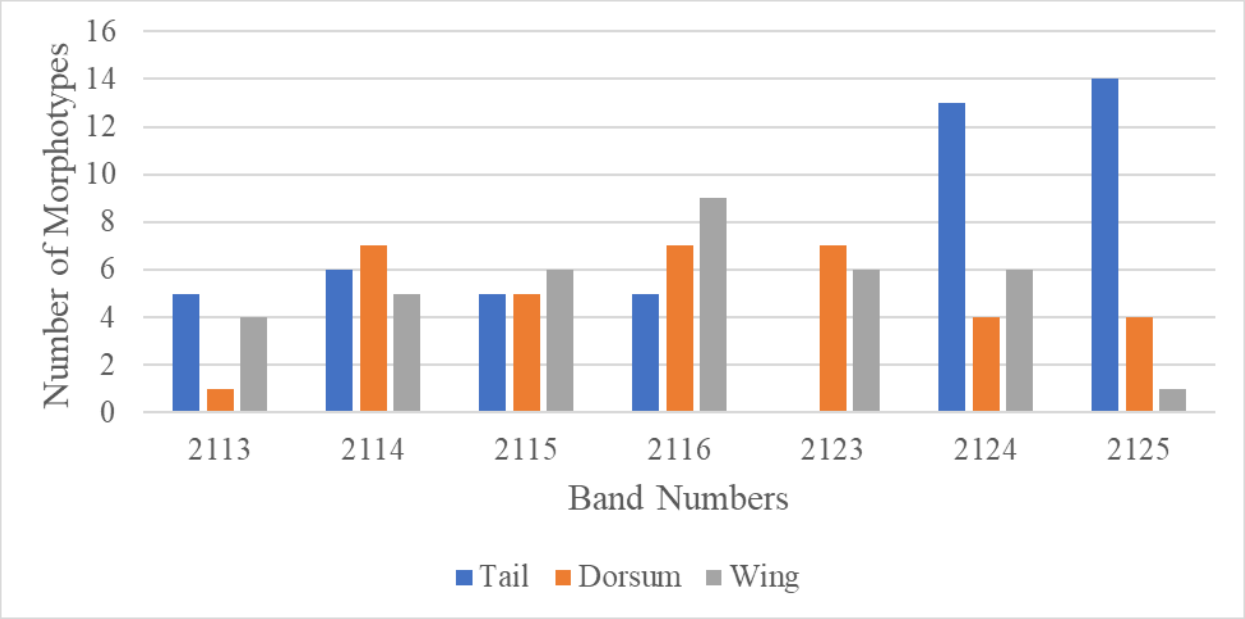

Figure 5. Graph of the number of fungal morphotypes counted and characterized from the initial sample plates by the owls' band number and location sampled.

There were 14 colonies that were found to likely be *B. licheniformis* and these were found on six of the seven owls sampled. However, the species of these colonies were not able to be confirmed through genetic sequencing and thus anytime *B. licheniformis* is used to refer to this study it should be understood to be possible and not confirmed colonies. Overall, there was

an 85.7% prevalence of *B. licheniformis* on the owls sampled and these 14 colonies represent 1.17% of the 1,189 colonies counted. 6 of these colonies were found on the dorsum, 5 on the tail, and 3 on the wings. The sample size was too small to perform statistical analysis to determine a difference in whether *B. licheniformis* is more likely to be found on a certain area of the owl. Owl 2116 had the most *B. licheniformis* colonies with a total of 5, owl 2113 had 4 colonies, owl 2115 had 2 colonies, and owls 2114, 2123, and 2125 had only 1 colony (Figure 6). Additionally, the samples with 0-50 bacterial colonies had the most *B. licheniformis* colonies and as the number of total colonies increased (Figure 7). Similarly, the samples with 0-10 fungal colonies represented half of the samples that *B. licheniformis* was isolated from (Figure 8).

