UNIVERSIDAD DE GUADALAJARA

Centro Universitario de Ciencias Biológicas y Agropecuarias

Bacterias e interacciones en las plumas de aves silvestres, así como la composición bacteriana asociada a las plumas del colibrí corona violeta (*Leucolia violiceps*) en tres ambientes contrastantes

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que para obtener el grado de

Maestro en Ciencias en Biosistemática y Manejo de Recursos Naturales y Agrícolas

Presenta
Lizeth Nohemi Raygoza Alcantar

Zapopan, Jalisco **Julio de 2021**

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Por

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Maestría en Ciencias en Biosistemática y Manejo de Recursos Naturales y Agrícolas

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Por este conducto el (la) abajo firmante, autor(a) del Trabajo Recepcional (Tesis) titulada: "Bacterias e interacciones en las plumas de aves silvestres, así como la composición bacteriana asociada a las plumas del colibrí corona violeta (*Leucolia violiceps*) en tres ambientes contrastantes", declaro que el contenido del mismo constituye un documento inédito y original por lo que cumple con los términos de originalidad a los que se hace mención en el Artículo 73 del Reglamento General de Posgrado y el Artículo 147 Fracción I del Reglamento General de Titulación de la Universidad de Guadalajara.

A T E N T A M E N T E

Guadalajara, Jalisco, a 26 de mayo de 2020

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"Una ley natural que olvidamos es que la versatilidad intelectual es la compensación por el cambio, el peligro y la inquietud. La naturaleza nunca apela a la inteligencia hasta que el hábito y el instinto son inútiles. No hay inteligencia donde no hay cambio ni necesidad de cambio. Sólo los animales que cuentan con inteligencia tienen que hacer frente a una enorme variedad de necesidades y de peligros."

H.G. Wells

La Máquina del Tiempo, capítulo 10.

Para mi familia, quienes también sufrieron la realización y culminación de esta etapa de mi vida, por estar siempre conmigo, porque me enseñaron que lo imposible era posible y Porque siempre creyeron en mí

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ÍNDICE

ÍNDICE DE FIGURAS

ÍNDICE DE CUADROS

CAPÍTULO 1

CAPÍTULO 2.

RESUMEN

El estudio de la microbiota asociada a las plumas de las aves silvestres se ha incrementado en los últimos años. Debido a que, se han registrado vínculos entre estas comunidades bacterianas y las condiciones del hospedero. El plumaje de las aves está directamente expuesto al ambiente que las rodea, por lo que alberga comunidades bacterianas naturalmente diversas. En este estudio se realizaron dos capítulos los cuales abordaron 1) Una revisión de las investigaciones que ayudan a entender las características de la comunidad microbiana de las aves silvestres, principalmente las bacterias que se encuentran en las plumas; 2) La composición bacteriana de las plumas del colibrí *Leucolia violiceps* tres ambientes en relación con las variables bióticas y abióticas. El primer capítulo destacó que las plumas constituyen el hábitat de diversas comunidades bacterianas, entre las que se encuentran bacterias degradadoras de plumas, bacterias productoras de bacteriocinas y bacterias patógenas en diferentes especies de aves silvestres. Los estudios indicaron que las plumas que mostraron menor degradación son las de las especies nectarívoras; esto está relacionado con la melanización, la tinción iridiscente y las estrategias de vida. Así también, la glándula uropigial produce una secreción aceitosa que se extiende por el plumaje durante el acicalamiento, el cuál las protégé contra el desgaste causado por bacterias. El Segundo capítulo reportó que la abundancia y la composición bacteriana en el plumaje de *L. violiceps* cambiaron en diferentes ambientes y sitios, junto con las características bióticas y abióticas específicas del hábitat. Los adultos presentaron una mayor riqueza y carga bacteriana en sus plumas debido al aumento de las actividades necesarias para la supervivencia. La mayoría de los aislados eran simbiontes beneficiosos para las plantas y el huésped.

ABSTRACT

The study of the microbiota associated with the feathers of wild birds has increased in recent years, because links between these bacterial communities and host conditions have been recorded. Bird plumage is directly exposed to the surrounding environment, thus harboring naturally diverse bacterial communities. In this thesis work, two chapters were conducted which addressed 1) A review of research that helps to understand the characteristics of the microbial community of wild birds, mainly the bacteria found in feathers; 2) The bacterial composition of the feathers of the hummingbird *Leucolia violiceps* three environments in relation to biotic, and abiotic variables. The first chapter highlighted that feathers constitute the habitat of diverse bacterial communities, including feather-degrading bacteria, bacteriocin-producing bacteria and pathogenic bacteria in different species of wild birds. The studies indicated that the feathers that showed less degradation are those of nectarivorous species; this is related to melanization, iridescent staining and life strategies. Also, the uropygial gland produces an oily secretion that spreads through the plumage during preening, which protects them against wear caused by bacteria. The second chapter reported that the abundance and bacterial composition in the plumage of *L. violiceps* changed in different environments and sites, along with habitat-specific biotic and abiotic characteristics. Adults have a higher richness and bacterial load in their feathers due to increased activities necessary for survival. Most isolates were symbionts beneficial to plants and host.

INTRODUCCIÓN

El conocimiento de los microorganismos de las plumas de las aves tienen permiten comprender los mecanismos de adaptación del organismo huésped a partir de la asociación de su plumaje y su diversidad microbiana (Ross et al. 2019). Razón por la cual, se analiza el funcionamiento del sistema inmunológico del huésped (Gomez et al. 2013; Woodhams et al. 2014) y el origen de los microorganismos patógenos (Kulkarni y Heeb 2007).

Se ha comprobado que la coloración del plumaje de la especie huésped juega un papel importante en la microbiota de las plumas (Kyptová 2017). Cabe mencionar, que cada estructura de la coloración responde de manera distinta a la variación microbiana de la pluma (Jacob et al. 2014). Como es el caso de la coloración estructural iridiscente que funciona como una protección a la degradación bacteriana (Javůrková 2019b). Asimismo, la coloración a partir de melaninas con tonos más intensos la cual, no presenta afectaciones a la degradación del plumaje, ya que las aves tienen la capacidad de remplazar sus plumas mediante las mudas (Jacob et al. 2014). Además, la fisiología del huésped es también un factor que determina la composición bacteriana por medio de la producción del aceite de la glándula uropygial, que al estar en contacto con las plumas por la acción del acicalamiento, actúa como una barrera protectora que equilibra las comunidades microbianas (Jacob et al. 2018), produciendo enzimas inmunes que antagonizan cepas que pueden ser perjudiciales para el plumaje (Carneiro et al. 2020).

Las comunidades bacterianas de las plumas están fuertemente influenciadas por el hábitat en el que viven, la alimentación, la migración, el acicalamiento, la coloración del plumaje, las mudas y la temporada de anidación (Bisson et al. 2009; Saag et al. 2011; Goodenough et al. 2016; Jacob et al. 2018; Javůrková 2019a; Javůrková 2019b). Sin embargo, se conoce muy poco sobre los cambios que estas comunidades podrían presentar en ambientes más hostiles. El incremento de las consecuencias de los cambios globales, como el desarrollo de las ciudades y las actividades agrícolas comienzan a hacer preocupantes, debido a que se ha generado gran reducción de hábitats y la extinción de especies silvestres (Olivier et al. 2020).

Las aves son los organismos más aceptados por las personas debido a su colorido plumaje, el vuelo y sus cantos (Del Hoyo et al. 1992; Cocker y Tipling 2003). Son las especies más

estudiadas porque son de gran valor como indicadores del ambiente y son el mayor contacto que las personas tienen con la vida silvestre (Tietze 2018). Sin embargo, el aumento de áreas urbanas reducirá las posibilidades de que las poblaciones de la mayoría de aves silvestres sobrevivan en estos ambientes, y por consiguiente, una mayor interacción con los humanos (Hedblom y Murgui 2017). Como se mencionó anteriormente, la distribución y diversidad de las aves está fuertemente vinculada con la composición microbiana que habita en el ambiente (Ritter 2019), por lo que han sido utilizadas como un bioindicador de la alteración de hábitats en zonas urbanas y rurales (Giraudeau et al. 2017; Hassell et al. 2019).

El colibrí *Leucolia violiceps* tiene una distribución amplia desde el suroeste de Estados Unidos hasta el noroeste de México (Arizmendi et al. 2014). Una de sus principales características es que mantiene una corona azul-violeta, la parte del pecho es color blanco y la espalda color marrón. Prefiere vivir en bosques con zonas ribereñas. Pero también se encuentra en ambientes urbanos (Sibley 2000). Esta especie se alimenta principalmente del néctar de las flores y complementa su dieta con insectos, aunque se ha observado que se alimenta también de algunos frutos (Palacio 2019). Los colibríes son uno de los polinizadores más importantes de América porque son los encargados de polinizar cientos de plantas silvestres, donde algunas son de interés económico como el agave (Magaña et al. 2008)

Se ha comprobado la importancia acerca de la relación de las bacterias y su hospedero. Sin embargo, aún quedan varias cuestiones por responder. Por ello, esta tesis realizó una revisión amplia acerca de las investigaciones que se han publicado sobre la composición bacteriana asociada con las plumas en los últimos 20 años. Además, se generaron hipótesis nuevas acerca de los procesos coevolutivos entre aves y bacterias, y sugerimos análisis ecológicos acerca de los factores que influyen en estas comunidades bacterianas. Además, analizamos la composición bacteriana que se encuentran en el plumaje del colibrí Corona violeta (*L. violiceps*) con cultivos *in vitro*, se secuenció el gen 16S del RNA bacteriano para dentificar especies bacterianas. Asimismo, se hizo una caracterización funcional en los ambientes urbano, agrícola y natural, así como se analizó la influencia de las variables bióticas y abióticas en la diversidad y abundancia de estas comunidades bacterianas.

OBJETIVO GENERAL

- Realizar una descripción de las investigaciones sobre la composición bacteriana asociada al plumaje de las aves silvestres y analizar la composición bacteriana de las plumas del colibrí *L. violiceps* en ambientes contrastantes.

OBJETIVOS PARTICULARES

- Realizar una revisión de las investigaciones que existen acerca de la composición bacteriana en las plumas de las aves silvestres en los últimos 20 años, para sugerir hipótesis nuevas acerca de las interacciones ambientales aves-bacterias y los procesos coevolutivos que desarrollaron para protegerse contra el ataque microbiano.
- Determinar si la composición bacteriana de las plumas del colibrí *L. violiceps* difiere en los tres ambientes (urbano, agrícola y natural) en relación con las variables bióticas (disponibilidad de recurso, medidas morfológicas del colibrí) y abióticas (temperatura y humedad).

HIPÓTESIS

Las bacterias del plumaje están influenciadas por varios factores, entre ellos las características específicas del hábitat (variables bióticas y abióticas), por la cual, la composición bacteriana de las plumas de *L. violiceps* presentará variación entre los sitios y los ambientes estudiados.

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CAPÍTULO 1

Bacteria in body feathers of wild birds: a review of bacterial composition, their interactions with the environment and coevolutionary processes.

ABSTRACT

Study of the microbiota associated with wild bird feathers has increased in recent years since links have been recorded between these bacterial communities and features related to their host conditions. Bird plumage is directly exposed to the environmental conditions that surround the birds, and is host to naturally diverse bacterial communities as a consequence. This study reviews the research that helps further our understanding of wild bird microbial community characteristics, specifically the bacteria found in body feathers. We review 139 scientific studies published over the last 20 years. Three main topics are addressed: 1) the interactions of bacterial communities with their host and environment, 2) the importance of plumage coloration in terms of bacterial degradation, and 3) the effect antimicrobial of the oil produced by the uropygial gland on bacterial communities that inhabit the feathers. We highlight the fact that feathers constitute the habitat of diverse bacterial communities, including feather-degrading bacteria, bacteriocin-producing bacteria and pathogenic bacteria, in different wild bird species. The studies also indicated that the feathers of nectarivorous species showed lower degradation. This is related to melanization, iridescent staining and life strategies, since nectarivorous species invest more in plumage maintenance through secretion from the uropygial gland. This gland produces an oily secretion that is spread through the plumage during preening, containing chemicals that may function as a wide-spectrum antimicrobial defense, thus favoring the maintenance of a diverse microbiota. Although many advances have been made in the last 20 years, future research should include abiotic environmental variables (e.g., temperature, precipitation), in addition to evaluating changes generated by anthropogenic activities, such as habitat fragmentation and urbanization, as well as their relationship to underlying processes of bird-associated microbiota.

Keywords: bacteria, wild birds, feathers, interactions, microbiome, microbiota

INTRODUCTION

Research of microbiomes in different ecosystems and diverse animal taxa has found evidence for an apparent coevolution between microorganisms and their hosts (Javůrková *et al.* 2019a). Study of the microbiota associated with wild bird feathers has increased in recent years since links were recorded between these bacterial communities and features related to their host conditions (Fülöp *et al.* 2016, Leclaire *et al.* 2015). Feathers are the most external bird tegument, are composed of 90% protein and almost pure keratin (Echeverry-Galvis *et al.* 2013, Pap *et al.* 2015) and are involved in thermoregulation (Osváth *et al.* 2018), courtship display (Penteriani *et al.* 2017) and flight (Echeverry-Galvis *et al.* 2013, Pap *et al.* 2015). Bird plumage is directly exposed to environmental conditions and, as a result, hosts naturally diverse bacterial communities (Shawkey *et al.* 2005, Bisson *et al.* 2009, Javůrková *et al.* 2019a).

A range of techniques have been used to evaluate the bacterial communities associated with feathers, prominent among which are the culture-based methods. These features the use of culture media, containing the nutrients necessary to allow growth of different bacterial communities (Burtt & Ichida, 1999, Dille *et al.* 2016, Giorgio *et al.* 2018), or selective culture media such as the solid medium feathers, used only for the growth of keratin-degrading bacteria (Lucas *et al.* 2003). However, the use of agar media does not allow evaluation of the total bacterial diversity associated with feathers, since around 99% of these microorganisms are not culturable (Amann *et al.* 1995; Valenzuela-González *et al.* 2015). The agar media method is therefore used mainly when the research is focused on cultivable bacterial communities. Moreover, it represents a lower-cost tool for the study of associated microorganisms in wild birds. On the other hand, there are other independent culture methods, which are more precise and effective in obtaining a greater diversity of bacterial communities; for example, quantitative PCR (qPCR) targeting the 16S rRNA gene in the extracted microbial DNA, which allows the evaluation of several non-cultivable bacterial genera (Javůrková *et al.* 2019b). There are also new generation techniques including high-throughput sequencing of the 16S rRNA gene or, more precisely, metabarcoding per 16S rRNA gene, since this allows a greater number of bacterial genera and species to be

sequenced that would be otherwise impossible to obtain using culture-dependent methods (Valenzuela-González *et al.* 2015; Musitelli *et al.* 2018; Javůrková *et al.* 2019a).

Here, we review the research that has helped further our understanding of bird microbial community characteristics, mainly the bacteria found in body feathers and the interaction they have with their host. Studies were reviewed based on both culture-dependent and culture-independent techniques. We focus on studies that 1) describe the interactions of these bacterial communities with their host and environment, 2) the importance of plumage coloration in terms of bacterial degradation and 3) the effect antimicrobial of the oil produced by the uropygial gland on bacterial communities that inhabit the feathers. In addition to addressing these three approaches with the literature review, our main purpose in this review was to identify the lack of studies involving environmental abiotic variables, in many cases, as variables responsible for the formation of bacterial communities in wild birds.

DATA SOURCES

An extensive literature search was conducted on the research and advances in bacterial communities associated with the feathers of wild birds, as well as the influence of the environment on these communities, coloration of the plumage as a protective structure against microbial wear and the effect of oil from the uropygial glands on bacterial communities. Literature published from 1999 to 2020 was consulted (Figure 1) using specialized web search engines such as Web of Science, Google Scholar, BioOne, Springer link and SpringerNature. Different combinations of the following keywords were entered for the search: ´wild birds´, ´feather´, ´plumage´ ´bacterial´, ´ecology´ and ´preen gland´. Our criticisms and suggestions for new hypotheses presented in this review were formulated based on the different identification techniques used in each study. We found a total of 139 papers related to the topic of interest; however, not all of these studies are reviewed in detail in this paper (Supplementary 1).

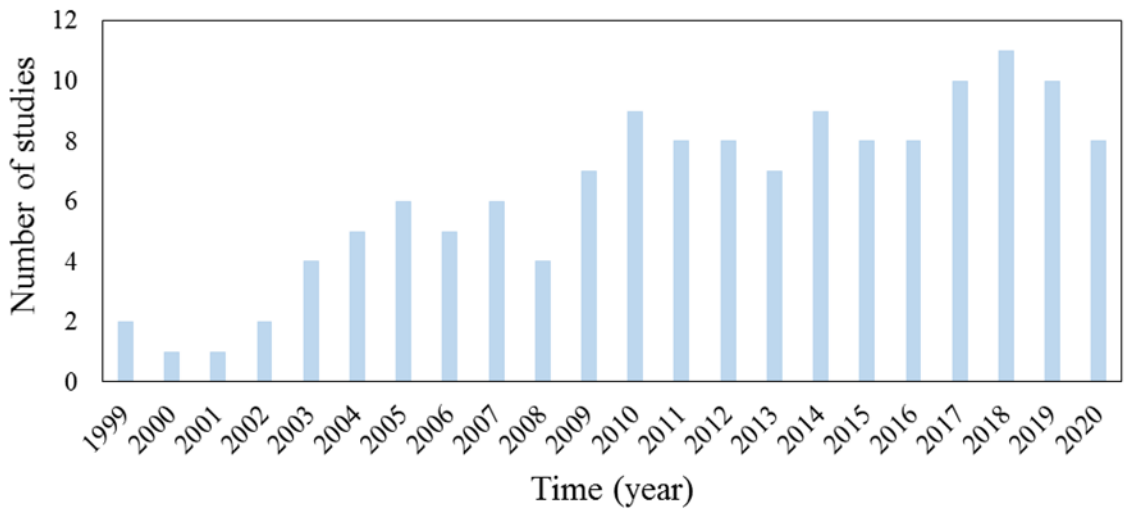

Figure 1. Number of scientific publications (139) reviewed regarding bacterial interactions associated with the body feathers of wild birds.

Bacterial composition associated with the feathers of wild birds and interactions between these bacteria and the environment.

Feathers constitute a habitat for diverse bacterial communities (Table 1) including feather-degrading bacteria (FDB), bacteriocin-producing bacteria (BPB) (Burtt & Ichida 1999; Shawkey *et al.* 2003, Kent & Burt 2016, Javůrková *et al.* 2019a), and pathogenic and beneficial bacteria (Bisson *et al.* 2009, Dille *et al.* 2016, Giorgio *et al.* 2018), in different wild bird species.

Feathers are composed of keratin, which protects birds from abiotic stress and biotic attacks. Nevertheless, microbial degradation of keratin is not a very extensive biochemical process in nature, and keratin can serve as an effective defense even against microbial attacks (Lingham-Soliar *et al.* 2010, Lange *et al.* 2016). The FDB include a wide diversity of genera, generally comprising bacteria found in the environment; however, when these bacteria are forced to live in an environment such as feathers, keratinase production increases (Lucas *et al.* 2003) and the bacteria become capable of extracting energy and nitrogen from the keratin. Groups of proteolytic enzymes, divided into serine proteases, metalloproteinases, thiol and aspartic keratinases (Lange et al. 2016), perform the biodegradation of this protein.