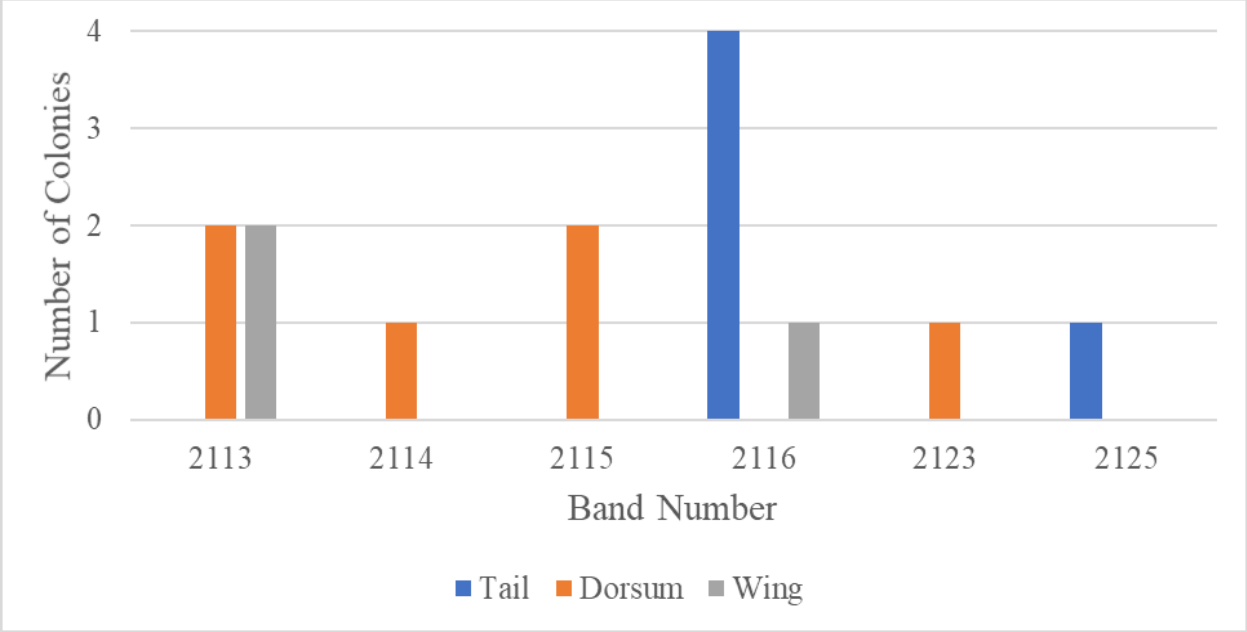

Figure 6. Graph of the number of *B. licheniformis* colonies isolated by owls' band number and location sampled.

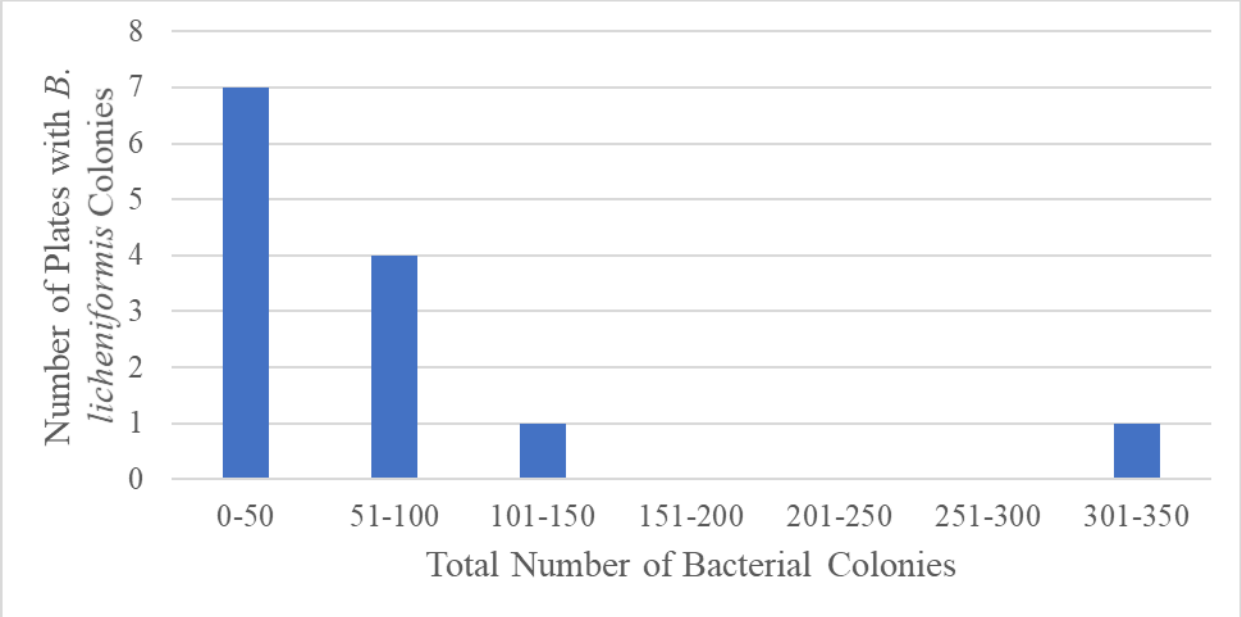

Figure 7. Number of source plates with possible *B. licheniformis* colonies by total number of bacterial colonies on the source plate that the *B. licheniformis* colony was isolated from.

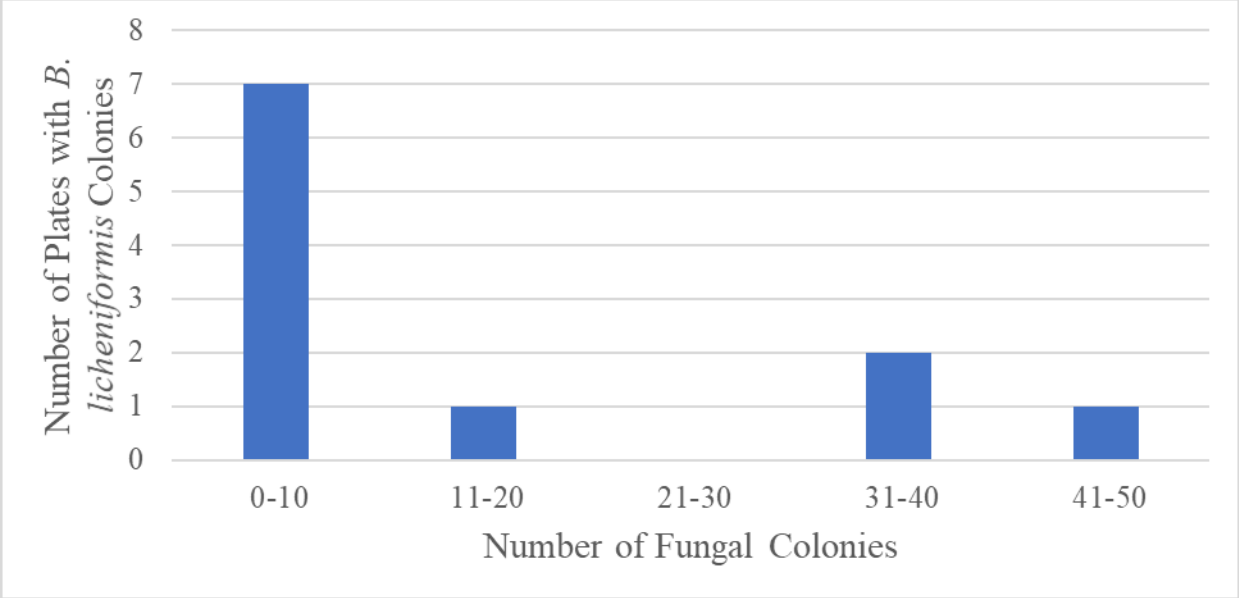

Figure 8. Number of source plates with possible *B. licheniformis* colonies by the number of fungal colonies on the source plate that the *B. licheniformis* colony was isolated from.