Table 1. Bacterial genera recorded in wild bird feathers

Bacterial genus	Bird taxa	Technique	Conclusion	Reference
Bradyrhizobium, *Rhizorhapis*, *Ralstonia*	*Malurus alboscapulatus* (*M. a. moretoni*, and *M. a. lorentzi*)	DGGE-PCR	Birds with iridescent plumage maintain lower load and high microbial diversity in the feathers in contrast to birds with melanized plumage.	Javůrková *et al.* 2019b
Bacillus, *Streptomyces*, *Kocuria*, *Staphylococcus*	Passerine	The scope of high-throughput sequencing	Bird feathers harbor species-specific bacterial communities that are likely to produce antimicrobials similar to mammalian skin.	Javůrková *et al.* 2019a
Bacillus, *Curtobacterium*, *Pantoea*, *Enterobacter*	*Muscicapa striata*, *Hippolais icterina*, and *Sylvia borin*	Culture-dependent (nutrient agar)	The transfer of bacteria, including pathogens, through bird migration between very distant countries.	Giorgio *et al.* 2018
Ralstonia, *Pseudomonas*, *Aeribacillus*, *Arenimonas*	*Apus apus*	The scope of high-throughput sequencing	High intraspecific variability in the composition of bacterial plumage communities, greater than interspecific variability.	Musitelli *et al.* 2018
Bacillus, *Lysinibacillus*, *Paenibacillus*, *Staphylococcus*, and *Clavibacter*	*Junco hyemalis*	Culture-dependent (nutrient agar)	Free-living migratory birds may carry bacteria throughout their geographic ranges and may transmit pathogens and beneficial bacteria to plants.	Dille *et al.* 2016
Lysinibacillus, *Bacillus*, *Paenibacillus*, *Cohnella*, and *Pseudomonas*	Trochilidae	Culture-dependent (feather-agar)	The feather's bacterial microbiome was mainly composed of members of the Bacillaceae family (sporulated bacteria) and a few members of the Pseudomonadaceae family.	Góngora *et al.* 2016
Bacillus, *Klebsiella*, *Enterobacter*, *Hafnia*, *Pseudomonas*	*Turdus nudigenis*	Culture-dependent (feather-agar)	Registration of new Gram-negative bacterial isolates capable of degrading keratin	Verea *et al.* 2014

Aeromonas, Bacillus, Enterobacter, Escherichia	*Cyanistes caeruleus* and *Parus major*	Culture-dependent (nutrient agar)	Substantial intraspecific variation in nest microflora, and significant interspecific differences, both in terms of individual microbes and overall.	Goodenough & Stallwood 2010.
Bacillus, Pseudomonas, Xanthomonas, Rhizobium	*Setophaga ruticilla, Geothlypis trichas, Vireo modestus* and *Cardinalis cardinalis*	LH-PCR	Migration and season play an important role in the dynamics of the microbial community in bird plumage and may reflect patterns of dispersal of pathogens by birds.	Bisson *et al.* 2009.
Bacillus, Microbacterium, Pantoea, Serratia	*Sialia sialis*	Culture-dependent (nutrient and feather agar)	Bacteria can alter the color of structural plumage by degradation, the brightness and purity were increased, but UV chroma was decreased, and did not affect the tone of the structural color.	Shawkey *et al.* 2007.
Bacillus, Streptomyces	*Branta canadensis, Oxyura jamaicensi,* and *Parkesia noveboracensis.*	Culture-dependent (nutrient agar)	Keratin degradation is greatest during the summer and this can contribute to feather deterioration and be a selective force in evolution and molting time.	Burtt & Ichida 1999.

The first studies related to the bacterial communities with the functionality of degrading the keratin of feathers in wild birds and the relationship between the bacteria and the host habitat began in 1999; when Burtt and Ichida (1999) described the prevalence of FDB in temperate forests of Ohio and Massachusetts in the United States of America, and in Canada. These authors recorded the prevalence of *Bacillus licheniformes*, *B. pumilus* and *B. subtillis*, which are capable of degrading keratin in 32 of 83 studied wild bird species. They also observed that ground-foraging birds had a high prevalence of FDB, indicating that the bacterial species capable of degrading keratin were those that lived in the soil and had changed their functionality in order to survive in the feathers. However, there was insufficient evidence to prove this. Furthermore, from this study, new unknowns were created with regard to bird-bacteria interactions. Lucas et al. (2003) cultured soil samples from a forest in Lausanne, Switzerland, using a medium enriched with pigeon feathers. They observed the growth of

bacterial groups such as Cytophagales, Actinomycetales and Proteobacteria capable of degrading keratin with an effectiveness of 98%. This proved the hypothesis that the bacteria came from the environment inhabited by the host and had adapted to the extreme conditions of the plumage. They also found that the ability to degrade keratin was not exclusive to bacteria of the genus *Bacillus*, but that in fact a large number of strains present this functionality. However, we must take into account that the research by Burtt and Iquida (1999), and another similar study by Kent and Burtt (2016), only addressed the prevalence of the genus *Bacillus* in their analyses, and this genus generates a low proportion of FDB in other trophic guilds such as aerial, bark-probing, and nectarivores. The importance of these two studies should be noted, since they showed that bacterial composition is influenced by both environment and lifestyle, and that the presence of environmental *Bacillus* is associated with wild bird feather degradation. It is necessary to analyze other bacterial genera or species capable of growing in feather culture media in order to find a higher prevalence of FDB in the trophic guilds mentioned above.

An approach that should be noted in the study of bird-bacteria interaction is that of determination of the influence of environmental factors, such as annual seasonality and changes in the habitat experienced by migratory birds when they travel to warmer areas in the non-reproductive stage, as well as that of residents in search of food. A study by Bisson et al. (2009) based on the culture-independent technique in feather samples of resident species of Red Cardinal (*Cardinalis cardinalis*) from a Nearctic zone in Maryland, a resident Jamaican Vireo (*Vireo modestus*) from the Neotropics, and the migratory species American Redstart (*Setophaga ruticilla*) and Common Yellowthroat (*Geothlypis trichas*) showed that, particularly at the beginning of these migrations, the bacterial diversity of birds with migratory routes differed significantly from the strains found in resident birds. Likewise, significant differences were recorded throughout the annual cycle in bacterial communities in the non-breeding season in autumn and winter and in the reproductive season, which confirms that both migration and climatic variation in resident birds act to change the bacterial diversity in the plumage. This result was similar to that reported by Burtt and Iquida (1999) in terms of the prevalence of the genus *Bacillus* (FDB) in the plumage, where they recorded a greater abundance in the winter season, explaining that *Bacillus* species form spores, which are adaptation structures for ensuring survival in times of environmental stress,

and that the birds in this season reduce the maintenance of their plumage, generating an increasing in the probability of survival and establishment of spores. For this reason, plumage degradation activity did not vary between seasons, which raises the following question: do all bacterial genera capable of producing spores have a greater advantage in terms of establishing themselves within the plumage? If so, we could suggest that pressure from these spore-forming bacterial assemblages that act to influence plumage wear may have influenced the activation of the complete or partial molting process in the reproductive season.

Moreover, Verea et al. (2014) recorded 14 new strains of FDB in the Spectacled Thrush (*Turdus nudigenis*). However, unlike the other studies, the effect of seasonality between the rainy and dry seasons was not found to significantly change the abundance and diversity of FDB. This was associated with the fact that they did not contemplate the effect of the winter season. Returning to the previous question about which other possible bacterial groups associated with plumage could participate in bacterial degradation, these authors recorded new species with high rates of keratin degradation, including *Bacillus cereus*, *Klebsiella oxytoca*, *Enterobacter cloacae* and *Hafnia alveiy*. These communities would be important to include when analyzing the factor of food guilds in wild birds. For example, Góngora et al. (2016) cultivated feather samples in Andean hummingbirds and recorded new bacterial genera with keratinolytic activity for this guild in addition to *Bacillus*, including *Lysinibacillus*, *Paenibacillus*, *Cohnella* and *Pseudomonas*. So what evidence is there to suggest that these strains are also involved in plumage degradation in nectarivores? and how differently might their abundance compare to that in other guilds? In addition, the question of whether non-culturable bacteria are present in birds with different habits remains to be addressed.

Other important, and perhaps less studied, groups recorded in the plumage of wild birds comprise bacteria that can be pathogenic or beneficial to other organisms. In resident and migratory birds, Dille et al. (2016) and Giorgio et al. (2018) recorded the presence of genera such as *Frigoribacterium* and *Kitasatospora*, which are known to be beneficial for host plants (Sessitsch et al. 2004, Haesler et al. 2008, Yashiro et al. 2011), and *Agrobacterium* and *Sphingomonas* bacteria, which are potentially pathogenic for plants (Garrity et al. 2005). Likewise, the genera of *Staphylococcus*, *Klebsiella*, *Enterobacter*, *Serratia*, *Arthrobacter* and

Pantoea ananatis have been recorded, and these are responsible for a wide array of infections in warm-blooded animals and humans (Ruoff et al. 1993, Mages et al. 2008, Smith et al. 2020). These studies suggest that there are possible multiple factors that lead to disease outbreak and that it must be considered that a single exposure may not necessarily cause disease in humans, animals, and plants. Moreover, it is hypothesized that these bacterial strains are only found temporarily in the feathers and that the birds act as reservoirs that can transport them through the geographical ranges in which they move, especially when these are migratory birds. Finally, they showed that certain bacterial species exist in strict association with a specific host, while others appear to be generalists (Dille et al. 2016; Giorgio 2018). It would be important to determine whether these bacterial groups are the cause of the microevolutionary changes that wild birds have developed in order to adapt to different habitats. More information is required on wild bird diseases associated with the bacteria on their feathers.

The scope of high-throughput sequencing

Two studies based on high throughput 16S rRNA sequencing and integrating a massive number of bacterial sequences that inhabit the plumage were reviewed. In the first, Musitelli *et al.* (2018) analyzed the bacterial composition in the feathers of the common Swift (*Apus apus*), a migratory bird in San Paolo, Italy. These authors determined a great diversity of Operational Taxonomic Units (OTUs), which indicated high intraspecific variability. This study described a high amount of *Bacillales* (keratinolytic activity) and other genera, such as *Virgibacillus* and *Lentibacillus*, which are moderately halophytic and found in saline or hypersaline habitats (Jeon et al. 2005, Zhang et al. 2016). They also found members of the genera *Hymenobacter*, *Methylobacterium*, and *Sphingomonas*, strains of which have been described colonizing plant leaves (Vorholt 2012, Bulgarelli et al. 2013, Gandolfi et al. 2017). It was also determined that the bacterial composition was unaffected by the corporal morphological characteristics of the common Swift, such as weight, body length, wing size and sex. This could be due to the fact that the two sexes share parental care duties. These results confirm, as previously described, how the life strategies of the host are a factor that

strongly influences microbial community dynamics. Likewise, they provided a broader view of the bacterial groups that inhabit the plumage, with 275 registered genera. However, it is not only important to determine what inhabits the feathers, but also to integrate the functionality of these strains, whether they are in the plumage for long or short periods, using bioinformatics tools (Langille, et al. 2013).

In the second study using high throughput sequencing and phylosymbiosis study, Javůrková et al. (2019a) analyzed the functionality of FDB and bacteriocin-producing bacteria (BPB) to analyze bird-bacteria interactions. The term phylosymbiosis refers to a significant association between host phylogenetic relationships and host-associated microbial community relationships, wherein 'phylo' refers to host clade and 'symbiosis' refers to the microbial community in or on the host (Lim & Bordenstein 2019). Javůrková et al. (2019a) described for the first time the presence of BPB in the feathers of wild birds. These are strains that produce bacteriocins or antimicrobial compounds that can be used as a defense system by various microorganisms (Riley & Wertz 2002; Chao & Levin 1981, Riley & Wertz 2002).

Javůrková *et al.* (2019a) described the microbiota associated with the feathers of seven free-living passerine birds, of which two were residents and five were long-distance migratory birds. This study reported the host species identity, phylogeny and geographic origin, concluding that these characteristics were important in terms of the bacterial composition of passerine birds, as well as the bacteriocin-producing capacity of the bacteria. They also reported a prevalence of 1.4% for FDB, 10.8% for both FDB and BPB and 13.7% for BPB only. The authors highlighted that the microbial assemblage associated with feathers was driven to a certain degree by specific environmental niche characteristics, but that specific factors of the host had a stronger effect. Likewise, a positive association was found between BPB and FDB host species, since the majority of the FDB (mainly from the *Bacillus* and *Pseudomonas* genera) are also capable of producing bacteriocins (Ghequire *et al.* 2017, Lim *et al.* 2016, Javůrková *et al.* 2019a). Population studies (i.e., of the same bird lineage) are required to corroborate these results.

A phylosymbiosis pattern was observed for feather microbiota in phylogenetically related species, which might indicate intensive coevolution between the host and their microbiota (Delsuc *et al.* 2014), although phylosymbiosis needs to be conditioned not only by

coevolutionary history, but also by environmental and specific factors of the host that may vary and contribute significantly to the microbiota assemblage (Carrier & Reitzel 2017, Brooks *et al.* 2016, Javůrková *et al.* 2019a). However, this research focused only on the diversity of FDB and BPB and it is necessary to increase the number of investigations based on high-throughput sequencing in different groups of birds and with holistic approaches in terms of bacterial composition to strengthen the hypotheses regarding the coevolutionary history of bird-bacteria interactions.

Composition of the bacteria that coexist in the feathers

The composition of the bacteria that coexist in the feathers is strongly influenced by environmental conditions, since significant differences have been recorded in both migratory and short-distance birds, as well as interaction with other organisms (ectoparasites, plants and animals). Likewise, the intra- and interspecific differences reinforce the importance of the life strategies of avian hosts as determinants in the bacterial composition in the feathers. However, there is a need to integrate other tools, such as landscape ecology analysis, which can be useful for understanding the distribution of microbial communities at the landscape level in order to determine the microbial core in different habitats. It is important to mention that increased research could generate a meta-analysis that includes the bacterial diversity recorded in different species of wild birds as well as the different environments in which they are distributed (urban, rural, riparian forest, temperate forest, rainforest, coastal and marine) (Hung-Ming et al. 2020). This would elucidate how the different environments in which birds move both long and short distances can bring about significant changes in the diversity of the bacterial assemblages associated with feathers.

Adaptation structures against bacterial-induced degradation of feathers

Plumage colorations

Birds have different adaptation mechanisms by which to achieve the coloration of their plumage, which can be classified into two groups: coloration based on pigments (carotenoids,

porphyrins, psittacofulvine and melanins), and structural colorations produced by the effect of microstructures in the keratin on incident light (iridescent, chromatic and white plumage) (Fox & Vevers, 1960; Finger, 1995; Siefferman & Hill, 2003).

Studies based on association of FDB and wild birds have reported a prevalence of 69 to 88% of these bacteria in their feathers in different habitats (Whitaker *et al.* 2005; Shawkey *et al.* 2007), indicating that the recorded bacterial strains capable of inhabiting extreme plumage conditions played an important role in the evolution of molt development and apparently in the plumage pigmentation of wild birds (Whitaker *et al.* 2005, Gunderson 2008). In recent years, several studies have evaluated resistance to bacterial degradation in melanized and iridescent feathers, and have observed that the life strategies of species and the environments they inhabit play a crucial role for melanin-degrading bacteria or FDB. Burtt (1986) and Bonser (1995) were the first to report that pigmented feathers were more resistant to FDB than non-pigmented feathers, but these authors did not know why the melanized feathers presented greater resistance. This result could most likely be explained by the fact that pigmented feathers are harder and tend to be more resistant to physical abrasion (Goldstein *et al.* 2004). Other studies have suggested an approach to coevolutionary behavior between FDB and feather coloration (Gunderson 2008). For example, *Bacillus licheniformis* strains isolated from a subspecies of the Song Sparrow (*Melospiza melodia morphna*) degraded non-pigmented chicken (*Gallus gallus domesticus*) feathers faster than *B. licheniformis* isolated from another subspecies of the Song Sparrow (*Melospiza melodia fallax)*. Based on this result, Burtt and Ichida (2004) suggested that an evolutionary "arms race", with progressive increases in bacterial efficiency, could be occurring through selection of birds with higher melanin deposition and vice versa. Shawkey *et al.* (2007) determined whether the structural plumage (not pigmented) could regulate degradation by bacteria with keratinolytic activity and other bacterial groups cultivated from adult male individuals of the Eastern Bluebird (*Sialia sialis*), and compared this with the activity observed in feathers of more opaque tonality in juvenile male individuals. The adult males presented a higher keratinolytic bacterial load, which increased plumage brilliance. These authors argued that this was because a large number of bacteria are beneficial and can exclude the growth of FDB, as is the case in the microbiota of human skin. However, years later, these bacteria were found to inhabit the commensal and symbiotic bacteria (BPB) that help maintain plumage (Javůrková

et al. 2019a). It was also observed that, under *in vitro* conditions, reflectance of ultraviolet light from the plumage decreased without affecting the structural color. It was argued that this process was due to wear of the feather bark induced by FDB. This wears left the spongy layer of the structurally colored tines exposed, possibly causing the bacteria to be less affected by UV radiation (Prum 2006, Örnborg et al. 2002, Shawkey *et al*. 2007).

We cannot be sure whether the results published by Shawkey *et al.* (2007) are present under *in vivo* conditions, since the effects of seasonality and biological interactions participate in the environment; for example, the study of Jacob *et al.* (2014) attempted to demonstrate for the first time whether keratinolytic bacteria could degrade feathers *in vivo* in a wild reproductive population of the Great Tit (*Parus major*). These authors collected 15 feathers from different body parts of both female and male individuals, measured the color, performed spectrophotometric analyzes and, during the following autumn, followed the same procedure with individuals that were under the same treatment in order to corroborate the longer-term effects on plumage coloration. To estimate bacterial composition, they used culture-dependent (nutritive agar and feather agar) and cultures-independent (automated approach for ribosomal intergenic spacer analysis, ARISA) techniques. Keratinolytic bacterial densities did not affect feather coloration during the nesting period or coloration of the recently molted feathers. The authors stated that keratin-degrading bacteria do not affect the feathers of live birds since birds can equilibrate these communities by preening. Likewise, molting in birds allows the replacement of old and worn feathers with faded colors with new feathers (Delhey *et al.* 2010, Giraudeau *et al.* 2010).

Conversely, these analyses only reflect the results for a single species; the results could be different if other groups of birds with different life strategies and plumage were to be integrated, as described by Kyptová (2017) who analyzed the bacteria of the plumage of 47 tropical bird species of Afromontane regions. This raises the question of whether the selected characteristics of tropical bird species and their life strategies affected the total bacterial load of the plumage and its capacity to resist bacterial degradation, with a suggestion that the resistance of the feathers to bacterial degradation shows a moderate dependence on the phylogenesis of the species. For example, it was observed that the feathers that showed lower degradation were those of nectarivorous species; this result is related to melanization,

iridescent staining (Shawkey *et al.* 2007), and life strategies (Kent and Burtt 2016; Bisson *et al.* 2009), since nectarivorous species invest more in plumage maintenance (e.g., preening) through the secretions of the uropygial gland (Giraudeau *et al.* 2010, Jacob *et al.* 2014).

The relationship between iridescent feathers and bacterial composition was studied in passerine birds (Javůrková *et al.* 2019b). These authors studied females and males of two subspecies of *Malurus alboscapulatus* (*M. a. moretoni* and *M. a. lorentzi*) in New Guinea, and discovered that microbial load was lower and microbial diversity of the feathers was significantly higher in the plumage of iridescent black males, compared to matte black females and brown individuals. Moreover, the males had different feather microbial communities than the females and brown individuals, which supported the conclusion that structural (i.e., iridescent) plumage coloration is a factor that may regulate feather microbiota, as has been studied by different authors (Goldstein *et al.* 2004, Burtt & Ichida 2004, Gunderson *et al.* 2008, Delhey 2018). Melanin deposition is one of the mechanisms used to protect feathers against degradation and the action of ectoparasites such as lice, FDB, and other bacteria. Given these results, it has been proposed that the iridescent nanostructure of feathers, which is based on sexually selected pigments, can reduce heat loss when reflecting solar energy since iridescent feathers often have reflectance peaks in the ultraviolet A spectrum (UVA) and B (UVB), which can be lethal to most microorganisms.

Regarding the increase in studies conducted, we could presume that the resistance of plumage to bacterial degradation is multifactorial (i.e., presence of ectosymbionts, production of bacteriocins, grooming, molting strategy, behavior, and plumage type); however, the effect of UV rays *in vivo* on pigment-based and structural plumage has not been extensively studied. More studies are therefore required in which comparisons are made between samples of feathers exposed to UV rays *in vitro* that simulate a "natural" environment (300 to 400 nm) and feathers exposed to solar radiation *in vivo* (measuring the times of exposure to UV light), and where the strategies of the species under evaluation are integrated (e.g., feeding habits, grooming rates, and molting). It is necessary to compare results in terms of the resistance to bacterial degradation between the different plumage coloration mechanisms (based on pigments and non-pigment or structural). While little is known about how each of the mechanisms of coloration function in terms of reducing degradation by bacteria, it is likely

that mechanisms based on carotenoids and psittacofulvins generate very different signals to melanin-based and structural colorations. For this reason, further studies are required using high-throughput sequencing (16S rRNA gene) to analyze the bacterial diversity that coexists in feathers, as well as to evaluate the effects of ultraviolet light in controlled environments.