Discussion

High quality feathers are essential to birds as feathers fulfill a variety of functions including flight, thermoregulation, and communication (Gunderson, 2008). The quality of a bird's feathers, including the coloration of feathers, also gives conspecifics information such as

age, immunocompetence, dominance, nutrition, and more (Gunderson, 2008). Feathers host a variety of ectoparasites, fungi, and bacterial species (Kent & Burtt, 2016). Within the feather microbiome, there are keratinolytic microorganisms capable of degrading β-keratin which composes more than 90% of a feather's mass (Gunderson, 2008). Bacteria from the Genus *Bacillus* are the most common feather degrading bacteria (Kent & Burtt, 2016). *Bacillus licheniformis* is one of the most prominent feather degrading bacteria both in terms of its ability to degrade feathers and the extent to which it has been studied (Burtt & Ichida, 1999). This study on Northern Saw-Whet Owls found an 85.7% prevalence of *B. licheniformis* meaning that 6 of the 7 owls sampled had at least one *B. licheniformis* colony. Overall, 14 possible *B. licheniformis* colonies were isolated which represents 1.18% of the 1,189 colonies counted (Figure 6). It is important to note that the true relative density is likely lower because there are many bacteria that are unable to be cultured in laboratory conditions. Most of the colonies were found on the dorsum, then the tail and the least were found on the wing. Interestingly, 7 of the *B. licheniformis* colonies were isolated from plates containing only 0-50 total bacterial colonies (Figure 7) and 0-10 fungal colonies (Figure 8). As the bacterial and fungal load increased in the samples, less *B. licheniformis* colonies were isolated from that source plate. Thus, owls with a higher bacterial and fungal load are less likely to host *B. licheniformis*. It is possible that this is due to other bacteria and fungi outcompeting *B. licheniformis* or by other bacteria and fungi producing antimicrobial substances against *B. licheniformis*. The uropygial gland contains its own unique microbiome that is spread onto the feathers while a bird is preening and some of these bacteria have been shown to have antimicrobial properties (Braun et al., 2014; Jacob et al., 2014; Bodawatta et al., 2020). However, only a small fraction of the uropygial microbiome has been cultured and identified, the cultured bacteria have not been able to limit the growth of *B.*

licheniformis, and the composition of the uropygial microbiome has been found to vary by species (Bodawatta et al., 2020). Therefore, if competition or antimicrobial substances are limiting the growth and colonization of *B. licheniformis* on the feathers, it is likely coming from bacteria that colonized the feathers through environmental contact and not from those that come from the uropygial gland.

The highest number of bacterial colonies were found on the dorsum, then the tail, and the least were found on the wing (Figure 1). This is similar to what was expected, but the tail was predicted to have the highest bacterial load. The dorsum may have the highest bacterial load because this would be the area most difficult for the owls to preen. There was not a significant difference in the number of morphotypes counted indicating that there is similar bacterial diversity across the three areas that were sampled (Figure 2). Although there was an observed trend with the tail having the most bacterial morphotypes, then the dorsum, and then the wing. Furthermore, a previous study noted that as bacterial diversity increased, abundance decreased (Saag et al., 2011). This trend was partially observed as the most diverse samples, those with the highest number of morphotypes, had less than 60 colonies and every sample that had more than 60 colonies, had less than 10 morphotypes, but this relationship was not significant (Figure 3).

Previous studies have reported a wide range of *B. licheniformis* prevalence levels. Burtt & Ichida, 1999 were the first to isolate *B. licheniformis* on wild birds and reported a prevalence of 6.7-10% from a mixed species study. They later conducted a study on Song Sparrows and found a 23% prevalence (2004). In 2005, Whitaker et al. published a multispecies study in which they found an overall prevalence of 26%, and a prevalence of 59% in Northern Saw-Whet Owls. Kent & Burtt found *B. licheniformis* in 39% of their multispecies study (2016). Two studies on

Eastern Bluebirds found rather high rates of 88% (Gunderson et al., 2008) and 99% (Gunderson et al., 2009). The results of this study (85.7%) are most similar to Gunderson et al., 2008.