Oil secretion from the uropygial gland could maintain an equilibrated bacterial community in feathers

Three main components have been strongly associated with the structure of the bacterial community in wild bird feathers: habitat, preening behavior, and the composition of the uropygial gland or preen gland oil (Shawkey *et al.* 2003, Bisson *et al.* 2009, Reneerkens *et al.* 2008). The uropygial gland has been described as a holocrine sebaceous gland of the avian integument that produces an oily secretion that is spread on the plumage during preening (Czirják *et al.* 2013). In a study of 14 domestic adult Sparrows (*Passer domesticus*) from Transylvania, Romania, the uropygial gland was removed from seven individuals while the other seven were used as a control group. Subsequently, feather samples were taken from each one of the 14 birds, and microbial culture performed (nutritive agar and feather agar). The results indicated that there was no change in keratin-degrading bacterial density, but the load of the other bacterial groups increased in the birds that had the uropygial gland removed (Czirják *et al.* 2013). This result demonstrated the significant effect of feather preening, as had been suggested by other research (Reneerkens *et al.* 2008, Giraudeau *et al.* 2010, Jacob *et al.* 2014).

The latest research on this topic has generated a new panorama with which to analyze the chemical composition of the uropygial gland oil and its possible antimicrobial effect. The relationships between the chemical production of the oily secretion in the uropygial gland of the host, the microbiota associated with feathers, and those of the nests of two Great Tit populations were studied (Jacob *et al.* 2018). It was determined that the chemicals in the oil may function as a wide-spectrum antimicrobial defense and could favor maintenance of a diverse microbiota (Møller *et al.* 2009, Ruiz-Rodríguez *et al.* 2009, Fülöp *et al.* 2016), since the oil is composed of 14 esters and 2 acids. The authors suggested that the esters do not have a direct antimicrobial effect, but they can form an oily physical barrier that limits the growth

of environmental bacteria, while the presence of acid compounds could explain the antimicrobial action (Jacob *et al.* 2018).

More research focusing on the specific antimicrobial effect of each of the chemical products in different bacterial strains, increasing the knowledge of the chemical composition of uropygial gland oil in different bird groups, is very important in order to determine whether interspecific relationships exist in the microbiota associated with feathers and chemical oil composition (Spor *et al.* 2011, Ezenwa *et al.* 2012, Sullam *et al.* 2012, Yatsunenko *et al.* 2012, Jacob *et al.* 2018). However, the response to antimicrobial activity can also be subject to immune system proteins responsible for inhibiting the growth of FDB. Immunoglobulin Y (IgY), an important antibody in the blood of birds, reptiles, and lungfish, has been recently proposed as responsible for inhibiting bacterial growth, since it is found in a wide variety of secretions (Carneiro *et al.* 2020). The presence of lysozymes in the oil of the uropygial gland has been proposed because these enzymes present antibacterial activity, mainly against gram-positive bacteria, confirming the effect of immune defense proteins on FDB. Finally, these authors propose measuring the effect of enzymatic peptides such as lysozymes on balancing the microbiota in feathers through the action of grooming.

The uropygial gland plays a key role in the adaptation mechanisms developed by birds to protect themselves from external pathogens. All species of birds are known to groom themselves in order to keep their plumage healthy, so study of this gland tends to be very important in evolutionary contexts. The biochemical composition of the uropygial gland oil has not been characterized in the different groups of birds and with different habits and strategies, which would bring new knowledge. For example, would individuals with a carrion feeding strategy invest more energy in the production of enzymes or proteins present in the oil to inhibit bacterial growth, compared to birds with a seed, insect, or nectar-feeding strategy? Moreover, in an ecological context, we assume that the environment can play an important role in the composition and production of oil in the uropygial gland. In this case, would the amounts of uropygial oil production vary in different environments with extreme climates? Does the uropygial oil have any relationship with the bacterial composition exposed in urban environments, as it does in natural environments? This is most likely true given the efficient strategies birds have to protect their plumage. However, further study is

necessary to evaluate the oil production rate of the uropygial gland, as well as the grooming behavior in different groups of birds with different strategies.

FUTURE PERSPECTIVE

Culture-independent and high throughput sequencing technologies have allowed the development of studies of the interactions between microorganisms and host, and have helped, through identification and functionality of bacterial groups using DNA and RNA sequences, to address questions such as what metabolic pathways do bacteria follow in order to adapt to each host. However, further research should include environmental abiotic variables (e.g., temperature, precipitation) and the changes these generate together with the anthropogenic activities, such as habitat fragmentation for agriculture and urbanization, as well as their relationship to underlying processes of bird-associated microbiota. Furthermore, it is important to add that, in response to abrupt climate change, the environmental characteristics of ecosystems (habitats) can change rapidly and thus alter adaptive microbiota behavior. These studies will help us to generate a database of current microbial communities and the changes in biochemical functionality that these could present as a consequence of the aforementioned factors (rapid climate change and/or changes in habitat due to anthropogenic activities). Likewise, an early alert system could be created to monitor the effects of these changes in wild bird populations. Finally, although many advances have been made in the last 20 years, there is still much scope for research into this fascinating microscopic world and its interaction with wild birds.

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CAPÍTULO 2.

Bacterial composition on Violet-crowned hummingbird (*Leucolia violiceps*) feathers in contrasting habitats in central-western Mexico

ABSTRACT

Different studies have confirmed that the microbiota that inhabits the feathers of birds depends greatly on the ecology of the host species and environmental factors. The diverse microbiota on the surface of the feathers plays an important role in the fitness of the birds. In urban and rural environments, the bacterial load and community composition of the plumage have been modified. The Violet-crowned hummingbird (*Leucolia violiceps*) is an important pollinator of western Mexico, and it is widely distributed in urban, agricultural, and natural environments. This study aims to evaluate the bacterial composition associated with the plumage of *L. violiceps* in three different environments and its relationship with environmental variables. Feather sweep samples with sterile swabs were taken from hummingbirds in all three environments. The strains were isolated and identified from the 16S rRNA gene sequencing. The results of this study indicated that the abundance, and bacterial composition in the plumage of *L. violiceps* changed in different environments and sites, together with the habitat's specific biotic and abiotic characteristics. The adults contain a higher richness and bacterial load in their feathers due to the increase in the necessary activities for survival. Most of the isolates were beneficial symbionts for the plants and the host. In natural areas close to livestock and urban environments, hummingbirds are more exposed to the transmission of pathogenic bacteria due to interaction with domestic animals and humans. It is necessary to increase studies on microorganisms associated with wild birds and their relationship with biotic and abiotic variables, and eto use metagenomic techniques for the analysis of microbial diversity associated with hummingbird plumage.

Keywords: hummingbird, bacterial composition, environment, age, variables

INTRODUCTION

Birds are the most abundant class of tetrapod vertebrates (Lovette and Fitzpatrick, 2004), they are warm-blooded, their body is covered with feathers (wings and tail), filoplumes, and

body downs (Jenni and Winkler, 2020) which are used for flight, thermal regulation, visual communication, camouflage, and swimming (Bostwick, 2004). The feathers are mostly composed of keratin (90%), a very strong and light protein (Lange et al. 2016). It has been recorded that a great diversity of bacterial communities living in the feathers, which are fundamental for the evolutionary and ecology study of birds (Musitelli et al. 2018; Azcárate-García et al. 2020).

The microbiota inhabiting the bird's feathers highly dependent on host species ecology and environmental factors (Burtt and Ichida 1999; Bisson et al. 2009; Saag et al. 2011; Javůrková et al. 2019). Studies using culture-based methods have discovered that the diverse microbiota of the feather surface playing important roles in bird fitness (Bisson et al. 2009; Dille et al. 2016; Giraudeau et al. 2017; Giorgio et al. 2018; Javůrková et al. 2019). Likewise, bacteria are an important component because they are exposed to environmental changes in ecosystems, and it has been shown that in urban and rural environments, the load and the composition of bacterial communities of the plumage have been modified (Giraudeau et al. 2017; Giorgio et al. 2018).

There are bacterial groups in feathers that are commensal residents producing bacteriocins (antimicrobial peptides) (Javůrková et al. 2019), and degrading bacteria of feathers which are of environmental source and are capable of produce energy and nitrogen from keratin of feathers (Burtt and Ichida, 1999; Lucas et al. 2003; Lange et al. 2016). Beneficial bacteria for other organisms, and pathogenic bacteria, so named because they have been reported to cause various diseases generated in birds (Smith et al. 2020). The best-known cases involve three enteric bacteria, *Salmonella* spp., *Escherichia coli*, and *Campylobacter* spp. that come from human waste, livestock, agriculture, and wildlife (Havelaar et al. 2015; Goodenough et al. 2017; Hassell et al. 2019).

Bacterial communities associated with feathers come from the different origin, such as contact with other birds (ectoparasites, courtship, interspecific, and intraspecific competition) (Glowska et al. 2020), themselves (cloacal contact and fecal material) (Hassell et al. 2019; van Veelen et al. 2020), and local environment (vegetation, bird feeders, waterers, and water bodies) (Goodenough et al. 2017; Giraudeau et al. 2017). Additionally, geographic location and climate may influence the growth of bacterial communities on feathers (Bisson et al.

2007; Saag et al. 2011; Sotnychuk et al. 2019). Burtt and Iquida (1999) and Kent and Burtt (2016) analyzed the seasonality in the bacterial composition of different wild birds in USA and Canada. They found that the bacterial load of the *Bacillus* genus (feather degraders) increases during the winter season because these bacteria form spores that make them adapt to low temperatures; unlike spring and the summer season, bacteria are more affected as birds are exposed to solar radiation for a longer time. On the other hand, Bisson et al. (2009) found that in resident birds, the bacterial diversity varied significantly throughout the annual cycle due to climatic changes in the environment where they live.

In Jalisco state (central-western Mexico), one of its main economic activity is agriculture, and is considered the most important producer of corn and agave to obtain tequila (Macías 2007). In addition, Guadalajara city is one of the largest and most populated cities in the country due to its urban and industrial development (Nodhal et al. 2016). These anthropogenic activities have generated the fragmentation of the habitat of different plants and animal species, including birds (Dille et al. 2016). Therefore, it induces the species to adapt to these environments, enhancing the underlying processes in the wildlife-human interface (Hassell et al. 2019). Finally, the state has fifteen protected natural areas, for example, Sierra de Quila reserve, which has great flora and fauna diversity (López-Gómez and Bastida-Izaguirre 2018).

Different hummingbird species inhabit agricultural, natural, and urban environments. However, in this study, we chose the Violet-crowned hummingbird (*Leucolia violiceps*) because it is a dominant semi-endemic pollinator for western Mexico and the southwestern United States (southern of Arizona) (Arizmendi and Berlanga 2014). It is a specialized nectarivore and pollinator bird, and its diet is based mainly on different flowers of trees and shrubs and complements it with insects (Yanega and Rubega 2004) and fruits (Palacio 2019). Violet-crowned is widely distributed in forests, cultivated fields, and in urban parks in the state of Jalisco (DeSucre-Medrano et al. 2016).

Environmental factors, as mentioned above, influences the bacterial composition of the plumage in wild birds. Then, we hypothesize that bacterial composition associated with the feathers of the violet-crowned hummingbird (*L. violiceps*) will present variation between environments and localities in terms of resource availability and the influence of

environmental variables. The objective of this work was to analyze the cultivable bacterial composition of hummingbird feathers through its biochemical characterization and molecular identification (i.e. 16S rRNA gene) in agricultural, urban, and natural environments. In addition, we consider the relationship with the morphological body measurements, resource availability, and the environmental variables (temperature and humidity).

MATERIAL AND METHODS

Study area

We chose three contrasting habitats (i.e., natural, agricultural, and urban) within the Jalisco state. In the natural environment, the samples were collected in the municipality of Tecolotlán where the natural protected area of flora and fauna Sierra de Quila is located. This area has a great plant and fauna diversity (Romero 2013). Hummingbirds of three localities within from Sierra de Quila were collected, La Campana (20°14'N, -104°3'W), Potrero (20°14'N, -104° 6'W), and Cofradía (20°14'N, -103°59'W) all of them within the tropical deciduous forest (Fig. 1). For the urban environment, the samplings were carried out in three sites (urban parks) within Guadalajara city (MAG): Los Colomos (20°42'N, -103°23'W), Solidaridad (20°39'N, -103°16'W) and Metropolitano (20°40'N, -103°26'W) (Fig. 1). Finally, for the agricultural environment, the collects were in Los Altos region to the north of the state in the municipalities of Tepatitlán de Morelos and San Ignacio Cerro Gordo, in the sites named El Bajío del Indio (20°42'N, -102°32'W), Bajío Nuevo (20°41'N, -102°32'W) and Piedra Herrada (20°43'N, -102°36'W); all of them are part of the most important areas as producers of agave in Jalisco (INEGI 1997) (Fig. 1).

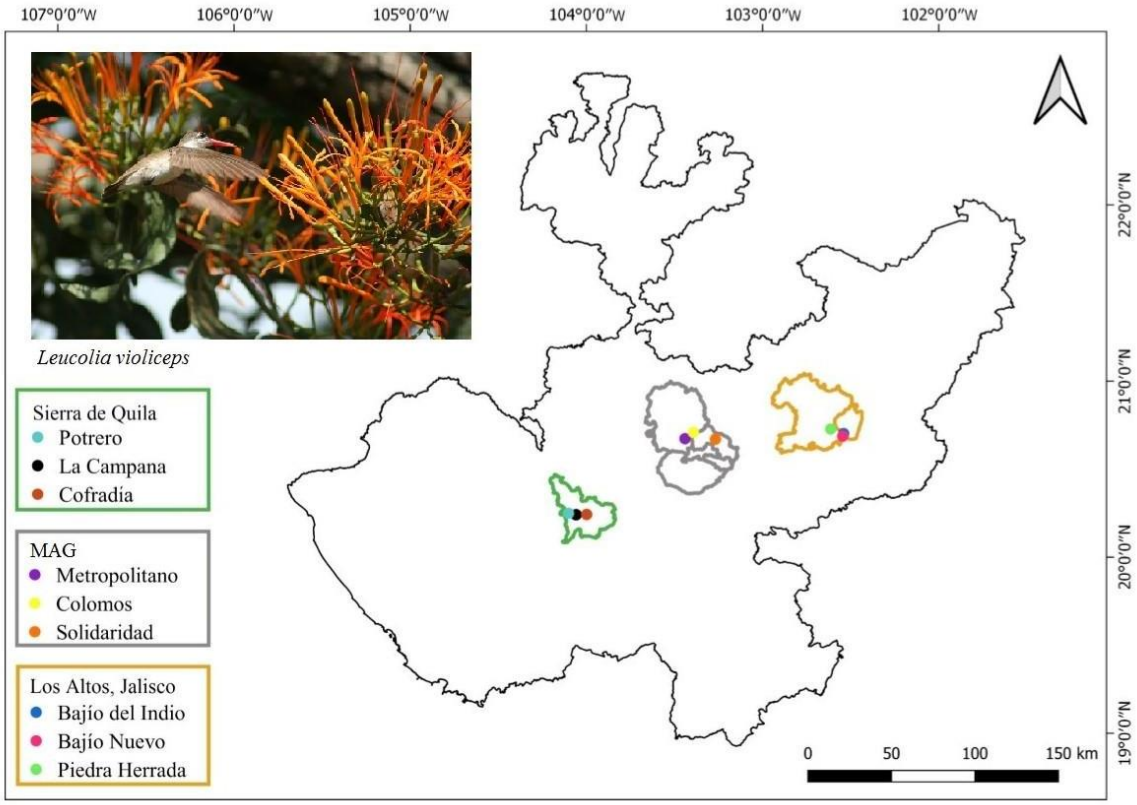

Figure 1. Map of the study area in Jalisco, Mexico. MAG = Metropolitan Area of Guadalajara. Green = natural, gray = urban, and orange = agricultural environments. Each color points on the map represent localities. Photo: Luis Ramírez.

Collection of samples

The samplings were carried out during the summer (June - September), autumn (October - November) in 2019, and winter (January - February), and summer (June - September) in 2020. Violet-crowned hummingbirds were captured using eight mist nets during the morning (Ralph et al. 1993). These were placed near patches of vegetation that showed hummingbird pollination syndrome mainly (Faegri and Van der Pijl 1979; Fenster et al. 2004). Sterile gloves were used to hold the hummingbirds; each individual underwent one gentle rub with a moistened sterile swab on the breast, abdomen, nape, and back (Fig. 2). These swabs were placed in a microtube (1.5 mL) with 1 ml of sterile buffered peptone water (1x). Age, morphological measurements such as wing, exposed culmen, tail, and weight were recorded. The environmental variables, temperature (°C), and humidity (%) were taken for each

sampling locality, and the type of floral resource where the nets were placed was recorded. All samples were stored at 4°C and were transported to the laboratory within 24 h.

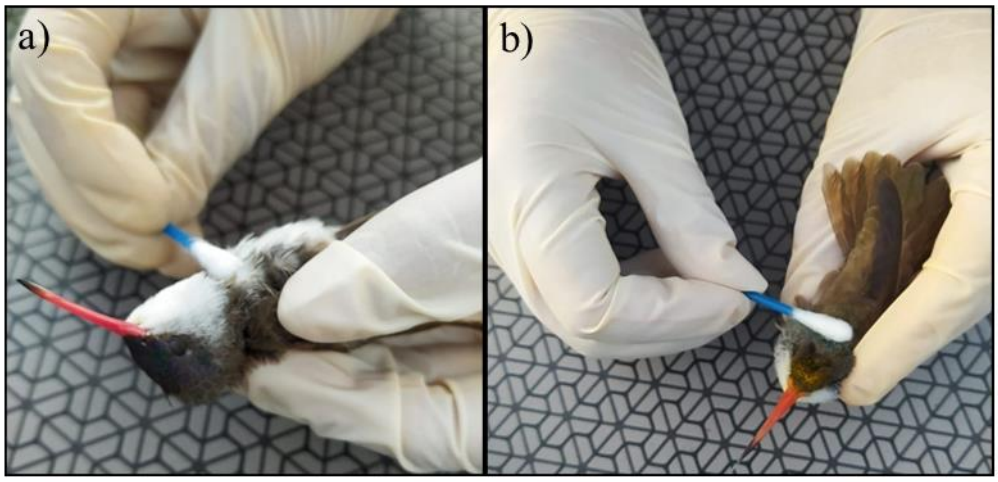

Figure 2: Swab sampling procedure of *L. violiceps* hummingbird. a) Breast and abdomen part, and b) Nape and back part.

Ethics Policy

Animal rights. No hummingbird was mistreated during the process; all the collections were carried out under government permits by Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) and state parks. The hummingbird species whose plumage was inspected is not listed as endangered by any national or international wildlife conservation program, and no particular type of maneuver or stress was generated for the birds.

Culture-based methods

The swab samples were vortexed for 1min to release the bacteria from the cotton. The swabs were removed with sterile tweezers and discarded. Subsequently, 100uL of cell suspension was taken from each vial and serial dilutions were made in microtubes (1.5mL), containing 900µl of distilled water, making dilutions from 10^{-1} to 10^{-4}. 100µL of each dilution were then taken and inoculated by the surface extension technique in plates, containing the nutrient-rich medium Zobell modified with distilled water (Zobell, 1940), and Standard Methods Agar (SMA) (Jett et al. 1997) separately. The plates were incubated for three to five days at 28°C for Zobell medium and from 24 to 48 hours for SMA at 28°C (Giorgio et al. 2018).

Morphological characterization of bacterial colonies and biochemical tests

To select the bacterial colonies for subsequent isolation, their morphological characteristics, colony shape, margin, elevation, size, texture, appearance, pigmentation, and optical property were taken into account. All the plates of the inoculated dilutions of each sample were checked; each colony was assigned an identification code for its conservation in the ceparium (Gephardt et al. 1981). Because the morphological characteristics of bacterial strains are quite subjective to classify them and that several species of bacteria may be similarly based on phenotypic traits, a series of biochemical tests were performed to determine them based on their metabolic characteristics. Gram stain to observe cell morphology, and the presence or absence of rigid cell wall, the oxidase test to determine if a bacterium produces certain cytochrome c oxidases, the catalase test to observe the presence of the enzyme catalase. Oxidative/fermentative glucose test (Hugh and Leifson test) to determine the utilization of carbohydrates by oxidative or fermentation pathway, and facultative (both pathways), salt tolerance test at different concentrations (3.4, 6 and 8%), and motility. Likewise, growth on a MacConkey selective medium to determine the presence of lactose-fermenting or non-lactose-fermenting enteric bacteria, differential medium TCBS to sucrose-fermenting or non-sucrose-fermenting bacteria. Methyl red test to see if bacteria have the ability to produce enough acid during glucose fermentation, Voges-Proskauer test to detect acetoin production, and Indole test to determine the ability of bacteria to convert tryptophan to indole (Bergey et al. 1994).

Furthermore, blood agar was used to see the hemolytic capacity of the bacteria. The reason isolated bacteria were inoculated on the blood agar medium was for the possible identification of pathogenic bacteria. This method has previously been used in other studies for the identification of pathogens (Smyth and McNamee 2008; Goodenough et al. 2017). However, not all bacteria that produced hemolysis are pathogenic. Finally, to find which bacterial strains had similar biochemical functions, a hierarchical clustering complete linkage based on Gower's distance was performed for categorical data (Gower 1967). The analysis was performed in the PRIMER® v 6.1 statistical software.

DNA extraction

DNA extraction was performed by suspending one to two isolated colonies in 100 µL of Milli-Q water in 1.5 ml tubes. The MagMax™ DNA Multi-Sample Ultra Kit (ThermoFisher) extraction kit was used following the manufacturer's protocol. The concentration and quality of the DNA of the samples were calculated by absorbance at wavelengths 260 and 280 nm with a NanoDrop™ 2000/2000c Spectrophotometers (ThermoFisher).

16S rRNA gene characterization

The working concentration for the 16S rRNA gene amplification was calculated at 10 ng / µL by diluting the pure DNA with Milli-Q water (Zúñiga 2012). The polymerase chain reaction (PCR) by each sample was performed in a Swift™ MiniPro Thermal Cyclers, with a reaction volume of 25 µL Containing 12.5 µL of Master mix of Thermo Scientific DreamTaq DNA Polymerases (Buffer KCL, $MgCl^2$, dNTPs, and Taq DNA Polymerase), 9.3 µl of Milli-Q water, 0.6 µL of primer 27F, 0.6 µL of primer 1492R and 2 µL of bacterial DNA. The sequences of the universal primers were: 27F 5´AGA GTT TGA TCM TGG CTC AG -3´and 1492R TAC CTT GTT ACG ACT T -3´. The DNA templates were amplified by initial denaturation at 94ºC for 4 min followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 58ºC for 45 sec, extension at 72ºC for 35 sec, and a final extension at 72ºC for 10 minutes. Negative controls were simultaneously included in the amplification process. The integrity of the PCR products was confirmed by the appearance of single bands following electrophoresis for 20 min at 80 V in 1% (w/v) agarose gels in Tris-borate EDTA buffer. The bands were visualized by SYBR® Safe staining, and with the transilluminator UV light, amplification of the V1-V9 region (1,500 bp) corresponding to the 16S rRNA gene was observed. According to the manufacturer's instructions, the PCR samples were purified with the DNA Clean and ConcentratorTM-5 purification kit.

Once the presence of the 1500 bp bands of all the bacterial isolates has been verified, volumes for a final 5 µL sequencing reaction mix were as follows: 0.5 µL BigDyeTM Terminator v3.1 (Ready Reaction Mix), 1 µL BigDye Terminator v3.1 5x Sequencing Buffer, 0.5 µL of each primer, 1 µL of the purified PCR product, 2 µL of nuclease-free water. The samples were analyzed with the SeqStudio Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific).

The chromatograms obtained from the sequencing of the 16S rRNA genes were analyzed and assembled using the Sequencher v. 4.8 software (Gene Codes Corporation, USA). The sequences were compared with the National Center for Biotechnology Information database, using BLAST (Basic Local Alignment Search Tool). In addition, we use morphological characteristics and biochemical tests to identify the strains (Supplementary A, Table A2).

Statistical analysis (Bacterial composition)

Abundance was calculated as colony-forming units (CFU) per milliliter (mL) in each sample, and richness corresponded to the total number of species per sample. The bacterial composition structure was characterized using the frequency of every observed species by sample, calculated as a relative species occurrence to the total number of species in the sample. Analyzes were based solely on species as the Operational Taxonomic Units (OTU). Sampling effort was assessed using sampled-based rarefactions according to the following non-parametric estimators (Colwell 2006): Chao 2 takes into account the unique species recorded exactly in one replicate or sampling unit, as well as the duplicates species found in two replicates; Jackknife 1 is sensible of the number of unique species, while Jackknife 2 considers both unique and duplicates species. Rarefaction curves were built using 10,000 permutations.

The spatial variation of the bacterial composition was evaluated with a two-way fully nested factors experimental design:

$$Y = \mu + En_i + Si_j(En_i) + e_{ij} \quad (1)$$

where En_i is the Environment as a fixed factor with three levels (i.e., natural, agricultural, and urban), Si_j corresponds to Sites nested into Environment with six sampling sites and random effect, and e_{ij} is the accumulated error.

The environment and the bird's age (adults and juveniles) effect on the bacterial composition was evaluated using a two-way crossed factor design:

$$Y = \mu + En_i + Ag_j + En_i*Ag_j + e_{ij} \quad (2)$$

Where En_i is the Environment as a fixed factor with three levels (i.e., natural, agricultural, and urban), Ag_j corresponds to the bird's age with two levels (i.e., adults and juveniles) and a fixed effect, and e_{ij} is the accumulated error.

The bacterial composition structure was assessed based on the two experimental designs mentioned above. The bacterial richness and abundance changes were individually evaluated with permutational analyses of variance (permutational ANOVA), using Euclidean distances and following the criteria of Anderson et al. (2008). Only fourth root transformation was applied in the abundance matrix of the nested design. Instead, the bacterial composition (i.e., absence and presence matrix) variation was analyzed with permutational multivariate analyses of variance (PERMANOVA) using a Sorensen similarity matrix (Anderson et al. 2008). The statistical significance of permutational ANOVA and PERMANOVA was tested with a type III sum of squares and 10,000 permutations based on a reduced model. When the statistical differences were found, several pairwise tests were calculated to identify the factor levels that explained the variation.

The species contribution to the average dissimilarity of the terms of the two experimental designs described above was estimated with a percentage similarity analysis (SIMPER) based on Bray-Curtis similarity and binary matrices, which generates an estimate analogous to Sorensen similarity (Dowing 1979; Clarke and Warwick 2001; Clarke and Gorley 2006). Resemblance patterns for the bacterial composition in both experimental designs were visualized using principal coordinate analysis (PCoA) based on Sorensen similarity (Dowing 1979; Anderson et al. 2008). All analyzes (sampled-based rarefactions, permutational ANOVA, PERMANOVA, SIMPER, and PCoA) were performed in PRIMER® v6.1 + PERMANOVA 1.1 software.

A canonical additive partition was carried out based on canonical redundancy analyses (TDA) for explaining the linear relationship of the bacterial composition and several

environmental variables subsets for the spatial variation model (sites nested within environments) (Legendre and Legendre 2012). The predictive variables subsets were: a) Abiotic variables (AV) as temperature and humidity; b) Biotic variables (BV) corresponded to the food resource availability as next plant species *Bauhinia variegata, Callistemon citrinum, Leonotis nepetifolia, Podranea ricasoliana, Psittacanthus calyculatus, Lobelia laxiflora, Cascabela ovata, Hymenocallis littoralis, Stenocereus queretaroensis, Mirabilis jalapa, Ceiba aesculifolia, Spathodea campanulata* and *Hamelia patens*; c) Bird measurement (BM) as weight, culmen, wing, and tail. The multicollinearity between the predictive variables was reduced with Pearson correlations, eliminating the variables with $r \geq 0.90$ and considering a variance inflation factor (VIF) < 10. Statistical significance was tested with 10,000 permutations under a reduced model in the CANOCO v4.5 software (ter Braak and Šmilauer 2002).

RESULTS

The feathers of 60 hummingbird individuals were captured, from those 21 were in the urban environment: eight in the Parque Metropolitano, eight in Los Colomos, and five in the Parque Solidaridad. In the agricultural environment were 18 individuals, six for each site (Bajio del Indio, Bajio Nuevo, and Piedra Herrada). Finally, in the natural environment, we collected 20 individuals: eight in La Campana, seven in Cofradía, and six in Potrero. 212 bacterial isolates associated with plumage were recovered (Supplementary A, Table A1). Hierarchical clustering analysis showed 60 strains with different functionality (Supplementary A, Fig. A1). Sixty strains were sequenced with the 16S rRNA gene. The sequences obtained were 1300 bp, and sequences with 97-100% similarity were analyzed (Supplementary A Table A2).

Biochemical characterization

The salinity test (NaCl) recorded that 203 strains grew at 3.4% NaCl concentration, at 6% 143, and 8% 95. The urban environment maintains the highest number (46) of strains with greater resistance to salinity (Supplementary A, Fig. A2a-c). The Gram stain test and KOH (3%) registered 143 Gram-positive, and 69 Gram-negative strains were obtained. The natural

environment reported the highest presence of 71 Gram-positive and the urban 37 Gram-negative as the highest (Supplementary A, Fig. A2d). The biochemical tests of catalase (212) and oxidase (124) were detected in most of the strains; the natural environment maintains the highest number of strains that produce the enzyme cytochrome c oxidase (Supplementary Fig. 2e-f). We found 165 strains with mobility, of which 67 belong to the urban environment (Supplementary A, Fig. A2g).

The glucose test registered a high number of facultative strains in the natural environment (40), while fermentative strains with a high number were in the urban environment (20), only 10 strains are oxidative in the three environments. In contrast, 100 strains do not use glucose (Supplementary A; Table A2h). For the three environments, they registered one hundred twenty-two strains that produce acetoin (Voges-Proskauer), 110 produce organic acids (Methil-red), and only 1 produces indole (Supplementary Table 2i). The culture medium TCBS registered the highest number of strains in the natural environment (7); these bacteria can grow in bile salts, citrate, and ferment sucrose. In MacConkey Agar, the growth of 21 strains capable of fermenting lactose, again more abundant in the natural environment, was observed (12) (Supplementary A, Fig. A2j). Finally, the strains with β-hemolytic activity were 74 with the highest prevalence in the natural environment (39) and α-hemolysis with 8 (Supplementary A, Fig. A2k).

Culture-dependent bacterial composition

Spatial variation

The culture-dependent bacterial composition of the hummingbird plumage recorded was 13 genera and 51 OTUs. Among these, *Bacillus* was the genus with the highest number of OTUs registered (22), followed by *Pseudomonas* (10), *Paenibacillus* (5), and *Moraxella* (3) (Supplementary A; Table A2). The OTUs with the highest incidence in the total of the samples were *Bacillus pumilus* present in 24 individuals, *Bacillus* sp. 11 and *Bacillus subtilis* 18 individuals, and *Achromobacter* sp. 11 individuals (Supplementary A, Table A2).

The sampled-based rarefaction curve showed that the total number of cultivated species (50 OTUs) was less than the expected number of OTUs present (63). The observed bacterial richness reached 69% of the total expected OTUs in the three environments. With the Chao 2 estimator, the 50 OTUs observed in the study represented 63% of the total number expected

by chance, while Jackknife 2 estimated that only 67% was detected. The shape of the curve confirmed that OTUs detection possibly augments as the number of samples increases (Supplementary A Fig. A3).

The permutational ANOVA results did not show significant differences in richness. However, the abundance at the level of environmentswas significant, the coefficient of variation of the model explained 42% (Table 1). The differences correspond between the urban and natural environment according to the *post-hoc* test. (Supplementary A, Table A3). The difference in the bacterial load between the two environments (urban *vs.* natural) is observed (Fig. 3). The PERMANOVA outputs showed differences in the composition, of bacterial species at the environment level (En) 22%CV, and at the site level (Si) 20%CV (Table 1). *Post-hoc* tests demonstrated the variation between the natural and urban environments (Supplementary A, Table A3).

Table 1. Permutational ANOVA and PERMANOVA test for measuring the spatial variation of the richness, abundance and bacterial composition in urban, agricultural, and natural environment. En = Environment, Si = Sites, coefficient of variation = CV%, and bold $P \leq 0.05$.

	Richness			Abundance			Bacterial composition		
Factors	Pse-F	*P*-value	%CV	Pse-F	*P*-value	%CV	Pse-F	*P*-value	%CV
En	0.81	0.48	-10	10.18	**0.005**	42.53	2.54	**0.0013**	21.78
Si(En)	1.61	0.16	25.78	0.91	0.49	-7.33	1.77	**0.0003**	19.99
Residuals			84.45			64.79			58.21

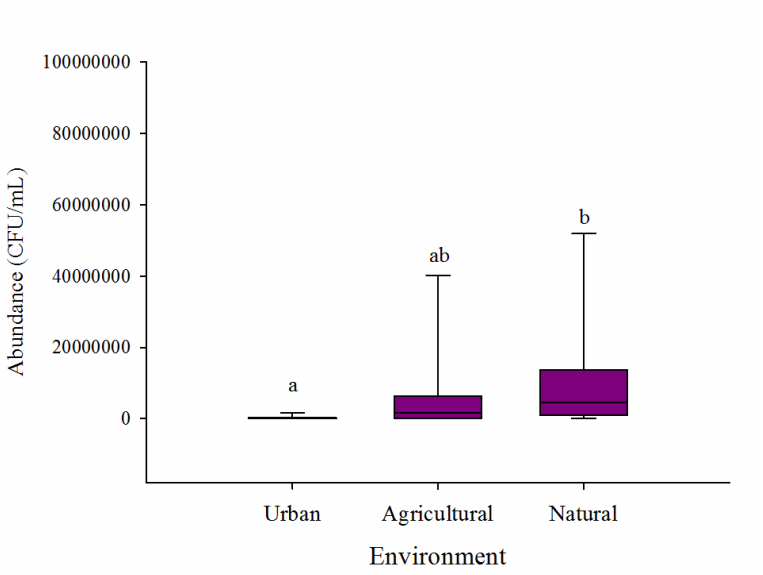

Figure 3. Box plot of the abundance representing the two-way nested design. The difference between urban environment *vs.* natural and agricultural environment is observed (letters indicate significant differences $P < 0.05$).

In the principal coordinate analysis (PCoA) of the nested design, the first two axes explained 56.8% of the total variation (Fig. 3). The axis 1 (PCo1) explained 32.3%. and the axis 2 (PCo2) explained 24.5%. The Metropolitano and Colomos sites of the urban environment tended to cluster and are more distant from La Campana and Potrero sites of the natural environment. However, Bajio del Indio and Piedra Herrada sites of the agricultural environment were grouped with the Potrero site of the natural environment and Bajio Nuevo site are close to the urban environment sites (Fig. 4)

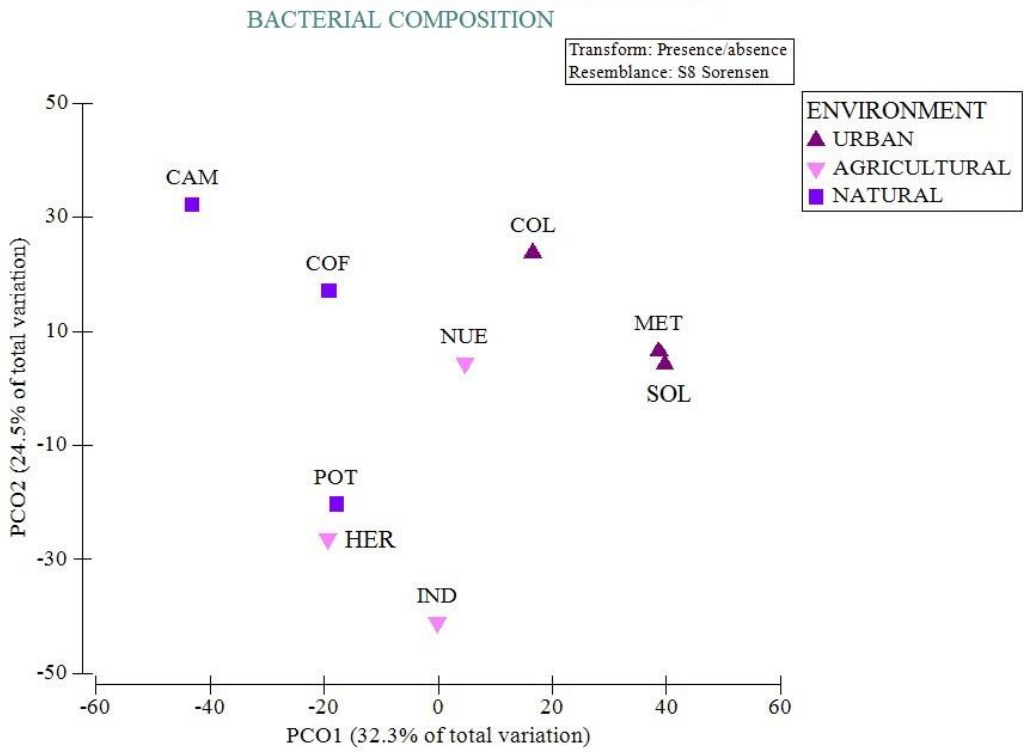

Figure 4. Principal coordinate analysis (PCoA) ordination of two-way nested spatial design. Bacterial composition registered significant differences between the environments and sites (natural and urban). Sites: CAM = La Campana, COF = Cofradía, POT = Potrero, NUE = Bajío Nuevo, IND = Bajío del Indio, HER = Piedra Herrada, COL = Colomos, MET = Metropolitano, SOL = Solidaridad.

The SIMPER results showed the dissimilarity of OTUs between the urban and agricultural environments (89%) *Bacillus subtilis* (11%), *B. pumilus* (9%), and *Achromobacter* sp. (7%) they are the species that contribute the most to dissimulation. While, the urban and natural environment (52%), the species that contributed the most were *Bacillus subtillis* (10.78%), *B. pumilus* (9%), *Achromobacter* sp. (7%), and *Paenibacillus glucanolyticus* (6%) (Table 2). Lastly, the natural environment vs. agricola were dissimilar (82%) with the highest contribution *Bacillus* sp. 11 (12%), *B. subtilis* (9%), and *B. cereus* (8%) (Table 2). Likewise, the recorded dissimilarity between environments was generated by the *Bacillus* genus with the highest incidence (60%) because it is found in all studied environments (Supplementary A, Table A2). The most common species were *Bacillus subtilis* and *Achromobacter* sp. in

the urban environment, and *B. subtilis* and *B. pumilus* in the agricultural, and by the natural *Bacillus* sp. 11 and *B. cereus* (Supplementary A, Table A4).

Table 2. Results of the similarity percentage analysis (SIMPER) between environments. Only the bacterial species collectively contributing 50% of the average dissimilarity are presented. "Contrib%" = percent contribution and "Cum%" = cumulative percent contribution.

Groups urban *vs.* agricultural			Groups urban *vs.* natural		
Average dissimilarity = 88.88			Average dissimilarity = 94.03		
Species	Contrib%	Cum.%	Species	Contrib%	Cum.%
Bacillus subtilis	10.78	10.78	*Bacillus* sp.11	10.79	10.79
Bacillus pumilus	9.52	20.3	*Bacillus cereus*	7.31	18.1
Achromobacter sp.	6.84	27.14	*Bacillus subtilis*	7.00	25.1
Paenibacillus glucanolyticus	5.79	32.94	*Bacillus pumilus*	6.78	31.88
Bacillus sp. 11	4.78	37.72	*Achromobacter* sp.	5.47	37.35
Sphingomonas taxi	4.34	42.06	*Sphingomonas taxi*	4.25	41.6
Bacillus sp. 3	3.78	45.84	*Bacillus altitudinis*	4.13	45.72
Paracoccus fontiphilus	3.33	49.17	*Bacillus* sp. 14	3.62	49.34
Bacillus thuringiensis	3.20	52.38	*Bacillus* sp. 3	3.25	52.6
Groups agricultural *vs.* natural					
Average dissimilarity = 88.91					
Species	Contrib%	Cum.%			
Bacillus sp. 11	11.86	11.86			
Bacillus subtilis	8.91	20.77			
Bacillus cereus	8.51	29.28			
Bacillus pumilus	8.38	37.66			
Paenibacillus glucanolyticus	6.40	44.06			
Bacillus sp. 3	6.04	50.1			

Age effect

The results of the permutational ANOVA in bacterial richness showed significant differences only in the interaction Environment and Age and the variation coefficient explained 55% (Table 3). Pairwise tests showed that the bacterial richness was lower in adult hummingbirds of the agricultural environment than in urban and natural environments (Supplementary A, Table A3). Furthermore, the adults were different from the juveniles within each urban and natural environment (Fig. 5a). Bacterial abundance was significant between environments and between ages. Furthermore, the variation of the model explained 58% (Table 3). The

environments that generated the main differences were natural and agricultural, as they maintain the highest number of CFU / mL in contrast to urban (Supplementary A, Table A3). Likewise, adult hummingbirds have the highest bacterial load compared to juveniles (Fig. 5b).