There are many possible explanations for the wide variety of reported prevalence levels of *B. licheniformis* in these studies. One is the way in which the birds are sampled. For example, whether the swab used is run over the plumage, swabs are run deeper into the plumage, or a mix of swabbing and pressing the plate directly onto the bird. By doing this, different feathers are sampled. To elucidate, birds have several different types of feathers on their body that all have different structures and functions (Foth, 2020). Contour feathers have a long rachis with many stiff, hooked barbs (Foth, 2020). These are the feathers located on the outermost portion of the bird and include flight and tail feathers (Foth, 2020). Under the contour feathers are down feathers which have a very short rachis and long, soft barbs and are mainly used for thermoregulation (Foth, 2020). The outer contour feathers are the feathers that have been most highly sampled and examined, however any study that runs swabs deeper into the plumage are likely to also sample the down feathers. Due to the difference in structure, these feathers are likely to be impacted differently by feather degrading bacteria and may even have different microbiomes from each other.

Interestingly, the variation likely has little to do with what species are sampled as there are stronger differences in the prevalence of *B. licheniformis* between individuals of the same species than between different species (Bisson et al., 2006). There is a difference in prevalence in different foraging guilds but the relationship is not as simple as one would expect. *B. licheniformis* is a soil bacterium and thus it would be expected that ground foraging birds who have more contact with the soil would be more likely to have a higher prevalence of *B. licheniformis* (Burtt & Ichida, 1999). This is in fact what Burtt & Ichida, 1999 reported in their

first study. However, Kent & Burtt, 2016 found that ground foraging, fly-catching, aerial foraging, and foliage gleaning birds all had a similar high prevalence of *B. licheniformis* and that tree probing and nectivorous birds had the lowest prevalence of *B. licheniformis*. This difference can be partially explained by the fact the *B. licheniformis* is an endospore former and its spores can be spread through the air allowing for more opportunities for random environmental contact (Whitaker et al., 2005) although overall, the explanation for the observed differences still remains largely unknown.

There is also much variance in the plumage microbiome between seasons and time of year (Bisson et al., 2006) with a lower *B. licheniformis* prevalence found in the spring than in the late fall (Burtt & Ichida, 1999). This study collected samples in late fall when *B. licheniformis* is highly prevalent (Burtt & Ichida, 1999). Therefore, the time of year that the study is conducted may influence the reported prevalence.

In addition to determining a prevalence, Burtt & Ichida, 1999 also examined the distribution of *B. licheniformis* throughout different areas of a bird. The distribution of *B. licheniformis* in this study was slightly different to that reported by Burtt & Ichida, 1999 who found the highest prevalence of *B. licheniformis* on the venter region (44.9%), then the dorsum (30.2%), the tail (24.9%), and some *B. licheniformis* colonies were isolated from the wings but this was not a systematic sampling so no percentage was reported for the wing samples. However, in this study there were 6 *B. licheniformis* colonies on the dorsum (42.9%), 5 colonies were found on the tail (35.7%), and 3 *B. licheniformis* colonies on the wing samples (21.4 %, Figure 6). One explanation for these differences could be different tail sampling methods. In this study, both sides of the tail were sampled while Burtt & Ichida, 1999 only sampled the top of the tail. The difference can also be due to the vast difference in sample size: Burtt & Ichida, 1999

sampled 1,588 birds from many species and only 7 birds from the same species were sampled in this study. Ultimately, both studies support that *B. licheniformis* can colonize any area of many bird species and the wings have the lowest bacterial load.

The impact of *B. licheniformis* on live wild birds is still being determined. Overall, keratinolytic fungi and bacteria are being shown to have negative impacts (Rubiaee et al., 2017) but the extent of those impacts is uncertain. Keratinases degrade barb keratin which can result in decreased thermoregulation and flight maneuverability (Rubiaee et al., 2017). Furthermore, birds with higher fungal loads are more likely to be caught by predators and to have damaged feathers (Rubiaee et al., 2017). Birds that were preyed upon by a goshawk were found to have three times more feather bacteria than birds that were not preyed upon (Møller et al., 2012). However, *B. licheniformis* has not yet been shown to conclusively degrade feathers on live birds.