Table 3. Permutational ANOVA and PERMANOVA test to measure the variation of the environment and the age of the bacterial composition, the abundance and richness in the urban, agricultural and natural environment and the age. En = Environment, Ag = Age, coefficient of variation = CV%, and bold $P \leq 0.05$.

	Richness			Abundance			Bacterial composition		
Factors	Pse-F	*P*-value	%CV	Pse-F	*P*-value	%CV	Pse-F	*P*-value	%CV
En	2.52	0.0887	13	4.36	**0.0146**	17.90	4.55	**0.001**	25.2
Ag	3.98	0.0511	14.9	10.41	**0.0019**	24.46	1.47	0.13	7.55
En*Ag	4.21	**0.0217**	26.8	2.34	0.1022	15.99	1.27	0.17	9.94
Residuals			45.1			41.63			57.2

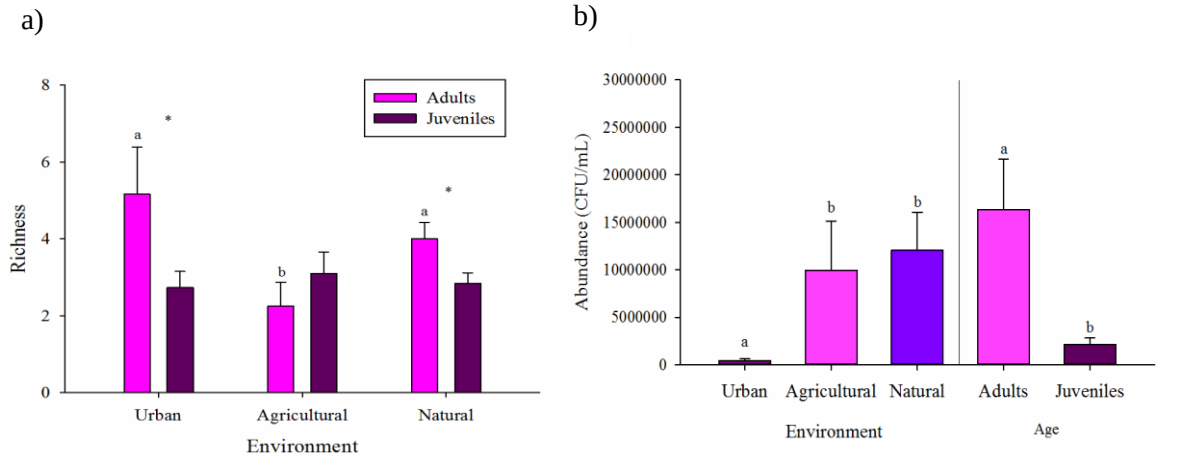

Figure 5. The figures represent the results of the permutational Anova test of the two-way crossover design for richness and abundance. a) The richness was significantly different in the interaction. Adult individuals from the agricultural environment *vs.* adults from the natural and urban environment are observed represented by letters. The asterisks (*) correspond to the significant differences between adults and juveniles in the urban environment. Likewise, among adults and juveniles of the natural environment. b) Bacterial abundance was different between environments and age. It is observed in the figure the differences between urban *vs*. agricultural and natural environment. Also, adults *vs*. juveniles differences are shown (letters represent $P < 0.05$).

The PERMANOVA outputs showed that the bacterial composition of the plumage of juvenile and adult hummingbirds varied only significantly among environments but not between ages or the interaction between these factors, and the coefficient of variation of the model was 43% (Table 3). Post-hoc tests check the differences between the three environments (Supplementary A, Table A3).

In the principal coordinate analysis (PCoA), based on the two-way crossover design (Enviroment and age), the first two axes explained 72.2% of the total variation. Axis 1 (PCo1) explained 47.2%, while axis two (PCo2) explained 25%. It is observed that both juveniles and adults are grouped by environment (urban, natural and agricultural) (Fig. 6).

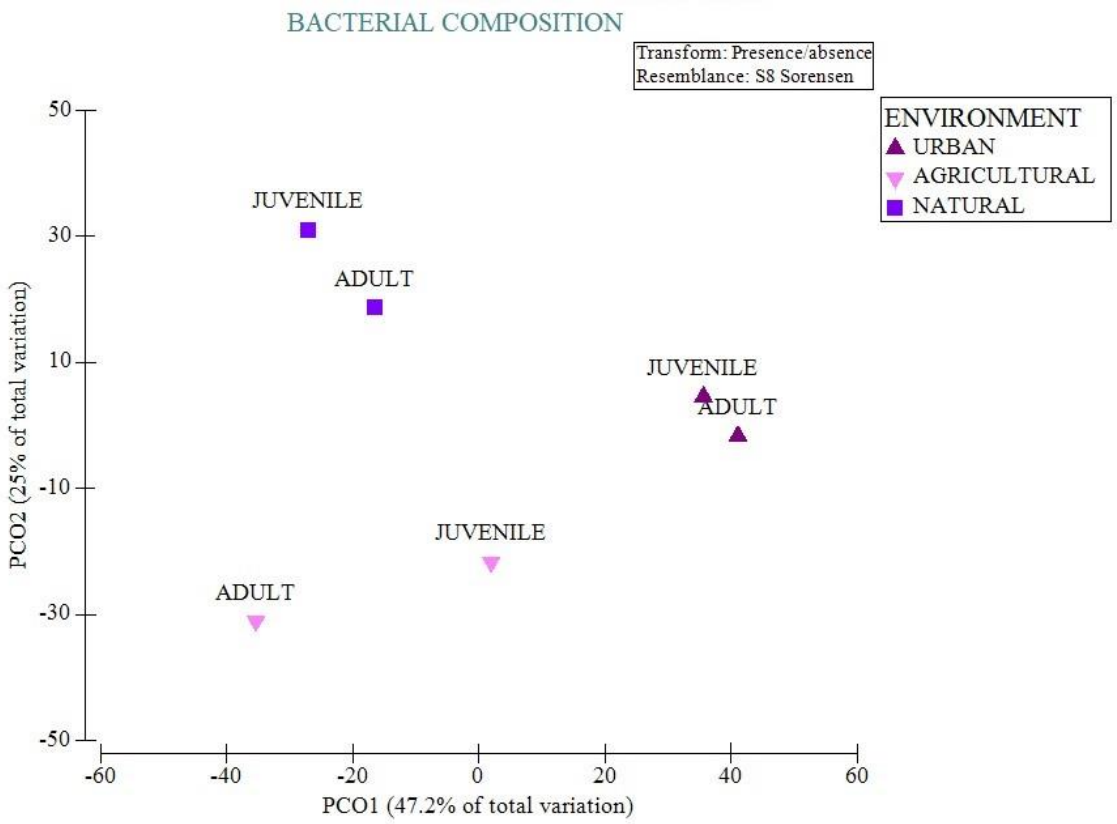

Figure 6. Principal coordinate analysis (PCoA) scatter plot represents two-way crossover design (environment-age). The bacterial composition show divergence between environments ($P < 0.05$).

The SIMPER dissimilarity analysis of the two-way crossover design showed that between the urban and agricultural environment (89%) the species that contributed to the dissimilarity

were *Bacillus pumilus* (10%), *B. subtilis* (8%), and *Paenibacillus glucanolyticus* (6 %) (Table 4). The urban environment *vs*. natural were dissimilar (95%) and the species that contributed the most were *Bacillus* sp. 11 (10%), *B. cereus* (7%), and *B. subtilis* (7%) (Table 4), and between the natural and agricultural environment (88%) the highest contribution was made by *Bacillus* sp. 11 (11%), *B. pumilus* (9%), and *B. cereus* (8%) (Table 4).

Table 4. Results of the similarity percentage analysis (SIMPER) between environments. Only the bacterial species collectively contributing 50% of the average dissimilarity are presented. "Contrib%" = percent contribution and "Cum%" = cumulative percent contribution.

Groups urban *vs* agricultural			Groups urban *vs*. natural		
Average dissimilarity = 89.53			Average dissimilarity = 94.72		
Species	Contrib%	Cum.%	Species	Contrib%	Cum.%
Bacillus pumilus	10.19	10.19	*Bacillus* sp. 11	9.78	9.78
Bacillus subtilis	8.28	18.48	*Bacillus cereus*	7.39	17.16
Paenibacillus glucanolyticus	6.89	25.37	*Bacillus subtilis*	6.63	23.8
Achromobacter sp.	6.61	31.98	*Bacillus pumilus*	6.47	30.27
Bacillus sp. 3	5.61	37.58	*Achromobacter* sp.	5.69	35.95
Sphingomonas taxi	4.56	42.14	*Sphingomonas taxi*	4.39	40.35
Bacillus sp. 11	4.36	46.5	*Bacillus altitudinis*	4.27	44.62
Bacillus thuringiensis	3.67	50.17	*Bacillus* sp. 14	3.79	48.41
			Acinetobacter sp.	3.49	51.9
Groups agricultural *vs.* natural					
Average dissimilarity = 88.50					
Species	Contrib%	Cum.%			
Bacillus sp. 11	11.4	11.4			
Bacillus pumilus	9.21	20.62			
Bacillus cereus	8.27	28.88			
Bacillus sp. 3	7.03	35.91			
Bacillus subtilis	6.97	42.89			
Paenibacillus glucanolyticus	6.71	49.6			
Bacillus altitudinis	4.69	54.29			

Relationship between bacterial composition and environmental variables

The results of the canonical partition analysis showed that the total variation explained by the model was 14.2%. Also, the relationship between the variables and the bacterial

composition was significant (Supplementary A. Figure A4). The variation of the bacterial composition at the site levels of environments and sites was mainly explained by BV [c] 8.9%, followed by the subset of AV [a] with .8%. Finally, the subset BM [b] explained only .4% (Supplementary A. Figure A4). The RDA triplot shows the influence of temperature on the bacterial composition at the La Campana and Cofradía sites within the natural environment. *Bacillus* spp. Strains were identified. *B. cereus, B. toyonensis, B. altitudinis, B. pumillus, Moraxella* sp., *Paenibacillus* spp., and *Rosenbergiella nectarea* was correlated with *Stenocereus queretaroensis, Hymenocallis littoralis* and *Cascabela ovata* more abundant food sources at the Cofradía and La Campana sites. Whereas, the Potrero site was correlated with humidity, and *Lobelia laxiflora* as an important food resource. Likewise, the presence of the bacterial colonies of *Bacillus* sp. 11, *B. pumillus, Moraxella osloensis*, and *Acinetobacter baumanni* (Fig. 7). The agricultural environment was correlated with the variable weight because the individuals of *L. violiceps* maintained a greater weight, while, *Psittacanthus calyculatus* and *Podranea ricasoliana* were the most abundant plants as a source of food for hummingbirds, coinciding with the bacterial communities *Sphingomonas* spp., *Bacillus* spp., *Paenibacillus glucanolyticus*, and *Curtobacterium* sp. (Fig. 7). Finally, the sites of the urban environment were negatively correlated with temperature and humidity, and correlated with the most abundant resource in the parks, *Bauhinia variegata, Callistemon citrinum*, and *Leonotis nepetifolia* with the presence of *Bacillus* spp., *B. subtilis, B. thuringiensis, Pseudomonas* spp., *Pseudomonas cremoricolorata*, *P. oryzihabitans*, *Sphingobacterium* sp., *Pantoea agglomerans*. *Pantoea* sp., *Paenibacillus* sp. 1, *Sphingomonas taxi*, *Achromobacter* sp., *Acinetobacter* sp., and *Paracoccus fontiphilus* (Fig. 7

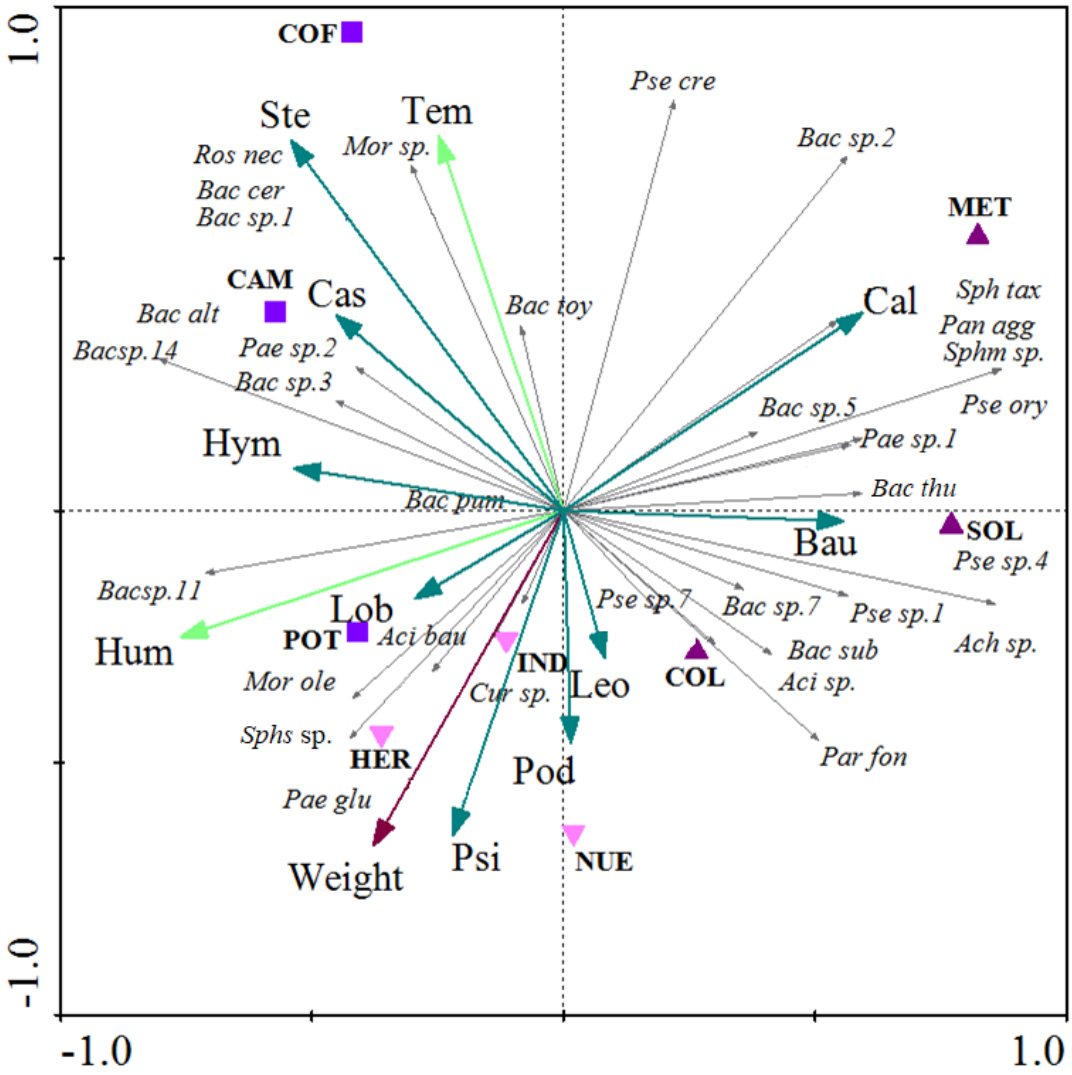

Figure 7. Canonical additive partitioning based on RDA ordinations of the bacterial composition at the sites nested by environments type (urban, agricultural, and natural). The RDA triplot. Three subsets of explanatory variables: abiotic variables (AV), bird measurement (BM), and biotic variables (BV). Sites: CAM = La Campana, COF = Cofradía, POT = Potrero, NUE = Bajío Nuevo, IND = Bajío del Indio, HER = Piedra Herrada, COL = Colomos, MET = Metropolitano, SOL = Solidaridad. AV: Tem = temperature, Hum = humidity. BV: BAU = *Bauhinia variegata*, CAL = *Callistemon citrinum*, LEO = *Leonotis nepetifolia*, POD = *Podranea ricasoliana*, PSI = *Psittacanthus calyculatus*, LOB = *Lobelia laxiflora*, CAS = *Cascabela ovata*, HYM = *Hymenocallis littoralis*, STE = *Stenocereus queretaroensis*. Abbreviations of species are given in Supplementary A, Table A2.

DISCUSSION

This study identified the culture-dependent bacterial communities associated with the plumage of 60 individuals of Violet-crowned hummingbird in response to the different environments. The results found that the bacterial composition structure varied among environments and that it was strongly influenced by the relationship of abiotic (temperature and humidity) and biotic variables such as resource availability.

Bacterial functionality

The biochemical characteristics registered 60 OTUs; however, bacterial species include strains with different functionality, for this, they only represented 51 species. The importance of knowing the biochemical functionality in addition to helping us to identify also provides us with information to suggest hypotheses based on how the strains could behave cohabiting in the hummingbird plumage (Martiny et al. 2013). Therefore, it is inferred that according to the glucose oxide-fermentation test, the strains that did not grow in the presence of this monosaccharide are possibly strains that can degrade keratin and are capable of adapting to extreme plumage conditions. Some of them were *Bacillus subtilis, B pumillus, B. thuringiensis, B. toyonensis, Pseudomonas orizihabitans, Paenibacillus glucanolyticus*, and *Achromobacter* sp. (Supplementary A, Table A2), which have been identified in previous studies with the same functionality (Burt and Iquida 1999; Shawkey et al. 2003; Lucas et al. 2013; Czirják et al. 2013). Most of the bacterial genera isolated in this study use glucose to obtain carbon and energy (Varela and Grotiuz 2008), and in this case, it was perhaps replaced by the keratin protein used for the same purpose (Lange et al. 2016). However, it is necessary to investigate its growth on a selective agar-feather medium to corroborate the glucose test result (Burt and Iquida 1999; Verea et al. 2014).

Differential media were used to identify some strains with the ability to lodge in the host's intestine. The TCBS medium that contains the necessary nutrients for organisms that grow in acidic environments, the Macconkey medium for enteric bacteria in the intestinal tract, and blood agar which was used to find out which strains produce hemolysins and are capable of degrading the erythrocytes of their hosts if they are in optimal conditions. The pathogenic species that grew in these media were *Bacillus cereus* (Supplementary A, Table A2)

registered as a potential pathogen in domestic birds and wild animals (Zuo et al. 2019), and *Acinetobacter baumannii* (Supplementary A, Table A2), which has been registered as a nosocomial pathogen in humans that has developed resistance to antibiotics (Doi et al. 2015; Tacconelli et al. 2018). Likewise, it has been found in the digestive tract of migratory wild birds (Dahiru and Enabulele 2015). This result indicates that unsustainable poultry farming serves as a vector for pathogenic bacteria for wild birds, and therefore, transmits them to different environments through migration (Wilharm et al. 2017). The hummingbird *L. violiceps* most likely contract it from interaction with other hummingbird species that are long-distance migratory or from the short-distance movement of the same hummingbird by contact with areas where poultry farms are located. It should be borne in mind that it is not certain that the strains isolated in this study are the ones that cause the outbreak of the infection, but it is necessary to consider this registry for future research focused on bacterial pathogens of wildlife.

Bacterial composition

Spatial variation

The permutational ANOVA analysis registered that the abundances maintain significant differences between the urban and natural environment (Table 1). It is observed that the natural environment maintains the highest abundance (Fig. 3). Likewise, according to the analysis of the predictive variables, this environment was also positively correlated with the variables of humidity and temperature (Fig. 7), this result coincides with the study of Verea et al. (2014). Which they reported in summer, when rainfall and humidity increase, optimal conditions are produced (e.g., water content and organic matter in the soil, increased plant cover) (Angel et al. 2009; Tian et al. 2018) for the increase of bacteria, and consequently greater transmission to the plumage would be possible but the result was contrary to what was recorded by Burtt and Iquida (1999), and Kent and Burtt (2016). They reported that in the winter season the bacterial load increased, however, in this work the bacterial load in the winter season was not evaluated.