The first study to determine the effects of *B. licheniformis* on live birds examined Northern Cardinals and European Starlings and found no evidence of feather wear (Cristol et al., 2005). However, the Northern Cardinal experiment was conducted in the winter and *B. licheniformis* requires warm and moist conditions to be in a vegetative state (Cristol et al., 2005). Moreover, the experiment on European Starlings was conducted in warmer and moister conditions, but European Starlings are darkly colored birds and melanized feathers are more resistant to degradation than unmelanized feathers (Gunderson, 2008). A multi-species study did find a relationship between tail feather wear and presence of feather degrading bacteria and no relationship between tail feather wear and total bacterial load (Kent & Burtt, 2016). Due to this, it is likely that feather degrading bacteria at least slightly degrade feather keratin on live, wild birds but it is also important to note that tail wear can be due to multiple causes and this study was unable to conclusively show that the tail feather wear observed was due to feather degrading

bacteria. *B. licheniformis* may also be able to better colonize and degrade more worn feathers but this has yet to be tested (Kent & Burtt, 2016). Moreover, a study on Feral Pigeons found that Pigeons with lower bacterial loads had higher quality plumage (Leclaire et al., 2014).

Despite the lack of strong evidence for feather damage by feather degrading bacteria on live wild birds, there is ample evidence showing that feather degrading bacteria can impact feather coloration in some species which can have fitness consequences as color can be used by conspecifics to quickly gauge fitness. In addition to that, a change in feather coloration is thought to be one of the first signs of feather degradation due to bacteria (Shawkey et al., 2007). One major pigment in feathers is carotenoid-based pigments. These red. orange, and yellow pigments, are expensive for birds to maintain as carotenoids are also needed for immune function (Jacob et al., 2014) and birds are unable to synthesize carotenoids so they must consume them in their diet (Foth, 2020). A study on House Finches found that males with brighter red coloration, which is preferred by females, had similar bacterial loads but much lower feather degrading bacterial loads than duller males (Shawkey et al., 2009).

White is another color that is considered expensive to maintain because white feathers are more prone to both physical and bacterial degradation than melanized, dark feathers (Wails et al., 2018). In Pied Flycatchers, the white patches in males experienced degradation faster than in females and males with smaller white patches were males of lesser quality (Ruiz-De-Castañeda et al., 2012). In Northern Saw-Whet Owls, the bird used in this study, it has been found that individuals with a wider band of white around the facial disk had higher keel scores and fat scores which indicates high short-term and long-term fitness (Wails et al., 2018). During boom years when food is plentiful and many young are successfully hatched and raised, there are more owls with wide white facial bands around the eyes and during bust years when food is scarce,

there is less white around the eyes overall (Wails et al., 2018). Owls with a thinner white band around the eyes were found to have lower keel scores and fat scores (Wails et al., 2018). Thus, birds that are able to maintain expensive white coloration or colors due to carotenoid pigments are generally more fit than their conspecifics that lack these colorations or are duller.

Birds with blue coloration do not show the same trend with increasing bacterial loads or increasing feather degrading bacterial loads as birds with mainly melanin or carotenoid pigments do. In fact, increased bacterial loads are associated with brighter neck spots in pigeons (Leclaire et al., 2014) and brighter male Eastern Bluebirds (Shawkey et al., 2007). In Eastern Bluebirds, brighter males are known to have more success when competing for nest boxes and males with higher loads of feather degrading bacteria were also found to have higher body conditions (Gunderson et al., 2009). Therefore, it is possible that males with a higher bacteria load may be perceived as more fit. The explanation for this odd trend is that blue coloration and brightness are created mainly by the structure of the feather instead of diet like carotenoid pigments are (Foth, 2020). As a result, *B. licheniformis* degrades feathers in a way that enhances their ability to reflect blue wavelengths of light (Shawkey et al., 2007). Interestingly, female Eastern Bluebirds with higher bacterial loads were duller and of lower body condition which is opposite of how the males were affected (Gunderson et al., 2009). This is likely due to different structural compositions of the feathers sampled in male and female Eastern Bluebirds but could also be due to behavioral differences (Gunderson et al., 2009). Thus, the relationship between coloration and bacterial loads is complex and different for different pigments and colors.