The bacterial composition structure recorded that the urban environment was dissimilar in contrast to the natural environment (Table 1), the species that contributed the highest percentage to the differences were *Bacillus cereus* and *Bacillus* sp. 11, registered in the natural environment (Table 2), these bacteria are commonly found in soil and water.

However, *Bacillus cereus* strains could be pathogenic in warm-blooded animals and humans, as mentioned previously (Cardoso et al. 2019). It is possible to suggest that the appearance of this strain is because *L. violiceps* hummingbirds tend to move at longer distances in natural environments in search of food. Besides, in the Sierra de Quila reserve (natural environment) surroundings, there are cattle and small farms, which directly exposes them to interaction with cows, chickens, and human's waste (Hassell et al. 2019; Smith et al. 2020).

The species of the genus *Bacillus* are part of the central microbiome that coexists in the plumage of hummingbirds because the species *Bacillus pumillus*, *B. subtilis*, *Bacillus* sp. 11 and *Bacillus* sp. 3 coincided in at least two or three sites (Supplement A, Table A4) coinciding with the study by Javurkova et al. (2019) who confirm that some species of the genus *Bacillus* are commensal plumage bacteria in birds, because not only are they keratin degraders, they also produce bacteriocins that work for them to antagonize strains that can be harmful to the host and are part of the degrading bacteria. of keratin such as *Bacillus subtilis,* and *B. pumilus*, the species with the highest incidence in the three environments.

Age effect

The effect of age registered differences in richness, and abundance the natural and urban environment and between adults and juveniles (Fig. 5a-b). While, the bacterial composition varied among the three environments (Fig. 6). Likewise, these environments maintain different characteristics such as the type of vegetation and changes in temperature and humidity as shown in the RDA arrangement of the predictive variables where the natural environment is positively correlated with these variables in contrast to the urban environment (Fig. 7). This result agrees with that reported by Bisson et al. (2007), Saag et al. (2011) and Sotnychuk et al. (2019) in various wild birds, the bacterial composition varied due to the conditions of the different habitats (mangrove, dry vegetation, deciduous and coniferous forest). Regarding the significant differences between adult and juvenile hummingbirds, adults registered the highest bacterial richness and abundance, this result could be given that adults begin to carry out other activities such as looking for insects to complement their diet, courtship display, collection of nest materials; and parental care. Therefore, they are more exposed to generating the optimal conditions for the increase of the bacterial load and the

transmission of different taxa through the contact of different substrates (Dille et al. 2016; Goodenough et al. 2017; Musitelli et al. 2018).

Relationship between bacterial composition and environmental variables

The canonical additive partition showed that the predictive variables that had the greatest contribution were the biotic variables (*Bauhinia variegata, Callistemon citrinum, Leonotis nepetifolia, Podranea ricasoliana, Psittacanthus calyculatus, Lobelia laxiflora, Cascabela ovata, Hymenocallis* and *Steberno littoralis*) which are related with some bacterial taxa recorded in the plumage, such as *Pseudomonas* sp. 7, *Rosenbergiella nectarea* and *Pseudomonas cremoricolorata* (Fig. 7) associated with nectar and flower petals (Alvarez-Perez and Herrera 2013; Halperd et al. 2013; Hayes et al. 2021). The cause of their appearance is that hummingbirds spend most of their time-consuming nectar (Suarez and Welch 2017), so they maintain direct contact with the vegetation where they live.

In the natural environment, the presence of *Rosenbergiella nectarea* was observed at the Cofradía site and it was correlated with the food resource of the *Stenocereus queretaroensis* plant. Likewise, the species *Pseudomonas* sp. 7, and *Pseudomonas cremoricolorata* were recorded in urban parks related to *Bauhinia variegata, Callistemon citrinum,* and *Leonotis nepetifolia* as a food resource available to captured individuals (Fig. 7). It will be necessary to analyze the bacterial composition of the nectar to confirm whether these bacteria inhabit this type of vegetation. González-Teuber and Heil (2009), Lee et al. (2019), and Lamb et al. (2020) have recorded that plants have mutualistic relationships with different bacterial strains that help them attract pollinators through biochemical signals. In addition, the La Campana, Cofradía and Potrero sites were correlated with temperature and humidity with the presence of *Bacillus* spp., *Moraxela* sp. 2, *Acinetobacter baumanni, Paenibacillus* spp., and *Sphingomonas* sp. most are symbiotic bacteria that inhabit water and soil with the exception of *Bacillus cereus* and *Acinetobacter baumanni* which have been reported to be opportunistic for birds and mammals as mentioned above (Fig. 7) (Doi et al. 2015; Tacconelli et al. 2018; Zuo et al. 2019). The increase in temperature and humidity at these sites was due to the fact that these sites were visited in the rainy season.

The weight variable did not influence bacterial composition. However, it was positively correlated with the agricultural environment sites, Bajío del Indio and Piedra Herrada (Fig. 7a-b) while it was observed that the urban environment was negatively related to the variable weight, due to the hummingbirds maintained a decrease in weight in contrast to the weight of hummingbirds in the agricultural environment. This result could indicate a negative aspect mediated by the type of resource available in the environment, since hummingbirds in the city tend to feed on feeders where artificial nectar does not provide the same nutrients as nectar (Roguz et al. 2019) and one of the main symptoms is the loss of body mass. Additionally, exposure to dirty feeders increases the risk of infection by pathogenic bacteria (Giraudeau et al. 2017; Lee et al. 2019). On the other hand, in agricultural sites the most abundant was correlated with the biotic variables *Psittacanthus calyculatus*, which is known as a source of nutritional nectar for hummingbirds (Rodríguez-Mendieta et al. 2018; Quintana-Rodríguez et al. 2018), followed of *Leonotis nepetifolia* and *Podranea ricasoliana* also recorded in these sites and correlated with the presence of the bacteria *Bacillus* spp., *Paenibacillus glucanolyticus*, and *Curtobacterium* sp. (Fig. 7). One hypothesis according to the result is that some registered strains could be related to the microbiota associated with nectar and that they can be symbiotic for plants because they help attract the pollinator and consequently, the hummingbird increases its nectar consumption. It is important to mention that the analysis of this study is only exploratory and the effect of the variables weight and vegetation in relation to the microbiota of the hummingbird plumage depends on the specific characteristics of the host, the type of feeding, reproductive stage and geographical distribution (Stanton, 2020).

In agricultural and urban environments, they were correlated with the presence of *Curtobacterium* sp., and *Sphingomonas taxi* (Eevers et al. 2015; Vimal et al. 2019) with beneficial importance in plants since they promote their growth (Fig. 7). Also, the species of *Pantoea* sp. and *Pantoea agglomerans* were also recorded, which includes both beneficial and pathogenic strains in plants (Soluch et al. 2021) (Fig. 7). These analyses agreed with Dille et al. (2016), which suggests that wild birds can transfer bacteria from different plants through local and long-distance movements. The *L. violiceps* hummingbird is an abundant species in the studied environments, and it is considered an important pollinator, especially for plants of economic interest in the agricultural environment., Thus, it could be a key

species in the dispersal of beneficial bacteria such as pathogens in different types of vegetation (Serrano-Serrano et al. 2017; Maruyama et al. 2019). In the agricultural environment analyzed in this study, the frequent use of pesticides was recorded, which cause phytopathogenic bacteria to generate resistance to these products and antibiotics. Likewise, affect the beneficial microbial communities for plants (Duke 2018) and consequently generate damage to the health of pollinators such as *L. violiceps*, and in general, to wild birds due to the consumption of pesticides through pollination and seed consumption (Goulson 2014).

This study verified that the culture-dependent bacterial composition associated with the plumage of *L. violiceps* changed in the different environments, related to the specific biotic and abiotic characteristics of the area and that the adults contain a higher bacterial load and richness in their feathers due to the increase in the necessary activities for survival. Furthermore, we support that the identified feather-degrading bacteria of the *Bacillus* genus were part of the central microbiota of *L. violiceps* plumage. In addition, it was found that in areas close to livestock, hummingbirds are more exposed to the transmission of pathogenic bacteria.

FUTURE PERSPECTIVE

This study contributes to the importance of native vegetation for pollinators in both agricultural, natural and urban environments because they are a better source of nutritious food for hummingbirds, would increase the interaction of bacterial strains with beneficial effects for both plants and host, and would help reduce wildlife-human interactions and, consequently, the transmission of strains with pathogenic potential from humans to wildlife a lower weight, probably due to the use of feeders as their main food source. It is necessary to increase studies on the microorganisms associated with wild birds and their relationship with biotic and abiotic variables such as the increase in the number of variables, for example: adding vegetation cover, abundance of plant species and precipitation. Also, their relationship with the microbial composition of native vegetation. On the other hand, it would be important to use metagenomic techniques for the analysis of microbial diversity associated with hummingbird plumage.

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SUPPLEMENTARY INFORMATION A

Table A1. Biochemical tests of the isolates. Cat = catalase, Oxi = Oxidase, NaCl = (3.4%, 6%, 8%), Mob = Motility, Glu = Glucose, Mac = Macconkey medium, Vog = Voges-Proskauer, Met = Methil red, Ind = Indole, and Cell = Cell shape.

Strains	Gram	Cat	Oxi	3.4	6	8	Mot	Glu	TCBS	Mac	Vog	Met	Ind	Cell
M5.1 z	1	1	1	1	1	0	1	2	0	0	1	1	0	2
M5.2 z	1	1	0	1	1	1	1	1	1	2	1	0	0	1
M5.3 z	1	1	0	1	0	0	1	4	0	0	0	0	0	2
M9.1 z	1	1	1	1	1	1	0	4	0	0	1	0	0	1
M9.2 z	0	1	1	1	0	0	1	4	0	0	0	0	0	2
M11.1 z	1	1	1	0	0	0	1	4	0	0	0	1	0	2
M12.2 z	0	1	1	1	0	0	1	4	0	2	0	0	0	1
M31.1 z	1	1	1	1	1	1	0	4	0	0	1	0	0	1
M31.2 z	1	1	0	1	0	0	1	4	0	0	1	0	0	1
M31.3 z	1	1	1	1	1	1	1	4	0	0	1	1	0	1
M31.4 z	1	1	0	1	1	1	1	4	0	0	0	1	0	1
M32.1 z	0	1	0	1	1	1	1	2	0	0	0	0	0	2
M32.2 z	1	1	0	1	1	1	1	4	0	0	0	1	0	1
M32.3 z	1	1	1	1	1	1	1	4	0	0	1	0	0	2
M32.4 z	1	1	1	1	1	1	0	4	0	0	1	0	0	1
M32.5 z	0	1	1	1	1	1	1	4	0	1	0	0	1	2
M32.6 z	0	1	1	1	1	1	1	4	0	1	0	0	0	1
M33.1 z	1	1	0	1	0	0	1	4	0	0	0	0	0	2
M33.2 z	1	1	0	1	1	1	0	4	0	0	0	0	0	1
M33.3 z	1	1	0	1	0	0	1	2	0	0	0	1	0	2
M33.4 z	1	1	0	1	0	0	1	2	0	0	0	1	0	2
M33.5 z	1	1	1	1	1	1	1	4	0	0	1	1	0	1
M33.6 z	0	1	0	1	1	0	1	3	0	1	0	0	0	1
M33.7 z	1	1	1	1	1	1	0	4	0	0	1	0	0	1
M33.8 z	0	1	0	1	1	1	1	2	0	0	0	0	0	2
M33.9 z	1	1	1	1	1	0	1	2	0	0	1	1	0	2
M33.10 z	1	1	0	1	1	1	1	4	0	0	0	1	0	1
M34.1 z	1	1	1	1	1	1	1	4	0	0	1	1	0	1
M34.2 z	1	1	0	1	1	1	1	4	0	0	0	1	0	1
C1.1 z	1	1	1	1	1	0	0	1	0	0	1	1	0	1
C1.2 z	1	1	1	1	0	0	1	4	0	0	1	1	0	1
C2.1 z	1	1	1	1	0	0	1	4	0	0	1	1	0	1
C7.1 z	1	1	1	1	0	0	1	2	0	0	0	0	0	2
C7.2 z	0	1	0	1	1	1	1	2	0	0	0	0	0	2
C8.1 z	1	1	1	1	1	1	0	4	0	0	1	0	0	1
C9.1 z	1	1	1	1	1	0	1	2	0	0	1	1	0	2
C9.2 z	0	1	1	0	0	0	1	4	0	0	0	1	0	1
C21.1 z	0	1	0	1	1	1	1	1	0	1	1	0	0	2
C21.2 z	0	1	1	1	1	1	1	1	1	0	1	0	0	2
C21.3 z	0	1	0	1	1	1	1	2	0	0	0	0	0	2
C21.1 a	0	1	1	1	1	1	1	1	1	0	1	0	0	2
C21.2 a	0	1	0	1	1	1	1	1	0	1	1	0	0	2
C21.3 a	0	1	0	1	1	1	1	2	0	0	0	0	0	2
C21.4 a	0	1	1	1	0	0	1	4	0	0	1	0	0	1
C22.1 z	0	1	0	1	1	1	1	2	0	0	0	0	0	1
C22.2 z	0	1	0	1	1	1	0	2	0	2	1	0	0	1
C23.1 z	1	1	1	1	1	1	1	1	0	0	0	0	0	2
C23.2 z	1	1	1	1	1	1	1	1	0	0	1	1	0	1
C23.3 z	0	1	0	1	1	1	1	2	0	0	0	0	0	2
C23.4 z	1	1	1	1	1	1	1	2	0	0	1	0	0	1

S4.1 z	0	1	0	1	1	1	1	2	0	0	0	0	0	2
S4.2 z	0	1	0	1	1	1	1	2	0	0	0	0	0	1
S4.3 z	0	1	0	1	1	1	0	3	0	2	0	0	0	1
S4.4 z	0	1	1	1	0	0	1	4	0	0	0	0	0	2
S4.5 z	0	1	0	1	1	1	1	3	0	0	0	0	0	1
S5.1 z	0	1	1	1	1	1	1	1	1	1	0	0	0	1
S5.2 Z	1	1	0	1	1	1	0	1	0	0	1	0	0	2
S5.3 z	0	1	0	1	1	1	1	2	0	0	0	0	0	2
S5.4 z	0	1	0	1	1	1	1	2	0	0	0	0	0	2
S7.1 z	0	1	1	1	1	1	1	1	1	0	1	0	0	2
S7.2 z	1	1	1	1	1	1	1	1	0	0	1	1	0	1
S7.3 z	0	1	1	1	0	0	1	4	0	0	1	0	0	1
S7.4 z	0	1	1	1	0	0	1	4	0	0	0	0	0	2
S7.5 z	1	1	0	1	0	0	1	4	0	0	1	0	0	1
S7.1 a	1	1	0	1	0	0	1	4	0	0	0	0	0	2
S8.1 z	1	1	1	1	1	1	1	4	0	0	1	0	0	2
S8.2 z	0	1	1	1	0	0	1	4	0	0	1	0	0	1
S8.3 z	0	1	0	1	1	1	1	2	0	0	0	0	0	2
S8.4 z	0	1	1	1	0	0	1	4	0	0	0	0	0	2
S8.5 z	1	1	1	1	1	1	0	4	0	0	1	0	0	1
S9.1 a	1	1	1	1	1	1	1	4	0	0	1	0	0	2
S9.2 a	0	1	1	1	0	0	1	4	0	0	1	0	0	1
S9.3 a	1	1	0	1	0	0	1	4	0	0	1	0	0	1
S9.1 z	0	1	1	1	0	0	1	4	0	0	1	0	0	1
S9.2 z	1	1	0	1	0	0	1	4	0	0	1	0	0	1
S9.3 z	0	1	1	1	0	0	1	4	0	0	0	0	0	2
S9.4 z	0	1	1	1	0	0	1	4	0	2	0	0	0	1
S9.5 z	1	1	0	1	0	0	1	4	0	0	0	0	0	2
B1.1 z	1	1	0	1	0	0	1	4	0	0	1	0	0	1
B2.1 z	1	1	0	1	0	0	1	4	0	0	1	0	0	1
B3.1 z	0	1	1	0	0	0	1	4	0	0	1	0	0	3
B4.1 z	1	1	0	1	1	1	0	1	0	0	1	0	0	2
B5.1 z	1	1	1	1	0	0	1	4	0	0	1	1	0	3
B5.2 z	1	1	1	1	1	1	1	4	0	0	1	1	0	1
B6.1 z	1	1	1	1	1	1	1	4	0	0	1	1	0	1
B6.2 z	1	1	0	1	0	0	1	2	0	0	0	1	0	2
B6.3 z	0	1	0	1	0	0	1	4	0	0	0	1	0	1
B6.4 z	1	1	1	1	0	0	1	4	0	0	1	1	0	3
B6.5 z	1	1	0	1	1	1	1	4	0	0	1	1	0	1
B6.6 z	1	1	1	1	1	1	1	4	0	0	1	1	0	2
PH3.1 z	1	1	0	1	0	0	1	4	0	0	1	0	0	1
PH3.2 z	1	1	1	1	1	1	1	2	0	0	1	0	0	1
PH4.1 z	1	1	1	1	1	0	0	1	0	0	1	1	0	1
PH4.2 z	0	1	0	1	1	0	1	4	0	0	0	1	0	4
PH4.3 z	1	1	0	1	1	1	1	4	0	0	1	1	0	1
PH4.4 z	0	1	1	1	0	0	1	4	0	0	0	1	0	3
PH6. 1 z	0	1	0	1	0	0	1	4	0	0	0	1	0	1
PH6. 2 z	1	1	1	1	1	0	0	1	0	0	1	1	0	1
PH6. 3 z	0	1	0	1	1	0	1	4	0	0	0	1	0	4
PH6. 4 z	1	1	1	1	1	1	1	2	0	0	1	1	0	3
PH8.1 a	1	1	1	1	1	1	1	1	0	0	1	1	0	2
PH8.2 a	1	1	1	1	1	0	0	1	0	0	1	1	0	1
PH9.1 a	1	1	1	1	1	1	1	2	0	0	1	0	0	1
PH9.2 a	1	1	0	1	1	1	1	2	0	0	1	0	0	2
PH9.3 a	0	1	0	1	1	0	1	4	0	0	0	1	0	4
PH9.4 a	0	1	0	1	1	0	1	4	0	0	0	1	0	4
PH9.5 a	1	1	1	1	1	0	1	4	2	0	1	1	0	2

PH9.6 a	1	1	1	1	1	1	1	4	0	0	1	1	0	2
PH10.1 z	1	1	1	1	1	1	1	4	0	0	1	1	0	1
PH10.2 z	0	1	0	1	1	0	1	4	0	0	0	1	0	4
BN1.1 z	0	1	1	1	0	0	1	3	1	2	0	0	0	2
BN1.2 z	0	1	1	1	0	0	1	4	1	1	0	0	0	1
BN2.3 z	0	1	1	1	0	0	1	3	1	2	0	0	0	2
BN2.4 z	1	1	1	1	0	0	1	2	0	0	0	0	0	2
BN2.5 z	0	1	1	1	0	0	1	4	0	0	0	0	0	2
BN2.6 z	0	1	1	1	0	0	1	3	0	2	0	0	0	2
BN2.7 z	0	1	0	1	1	1	1	2	0	0	0	0	0	2
BN2.8 z	0	1	1	1	0	0	1	4	0	0	1	0	0	1
BN3.1 z	0	1	1	1	0	0	1	3	0	2	0	0	0	2
BN4.1 z	1	1	1	1	1	1	1	2	0	0	1	0	0	1
BN4.2 z	1	1	1	1	1	0	0	1	0	0	1	1	0	1
BN4.3 z	0	1	0	1	1	0	1	4	0	0	0	1	0	4
BN4.4 z	1	1	1	1	1	1	1	4	0	0	1	1	0	1
BN4.5 z	1	1	1	1	1	1	1	4	0	0	1	1	0	4
BN5.1 z	1	1	0	1	1	1	1	4	0	0	1	1	0	1
BN5.2 z	0	1	0	1	1	0	1	4	0	0	0	1	0	4
BN5.3 z	1	1	1	1	1	0	0	1	0	0	1	1	0	1
BN6.1 z	1	1	1	1	1	1	0	4	0	0	1	0	0	1
QC1.1 z	1	1	1	1	1	0	0	2	0	0	1	1	0	2
QC1.2 z	1	1	1	1	0	0	1	2	0	0	0	0	0	2
QC1.3 z	0	1	0	1	0	0	1	4	0	2	0	1	0	1
QC2.1 z	1	1	1	1	0	0	1	2	0	0	0	0	0	2
QC2.2 z	1	1	1	1	1	0	0	2	0	0	1	1	0	2
QC4.1 a	1	1	0	1	1	0	0	1	0	0	1	1	0	1
QC4.2 a	1	1	1	1	1	1	1	1	0	0	0	1	0	3
QC4.3 a	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QC4.1 z	1	1	0	1	1	0	0	1	0	0	0	1	0	1
QC4.2 z	1	1	1	1	1	1	1	4	2	0	1	1	0	1
QC5.1 z	1	1	0	1	1	1	1	1	1	2	1	0	0	1
QC5.2 z	1	1	0	1	1	0	0	1	0	0	0	1	0	1
QC7.1 a	1	1	0	1	1	0	0	1	0	0	1	1	0	1
QC7.2 a	1	1	0	1	1	1	1	1	1	2	1	0	0	1
QC7.3 a	1	1	0	1	1	0	0	1	0	0	1	1	0	1
QC7.1 z	1	1	0	1	1	1	1	1	1	2	1	0	0	1
QC9.1 a	1	1	0	1	1	1	1	1	1	2	1	0	0	1
QC9.1 z	1	1	0	1	1	0	0	1	0	0	1	1	0	1
QC9.2 z	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QC10.1 z	1	1	0	1	0	0	0	2	0	0	0	1	0	3
QC10.2 z	1	1	0	1	1	0	0	1	0	0	0	1	0	1
QC10.3 z	1	1	0	1	1	1	1	1	1	2	1	0	0	1
QC10.1 a	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QC10.2 a	1	1	0	1	1	0	0	1	0	0	0	1	0	1
QC10.3 a	1	1	0	1	1	1	1	2	2	2	1	0	0	4
QC11.1 a	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QC11.2 a	1	1	0	1	1	0	0	1	0	0	0	1	0	1
QC11.3 a	1	1	1	1	1	1	1	1	0	0	0	0	0	2
QC11.4 a	1	1	0	1	1	1	1	1	1	2	1	0	0	1
QF1.1 a	0	1	0	1	1	0	1	3	0	1	0	0	0	1
QF1.2 a	1	1	0	1	0	0	1	4	0	0	0	0	0	2
QF1.3 a	0	1	0	1	0	0	1	4	0	2	0	1	0	1
QF1.1 z	0	1	0	1	0	0	1	4	0	2	0	1	0	1
QF1.2 z	0	1	0	1	0	0	1	2	0	0	0	0	0	1
QF1.3 z	0	1	0	1	1	0	1	3	0	1	0	0	0	1
QF2.1 z	1	1	1	1	0	0	0	4	0	0	1	0	0	3

QF2.2 z	1	1	1	1	1	1	1	1	0	0	0	1	0	3
QF3.1 z	1	1	0	1	1	0	0	1	0	0	1	1	0	1
QF3.2 z	1	1	1	1	1	1	1	2	0	0	1	0	0	1
QF3.1 z	1	1	1	1	1	1	1	1	0	0	0	1	0	3
QF3.1 z	1	1	0	1	1	0	0	1	0	0	1	1	0	1
QF3.2 z	1	1	1	1	1	1	1	1	0	0	0	1	0	3
QF3.3 z	1	1	0	1	0	0	0	2	0	0	0	1	0	3
QF4.1 z	1	1	1	1	0	0	1	4	0	0	1	1	0	1
QF4.2 z	1	1	0	1	1	0	0	1	0	0	1	1	0	1
QF4.3 z	1	1	0	1	1	1	1	1	1	2	1	0	0	1
QF4.4 z	1	1	1	1	1	1	1	1	0	0	0	1	0	3
QF5.1 z	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QF5.2 z	1	1	1	1	0	0	1	4	0	0	1	1	0	3
QF5.3 z	1	1	1	1	1	1	1	1	0	0	1	1	0	1
QF5.1 a	1	1	1	1	1	1	1	2	0	0	1	0	0	1
QF5.2 a	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QF5.3 a	1	1	1	1	1	1	1	4	2	0	1	1	0	1
QF5.4 a	1	1	1	1	0	0	1	4	0	0	0	1	0	1
QF6.1 z	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QF6.2 z	1	1	1	1	1	1	1	1	0	0	1	1	0	1
QF6.3 z	1	1	1	1	0	0	1	4	0	0	1	1	0	3
QF6.4 z	1	1	1	1	1	1	1	2	0	0	1	0	0	1
QF6.1 a	1	1	1	1	1	1	1	2	0	0	1	0	0	1
QF6.2 a	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QF6.3 a	1	1	1	1	0	0	1	4	0	0	1	1	0	3
QF6.4 a	1	1	1	1	1	1	1	1	0	0	1	1	0	1
QF7.1 a	1	1	1	1	1	1	1	4	0	0	1	1	0	2
QF7.2 a	0	1	1	1	0	0	1	4	0	0	0	0	0	2
QP1.1 z	0	1	0	1	1	1	1	2	0	0	0	0	0	1
QP2.1 a	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QP2.2 a	1	1	1	1	0	0	1	4	0	0	0	1	0	1
QP2.3 a	1	1	1	1	1	1	1	2	0	0	1	0	0	1
QP2.1 z	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QP2.2 z	1	1	1	1	1	1	1	4	2	0	1	1	0	1
QP2.3 z	1	1	1	1	1	0	1	4	2	0	1	1	0	2
QP3.1 z	0	1	1	1	1	1	1	4	0	1	0	0	0	1
QP3.2 z	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QP3.3 z	1	1	1	1	0	0	1	4	0	0	1	1	0	3
QP4.1 z	1	1	1	1	1	0	0	1	0	0	1	1	0	1
QP4.2 z	1	1	1	1	0	0	1	4	0	0	1	1	0	3
QP4.3 z	1	1	1	1	1	1	1	2	0	0	1	1	0	3
QP5.1 z	0	1	0	1	0	0	1	4	0	0	0	1	0	1
QP5.2 z	0	1	0	1	1	0	1	4	0	0	0	1	0	4
QP5.3 z	1	1	1	1	1	0	1	4	2	0	1	1	0	2
QP5.4 z	0	1	0	1	0	0	1	3	0	2	0	1	0	4
QP6.1 z	1	1	1	1	1	1	1	4	0	0	1	1	0	1
QP6.2 z	0	1	0	1	1	0	1	4	0	0	0	1	0	4
QP6.3 z	1	1	1	1	1	0	1	4	2	0	1	1	0	2

TCBS		MacConkey		Glucose		Cell shape		Positive 1
0	No growth	0	No growth	1	facultative	1	Cocci	Negative 0
1	green	1	White	2	Fermentative	2	Bacilli	
2	yellow	2	Pink	3	Oxidative	3	Streptococci	
				4	Inactive	4	Coccobacilli	

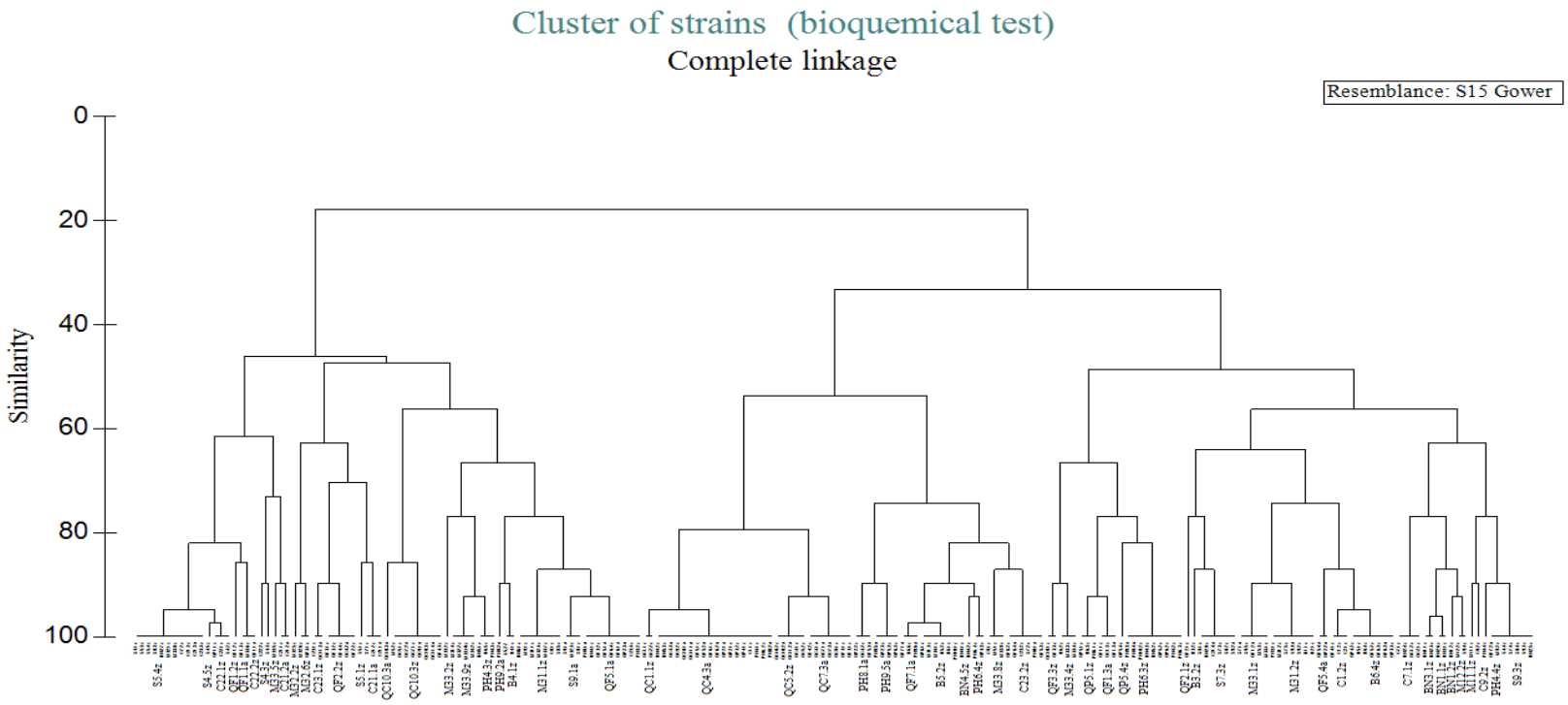


Figure 2. Complete hierarchical grouping linking the biochemical tests performed on the 212 isolates.

Table 2. OTUs identification table. Triplot Abbreviations, Sequence similarity in blast, incidence in the samples, habitat and functionality registered, and biochemical tests results. Positive (+) and negative (-).

Operational Taxonomic Units (OTUs)	Abbreviations	Similarity (%)	Incidence (%)	Habitat/Functionality	Biochemical test													
					Oxidase	Catalase	Voges-Proskauer	Methyl red	Indole	3% (NaCl)	6% (NaCl)	8% (NaCl)	TCBS	MacConkey	Glucose	Motility	Gram	Hemolysis
Achromobacter sp.	*Ach sp.*	99.56%	5.18867925	Soil and polluted environments/Feather-degrading	-	+	-	-	-	+	+	+	-	-	Fermentative	+	-	Inactive
Acinetobacter baumannii	*Acibau*	98.24%	0.47169811	Soil and water/feather-degrading and pathogen	-	+	-	+	-	+	-	-	-	+	Oxidative	+	-	β
Acinetobacter sp.	*Acisp.*	98.77%	1.41509434	Soil, water, and wild animals	-	+	-	-	-	+	+	+	-	-	Facultative	+	-	Inactive
Bacillus altitudinis	*Bac alt*	100%	3.77358491	Soil and vegetation/plant pathogen	-	+	+	-	-	+	+	+	+	+	Facultative	+	+	Inactive
Bacillus cereus	*Bac cer*	99.25%	2.35849057	Soil and water/pathogen	-	+	+	-	-	+	+	+	+	+	Facultative	+	+	Inactive
Bacillus cereus	*Bac cer*	99.95%	0.94339623	Soil and water/pathogen	+	+	+	+	-	+	+	-	-	-	Facultative	-	+	β
Bacillus pumilus	*Bac pum*	99.11%	3.30188679	Soil and water/Feather-degrading	+	+	+	+	-	+	+	+	-	-	Inactive	+	+	Inactive
Bacillus pumilus	*Bac pum*	99.33%	1.41509434	Soil and water	+	+	+	+	-	+	+	+	-	-	Facultative	+	+	β
Bacillus pumilus	*Bac pum*	99.57%	1.41509434	Soil and water/Feather-degrading	+	+	+	+	-	+	+	+	-	-	Inactive	+	+	Inactive
Bacillus pumilus	*Bac pum*	99.30%	2.83018868	Soil and water/Feather-degrading	+	+	+	+	-	+	+	+	-	-	Inactive	+	+	Inactive
Bacillus pumilus	*Bac pum*	99.13%	1.41509434	Soil and water/Feather-degrading	+	+	+	+	-	+	+	+	-	-	Inactive	+	+	β
Bacillus pumilus	*Bac pum*	97.74%	0.94339623	Soil and water	-	+	-	+	-	+	-	-	-	-	Fermentative	-	+	β
Bacillus safensis	*Bac saf*	97.45%	0.47169811	Soil and water	-	+	+	-	-	+	+	+	+	+	Fermentative	+	+	Inactive
Bacillus subtilis	*Bac sub*	99.55%	3.30188679	Soil and water/Feather-degrading	+	+	+	-	-	+	+	+	-	-	Inactive	+	+	Inactive
Bacillus subtilis	*Bac sub*	98.84%	4.24528302	Soil and water	+	+	+	-	-	+	+	+	-	-	Fermentative	+	+	β
Bacillus subtilis	*Bac sub*	98.27%	0.94339623	Soil and water	+	+	+	+	-	+	+	+	-	-	Fermentative	+	+	Inactive
Bacillus sp. 1	*Bac sp.1*	99.63%	2.35849057	Soil,water, and wildlife	-	+	-	+	-	+	+	+	-	-	Facultative	+	+	Inactive
Bacillus sp. 2	*Bac sp.2*	98.03%	2.35849057	Soil,water, and wildlife/Feather-degrading	-	+	-	-	-	+	-	-	-	-	Inactive	+	+	β
Bacillus sp. 3	*Bac sp.3*	99.55%	3.30188679	Soil,water, and wildlife/Feather-degrading	+	+	+	+	-	+	-	-	-	-	Inactive	+	+	Inactive
Bacillus sp. 4	*Bac sp.4*	99.49%	0.47169811	Soil,water, and wildlife/Feather-degrading	+	+	-	+	-	-	-	-	-	-	Inactive	+	+	Inactive
Bacillus sp. 5	*Bac sp.5*	98.59%	1.41509434	Soil,water, and wildlife	-	+	-	+	-	+	-	-	-	-	Fermentative	+	+	α
Bacillus sp. 6	*Bac sp.6*	97.26%	0.47169811	Soil,water, and wildlife/Feather-degrading	-	+	-	-	-	+	+	+	-	-	Inactive	+	+	Inactive
Bacillus sp. 7	*Bac sp.7*	98.99%	0.94339623	Soil,water, and wildlife	-	+	+	-	-	+	+	+	-	-	Facultative	-	+	Inactive
Bacillus sp. 8	*Bac sp.8*	98.09%	0.47169811	Soil,water, and wildlife/Feather-degrading	+	+	+	+	-	+	+	+	-	-	Inactive	+	+	Inactive
Bacillus sp. 9	*Bac sp.9*	98.63%	0.94339623	Soil,water, and wildlife	+	+	-	-	-	+	+	+	-	-	Facultative	+	+	β
Bacillus sp. 10	*Bac sp.10*	98.97%	1.88679245	Soil,water, and wildlife/Feather-degrading	-	+	-	+	-	+	+	+	-	-	Inactive	+	+	Inactive
Bacillus sp. 11	*Bac sp.11*	98.37%	8.49056604	Soil,water, and wildlife	+	+	+	+	-	+	+	-	-	-	Facultative	-	+	β
Bacillus sp. 12	*Bac sp.12*	97.53%	1.41509434	Soil,water, and wildlife/Feather-degrading	+	+	+	+	-	+	+	+	-	-	Inactive	+	+	Inactive
Bacillus sp. 13	*Bac sp.13*	98.71%	0.94339623	Soil,water, and wildlife/Feather-degrading	+	+	-	+	-	+	-	-	-	-	Inactive	+	+	β
Bacillus sp. 14	*Bac sp.14*	98.65%	1.88679245	Soil,water, and wildlife/Feather-degrading	+	+	+	+	-	+	+	+	+	-	Inactive	+	+	β

Operational Taxonomic Units (OTUs)	Abbreviations	Similarity (%)	Incidence (%)	Habitat/Functionality	Biochemical test													
					Oxidase	Catalase	Voges-Proskauer	Methyl red	Indole	3%(NaCl)	6%(NaCl)	8%(NaCl)	TCBS	MacConkey	Glucose	Motility	Gram	Hemolysis
Bacillus tequilensis	*Bac teq*	99.18%	0.47169811	Soil and water	-	+	+	-	-	+	+	+	-	-	Fermentative	+	+	β
Bacillus thuringiensis	*Bac thu*	99.12%	3.30188679	Soil and water/Feather-degrading	-	+	+	-	-	+	-	-	-	-	Inactive	+	+	β
Bacillus toyonensis	*Bac toy*	99.93%	1.41509434	Soil and feces of cattle/Feather-degrading	+	+	+	+	-	+	-	-	-	-	Inactive	+	+	β
Curtobacterium sp.	*Cur sp.*	96.05%	1.88679245	Vegetation	+	+	-	-	-	+	-	-	-	-	Fermentative	+	+	Inactive
Moraxella osloensis	*Mor osl*	99.33%	0.47169811	Damp environments/Feather-degrading	+	+	+	-	-	-	-	-	-	-	Inactive	+	-	Inactive
Moraxella sp. 1	*Mor sp.1*	99.12%	0.94339623	Soil, water, and vegetation	+	+	-	-	-	+	-	-	-	+	Oxidative	+	-	Inactive
Moraxella sp. 2	*Mor sp.2*	97.99%	0.47169811	Soil, water, and vegetation	-	+	-	-	-	+	-	-	-	-	Fermentative	+	-	Inactive
Paenibacillus glucanolyticus	*Pae glu*	99.49%	4.24528302	Vegetation/Feather-degrading	-	+	-	+	-	+	+	-	-	-	Inactive	+	-	α
Paenibacillus sp. 1	*Pae sp.1*	98.21%	1.41509434	Soil and vegetation	+	+	+	+	-	+	+	-	-	-	Fermentative	+	+	Inactive
Paenibacillus sp. 2	*Pae sp.2*	99.47%	3.30188679	Soil and vegetation	-	+	+	+	-	+	+	-	-	-	Facultative	-	+	Inactive
Paenibacillus sp. 3	*Pae sp.3*	98.44%	1.41509434	Soil and vegetation/Feather-degrading	-	+	-	+	-	+	+	+	-	-	Inactive	+	-	β
Paenibacillus sp. 4	*Pae sp.4*	99.21%	0.47169811	Soil and vegetation/Feather-degrading	+	+	+	-	-	+	-	-	-	-	Inactive	-	+	Inactive
Pantoea agglomerans	*Pan agg*	99.27%	0.47169811	Soil, water, and vegetation	-	+	+	-	-	+	+	+	-	-	Inactive	+	-	Inactive
Pantoea sp.	*Pan sp.*	99.29%	0.47169811	Soil, water, and vegetation	-	+	+	-	-	+	+	+	-	+	Fermentative	-	-	Inactive
Paracoccus fontiphilus	*Par fon*	99.40%	2.83018868	Freshwater/Feather-degrading	+	+	+	-	-	+	-	-	-	-	Inactive	-	-	β
Pseudomonas cremoricolorata	*Pse cre*	99%	1.41509434	Sediments and flowers	-	+	-	-	-	+	+	-	-	+	Oxidative	+	-	Inactive
Pseudomonas oryzihabitans	*Pse ory*	97.61%	0.47169811	Damp environments/Feather-degrading	+	+	-	-	+	+	+	+	-	+	Inactive	+	-	Inactive
Pseudomonas oryzihabitans	*Pse ory*	97.09%	0.47169811	Damp environments/Feather-degrading	+	+	-	+	-	-	-	-	-	-	Inactive	+	-	Inactive
Pseudomonas sp. 1	*Pse sp.1*	99.55%	1.41509434	Floral nectar	-	+	+	-	-	+	+	+	-	-	Facultative	+	-	Inactive
Pseudomonas sp. 2	*Pse sp.2*	99.25%	0.47169811	Soil and water/Feather-degrading	+	+	-	-	-	+	-	-	+	-	Inactive	+	-	β
Pseudomonas sp. 3	*Pse sp.3*	99.78%	0.94339623	Soil and water	+	+	-	-	-	+	-	-	-	+	Oxidative	+	-	Inactive
Pseudomonas sp. 4	*Pse sp.4*	98.85%	0.47169811	Soil and water	-	+	-	-	-	+	+	+	-	+	Oxidative	-	-	Inactive
Pseudomonas sp. 5	*Pse sp.5*	99.33%	0.47169811	Soil and water	-	+	-	-	-	+	+	+	-	-	Oxidative	+	-	Inactive
Pseudomonas sp. 6	*Pse sp.6*	98.49%	0.94339623	Soil and water/Feather-degrading	+	+	-	-	-	+	+	+	-	-	Inactive	+	-	Inactive
Pseudomonas sp. 7	*Pse sp.7*	100.00%	0.94339623	Floral nectar	-	+	+	-	-	+	+	+	-	+	Facultative	+	-	Inactive
Pseudomonas sp. 8	*Pse sp.8*	99.02%	0.47169811	Soil and water	+	+	-	-	-	+	+	+	+	+	Facultative	+	-	Inactive
Sphingobacterium sp.	*Sph sp.*	97.98%	0.94339623	Soil and polluted environments/Feather-degrading	-	+	-	-	-	+	-	-	-	+	Inactive	+	-	Inactive
Sphingomonas sp.	*Sph sp.*	95.63%	0.47169811	Soil, water, and vegetation/Feather-degrading	+	+	-	+	-	+	-	-	-	-	Inactive	+	-	Inactive
Sphingomonas taxi	*Sph tax*	99.84%	3.30188679	Soil and vegetation/Feather-degrading	+	+	-	-	-	+	-	-	+	-	Inactive	+	-	Inactive
Rosenbergiella nectarea	*Ros nec*	99.88%	1.41509434	Floral nectar	-	+	-	+	-	+	-	-	-	+	Inactive	+	-	Inactive
Percentage			100%															
Total trains			212															

Figure A2. Graphs of the results of the strains in the biochemical tests by environment.