It is also important to note that not every species studied thus far experienced a coloration impact from feather degrading bacteria. A study on Great Tits found no color changes between Great Tits on nests that were treated to favor bacterial growth, nests that were treated to inhibit

growth, and the control nests that were misted with water (Jacob et al., 2014). The study did find that Great Tits on nests treated to favor bacterial growth increased uropygial investment by modifying their preen oil composition and males increased the volume of their uropygial gland which might have resulted in the birds successfully preventing feather degradation (Jacob et al., 2014). Similarly, a study on Feral Pigeons also noted that pigeons with a higher bacterial load had increased preen oil production and secretion as well as time spent preening and pigeons with a lower bacterial load produced less preen oil and spent less time preening (Leclaire et al., 2014). Preen oil is composed of lipids and the waxes in preen oil protect feathers and skin by creating a barrier (Braun et al., 2018). In addition to that, it is possible that preen oil could also favor the growth of beneficial bacteria which would limit harmful bacteria, and potentially feather degrading bacteria, through competitive exclusion for both space and resources (Braun et al., 2018). In this study, it was found that owls with higher bacterial loads had less *B. licheniformis* colonies.

Birds also have other defenses against feather degrading bacteria including the general structure of feathers which are composed of tightly woven strands of β-keratin, melanized feathers which are structurally more resistant to bacterial and physical degradation (Gunderson et al., 2008) and sunbathing (Saranathan & Burtt, 2007). Feathers inoculated with *B. licheniformis* and exposed to sunlight had significantly less colony forming units than those that were not exposed to sunlight (Saranathan & Burtt, 2007). The reduction in the number of colony forming units is due to UV damage by sunlight (Saranathan & Burtt, 2007) The fact that sunlight inhibits *B. licheniformis* is consistent with previous studies that found lower *B. licheniformis* loads in the summer (Saranathan & Burtt, 2007). However, increased sunlight is not the sole explanation for lower *B. licheniformis* loads in the summer. Some birds also dust bathe which may further

reduce the abundance of feather degrading bacteria by drying out feathers and creating an unfavorable microenvironment (Gunderson 2008). Lastly, bird plumage in general does not provide optimal growth conditions for *B. licheniformis* and many bacterial species as it is not the moist, hot conditions many bacteria prefer (Cristol et al., 2005). During unfavorable conditions, *B. licheniformis* forms spores and is unable to degrade β-keratin in this state. Therefore, the keratinolytic ability of feather degrading bacteria may be strong, but birds possess many defenses to protect their feathers from degradation which may be why significant feather degradation by feather degrading bacteria is yet to be observed.

Conclusion

Ultimately, *B. licheniformis* and other feather degrading bacteria are widespread throughout many species and habitats (Kent & Burtt, 2016). However, they represent only a small subset of the entire feather microbiome. In this study, *B. licheniformis* colonies composed 1.17% of the culturable bacteria characterized. They can be found on any area on a bird but occur in higher abundances on the dorsum and tail, and lower abundances on the wings. Feather degrading bacteria have not been shown to conclusively degrade feathers on live, wild birds but they do alter the plumage coloration of many species (Gunderson et al., 2009; Leclaire et al., 2014; Ruiz-De-Castañeda et al., 2012; Shawkey et al., 2007; Shawkey et al., 2009). As other researchers have noted, it is important to remember that research on feather degrading bacteria has primarily focused on the effects of *Bacillus* species, especially *B. licheniformis*, and that other bacterial genera, fungi, and parasites have keratinolytic effects as well. Moreover, most studies, including this one, only examine culturable bacteria and there are innumerable unculturable bacteria that are yet to be studied. Thus, it is likely that the effects of keratinolytic organisms may be stronger than currently assumed because there are many keratinolytic organisms that are still poorly

understood and the total effect has not been investigated. Moreover, the vast majority of studies on wild birds have been conducted on songbirds from the Order *Passiformes*. In order to develop a more holistic understanding of the impact of keratinolytic microorganisms, birds from more Orders should be studied. This is because different birds have different behaviors and feather structure. As a result, feather degrading bacteria will likely impact Orders, Genera, and potentially even species differently.

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