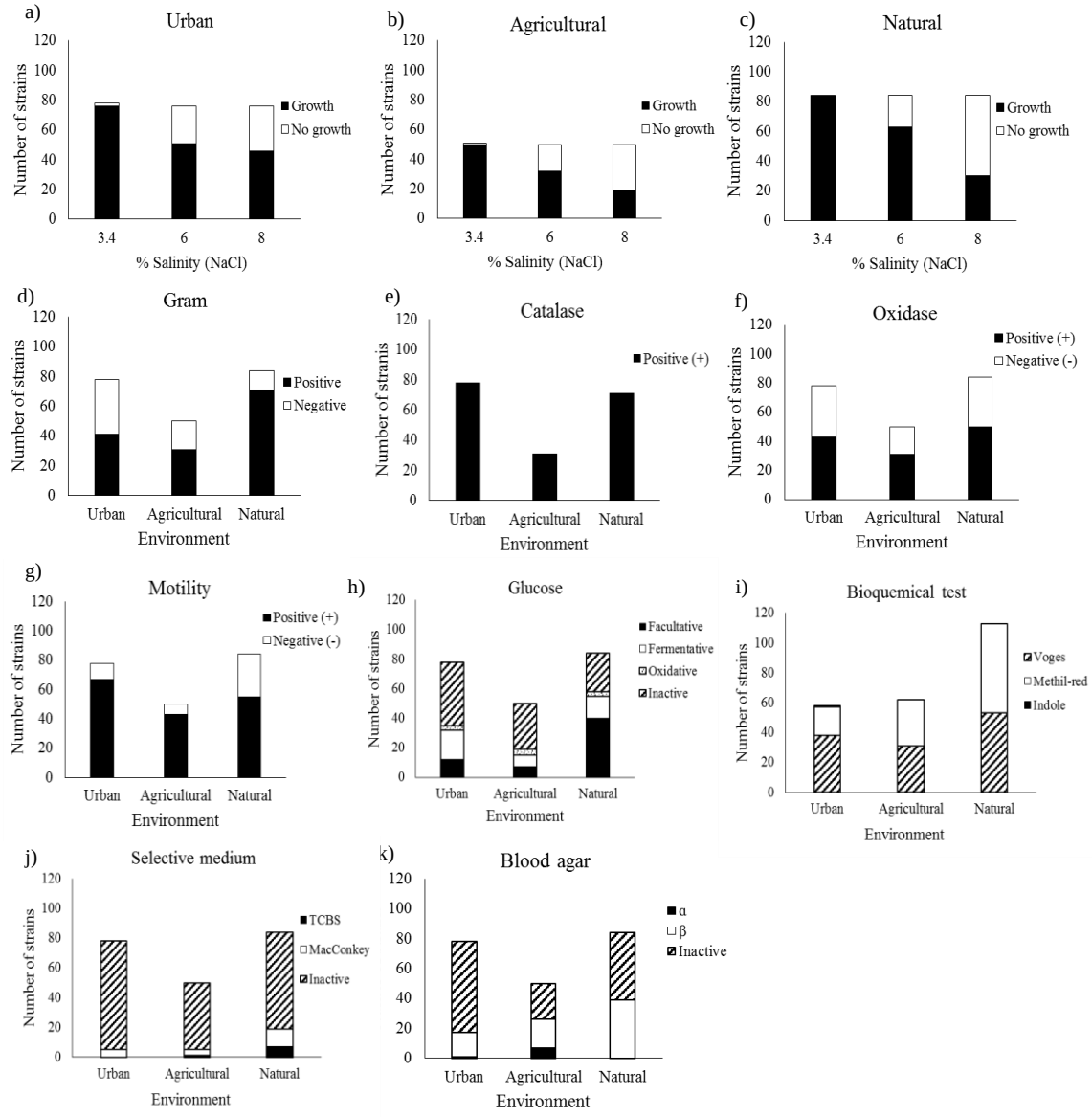
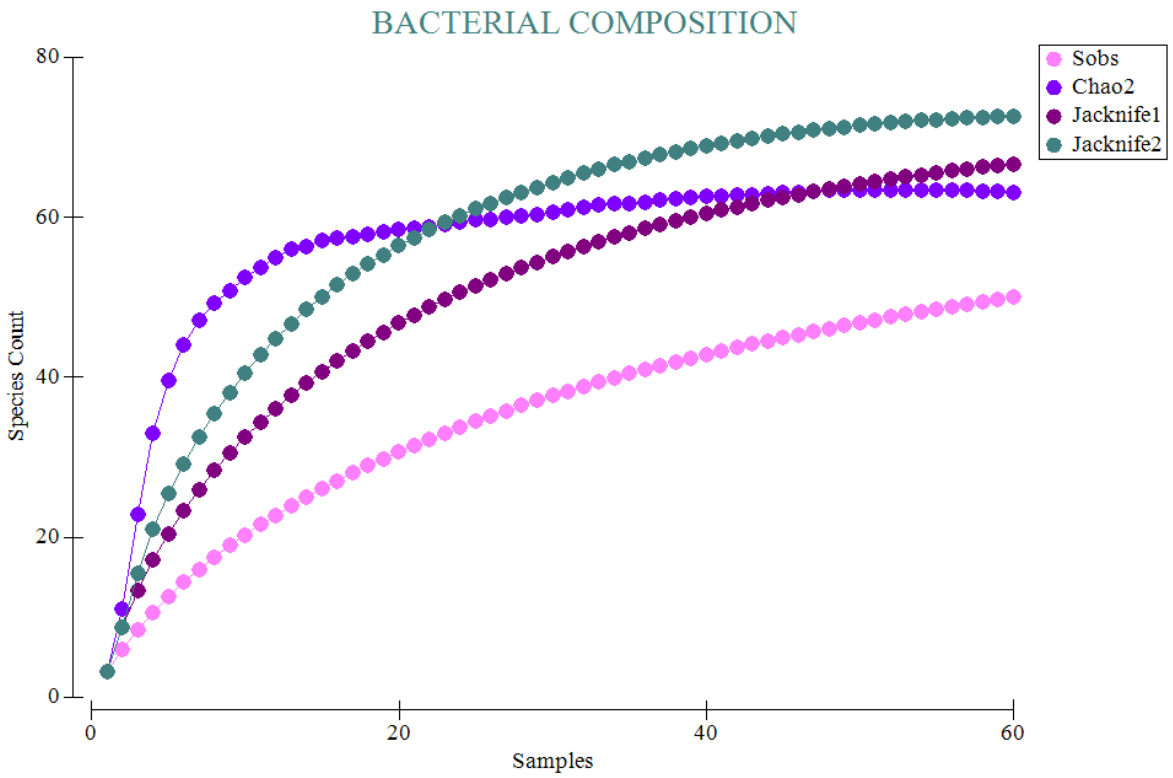


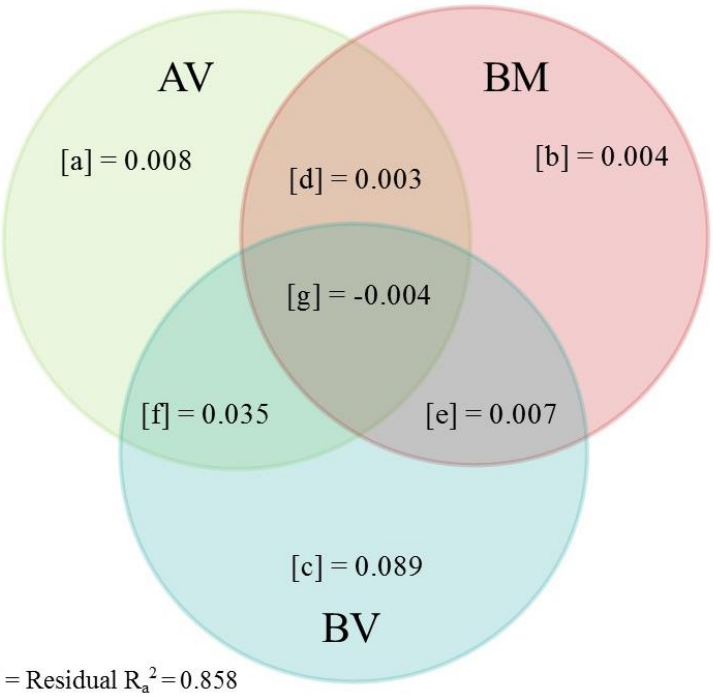
Figure A3. The species accumulation curve. Sampling effort was assessed using sampled-based rarefactions according to the following non-parametric estimators: Chao 2, Jackknife 1, and Jackknife 2. Sobs = Observed Species.



Sobs	Observed richness (%)	Stimated richness (%)	Stimators	Percentage (%)
50	69	73	CHAO 2	63
			JACKNIFE1	67
			JACKNIFE2	67

Table A3. Post-hoc permutational ANOVA, and PERMANOVA. MC = Monte Carlo

$Y=\mu+En+Sit(En)+e$					
Abundance			Bacterial composition		
Groups	t	P(MC)	Groups	t	P(MC)
URBAN, AGRICULTURAL	2.1444	0.09	URBAN, AGRICULTURAL	2.1444	0.09
URBAN, NATURAL	4.3001	**0.0118**	URBAN, NATURAL	4.3001	**0.0118**
AGRICULTURAL, NATURAL	2.5472	0.0614	AGRICULTURAL, NATURAL	2.5472	0.0614
$Y=\mu+En+Ag+ En*Ag+e$					
Richness (Interaction)					
Within level 'ADULT' of factor 'AGE'			Within level 'JUVENILE' of factor 'AGE'		
Groups	t	P(MC)	Groups	t	P(MC)
URBAN, AGRICULTURAL	2.297	**0.0402**	URBAN, AGRICULTURAL	0.53087	0.5988
URBAN, NATURAL	1.0106	0.3368	URBAN, NATURAL	0.21771	0.8284
AGRICULTURAL, NATURAL	2.3333	**0.0375**	AGRICULTURAL, NATURAL	0.4340	0.6629
Within level 'URBAN' of fator 'ENVIRONMENT'			Within level 'AGRICULTURAL' of factor 'ENVIRONMENT'		
Groups	t	P(MC)	Groups	t	P (MC)
ADULT, JUVENILE	2.4283	**0.0258**	ADULT, JUVENILE	1.0097	0.3274
Within level 'NATURAL' of factor 'ENVIRONMENT'					
Groups	t	P(MC)			
ADULT, JUVENILE	2.4031	**0.0274**			
Abundance			Bacterial composition		
Groups	t	P	Groups	t	P
URBAN, AGRICULTURAL	2.2132	**0.0131**	URBAN, AGRICULTURAL	1.5512	**0.0088**
URBAN, NATURAL	3.7365	**0.001**	URBAN, NATURAL	2.6076	**0.0001**
AGRICULTURAL, NATURAL	0.5928	0.5928	AGRICULTURAL, NATURAL	2.1238	**0.0001**



[h] = Residual $R_a^2 = 0.858$

Predictives variables	R^2_{adj}	*P*-value
Abiotic Variables (AV)	0.042	**0.0001**
Bird Measurement (BM)	0.010	**0.0388**
Biotic Variables (BV)	0.127	**0.0001**
AV+BM	0.053	**0.0001**
AV+BV	0.138	**0.0001**
BM+BV	0.134	**0.0001**
AV+BM+BV	0.142	**0.0001**

Figure A4: Venn diagrams illustrating the results of variation partitioning of the data (bacterial composition) among three subsets of explanatory variables: abiotic variables (AV), bird measurement (BM), and biotic variables (BV). Letters [a-h] identify the fractions of variation.

Table A4. Results of the similarity percentage analysis (SIMPER) between environments. "Contrib%" = percent contribution and "Cum%" = cumulative percent contribution.

Group URBAN		
Average similarity: 13.98		
Species	Contrib%	Cum.%
Bacillus subtilis	28.34	28.34
Achromobacter sp.	21.68	50.02
Bacillus pumilus	20.74	70.76
Sphingomonas taxi	7.09	77.84
Bacillus sp. 10	4.15	81.99
Paracoccus fontiphilus	3.92	85.92
Bacillus sp. 2	3.69	89.61
Paenibacillus sp.1	2.84	92.44
Group AGRICULTURAL		
Average similarity: 16.11		
Species	Contrib%	Cum.%
Bacillus subtilis	39.54	39.54
Bacillus pumilus	27.05	66.59
Paenibacillus glucanolyticus	16.42	83.01
Bacillus sp. 11	6.68	89.69
Bacillus sp. 3	4.88	94.57
Group NATURAL		
Average similarity: 23.13		
Species	Contrib%	Cum.%
Bacillus sp.11	51.74	51.74
Bacillus cereus	21.54	73.28
Bacillus altitudinis	6.18	79.45
Bacillus pumilus	5.01	84.46
Bacillus sp. 14	4.96	89.42
Bacillus sp. 3	3.6	93.03

DISCUSIÓN Y CONCLUSIÓN GENERAL

El trabajo realizado en este estudio fue la revisión global sobre las interacciones bacterianas asociadas al plumaje en las aves silvestres y analizar la composición bacteriana del plumaje del colibrí corona violeta (*L. violiceps*) en tres ambientes diferentes (urbano, agrícola y natural). En el trabajo de revisión se describieron y analizaron 139 investigaciones que existen acerca de la composición bacteriana de las plumas a partir de 1999 al 2020. Se clasificaron en dos líneas de estudio: a) La composición bacteriana asociada al plumaje en las aves silvestres y sus interacciones con el ambiente; b) Las estructuras de adaptación en aves silvestre contra la degradación del plumaje inducida por bacterias. En los próximos párrafos se presentan la discusión del primer artículo.

La composición bacteriana del plumaje está determinada por múltiples factores como el hábitat del hospedero, gremio alimenticio, migración y temporada de reproducción (Burtt y Iquida 1999; Bisson et al. 2007; Bisson 2009; Saag et al. 2011; Kent y Burtt 2016; Musitelli et al. 2018). Las aves silvestres pueden transmitir una gran diversidad de grupos bacterianos a la plantas por medio de sus movimientos migratorios y locales. Debido a que, se han registraron especies de género bacterianos como *Frigoribacterium*, *Kitasatospora*, *Agrobacterium*, y *Sphingomonas* las cuales incluyen especies tanto benéficas como patógenas para las plantas tanto de interés comercial como especies silvestres (Dille et al. 2016; Giorgio et al. 2018).

Las bacterias capaces de cohabitar en el plumaje por medio del uso de la queratina como fuente de carbono y energía (Lange et al. 2016), Además, producen compuestos antimicrobianos el cuál las convierte en simbiontes benéficos para el hospedero. Así mismo, las bacterias del plumaje tienen una asociación filosimbiótica con las aves silvestres como se ha comprobado con los microrganismos que habitan en la piel de los mamíferos (Javůrková et al. 2019a).

Las aves silvestres han desarrollado estructuras adaptativas que les sirven para el mantenimiento de su plumaje el cual les genera ventaja al momento del cortejo sexual, el vuelo y la termorregulación. Así también, se ha comprobado que funcionan como un mecanismo de protección contra ataques microbianos. Javůrková et al. (2019b) registraron que las aves con plumaje estructural mantenían menor carga bacteriana en contraste con aves de plumaje melanizado. Por otra parte, será necesario fortalecer este resultado, analizando, la microbiota del plumaje en diversas especies de aves con plumaje estructural. Otra de las características adaptativas en las aves, es el aceite producido en la glándula uropygial, además de que es una barrera protectora, contiene enzimas antimicrobianas que ayuda a mantener el equilibrio de las comunidades bacterianas del plumaje por medio del acicalamiento (Jacob et al. 2018; Carneiro et al. 2020).

En el trabajo acerca de la determinación bacteriana asociada al plumaje de *L. violiceps* en los tres ambientes se registraron los siguientes acontecimientos. Se comprobó que el ambiente urbano, agrícola y natural registraron difenrencias significativas en la composición bacteriana asociada al plumaje del colibrí aunado a las características específicas del lugar. Además, las variables bióticas como la disponibilidad de recurso juegan un importante papel en la composición bacteriana aislada del plumaje de los colibríes debido a su interacción con la vegetación del ambiente. De manera que, los colibríes son importantes polinizadores y están contacto directo con las plantas durante la mayor parte del día para satisfacer la demanda energética, se ha comprobado que el néctar floral además de ser una fuente nutritiva de alimento para los colibríes (Roguz et al. 2019), mantienen comunidades bacterianas simbióticas de la planta para atraer a los polinizadores (González-Teuber y Heil 2009; Lee et al. 2019; y Lamb et al. 2020).

La composición bacteriana y la carga bacteriana fueron más altas en el ambiente natural, el cual coincidió con el aumento de la temperatura y la humedad. Este resultado corroboró por lo reportado por Verea et al. (2014) donde en temporada de lluvias y el aumento de la temperatura en verano, las aves silvestres mantenían mayor carga bacteriana. Sin embargo, este resultado puede variar dependiendo de las características específicas del hospedero. En

los colibríes *L. violiceps* se ha observado que en un ambiente natural se desplazan a grandes distancias en búsqueda de alimento, lo que probablemente favoreció las condiciones ambientales en el plumaje para las bacterias (Angel et al. 2009; Tian et al. 2018). Además, mantienen mayor contacto con la vegetación y otros animales. Cabe resaltar, que los individuos adultos de *L. violiceps* registraron la mayor carga bacteriana, esto puede estar asociado al aumento de las actividades de supervivencia como la búsqueda de alimento a zonas más distantes, el cortejo sexual, construcción de nidos y cuidados parentales en contraste con los colibríes juveniles. Por último, se registró que los ambientes cercanos a sitios de ganado incrementan la exposición a la transmisión de bacterias patógenas en los colibríes (Zuo et al. 2019; Hassell et al. 2019).

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