The mammalian skin microbiome

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any final required revisions, as accepted by my examiners.

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Abstract

Skin constitutes the primary physical barrier between mammals and their external environment. Characterization of the microorganisms on skin is essential for understanding how a host evolves in association with its microbial symbionts, modeling immune system development, diagnosing illnesses, and exploring the origins of potential zoonoses that affect humans.

Distinct microbial communities inhabit individuals as part of the human skin microbiome, which are continually shed to the surrounding environment. Microbial communities from 17 skin sites of 10 sexually active cohabiting couples (20 individuals) were sampled to test whether cohabitation impacts an individual's skin microbiome, leading to shared skin microbiota among partner pairs. Amplified 16S rRNA genes of bacteria and archaea from a total of 340 skin swabs were analyzed by high-throughput sequencing and the results demonstrated that cohabitation was significantly associated with microbial community composition, although this association was greatly exceeded by characteristics of body location and individuality. Random forest modeling demonstrated that partners could be predicted 86% of the time ($p < 0.001$) based on their skin microbiome profiles, which was always greater than combinations of incorrectly matched partners. Cohabiting couples had the most similar overall microbial skin communities on their feet, according to Bray-Curtis distances. In contrast, thigh microbial communities were strongly associated with biological sex rather than cohabiting partner. Additional factors that were associated with the skin microbiome of specific body locations included the use of skin care products, pet ownership, allergies, and alcohol consumption. These baseline data identified links between the skin microbiome and daily interactions among cohabiting individuals, adding

to known factors that shape the human microbiome and, by extension, its relation to human health.

Although many studies have characterized the human microbiome, far less is known about the skin microbiome of non-human mammals. The objective of this research was to create a baseline skin microbiome dataset for the Mammalia class, testing the effects of species, location, hygiene, body region, and biological sex. The back, torso, and inner thigh regions of 177 non-human mammals and 20 human participants were collected to include representatives from 38 species and 10 mammalian orders. Animals were collected from local farms, zoos, households, and the wild. All samples were amplified using the V3-V4 16S rRNA gene region and sequenced using a MiSeq (Illumina). Human skin was significantly less diverse than all other mammalian orders according to Shannon indices (6.54 versus 3.96, $p < 0.001$). The factor most strongly associated with community variation for all samples was whether the host was a human (PERMANOVA, $F = 37.8$, $p < 0.001$). By analyzing all samples together, random forest modelling identified that human and animal samples could be distinguished correctly 98.5±1.2% of the time. This study represents the largest non-human mammalian skin microbiome project to date and is the first study to elucidate the skin microbiota for 32 unique species. Additionally, these findings are the first to demonstrate that human skin is distinct, not only from other Primates, but from all 10 mammalian orders sampled. Baseline data on the mammalian skin microbiome is crucial for making informed decisions for veterinarian research and conservation strategies, as well as providing implications for mammalian evolutionary history.

This thesis is dedicated to my late mother Heather, who taught me to always work hard, and to complete everything with integrity and enthusiasm. Your lessons continue to resonate with me daily.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

%	percent	HTP	high-throughput
°C	degrees celsius	IBD	inclusion body disease
μg	microgram	KWHS	Kitchener-Waterloo
μL	microlitre		Humane Society
μm	micrometer	m	meter
μM	micromolar	mg	milligram
3D	three dimensional	min	minutes
ALS	African Lion Safari	mL	milliliter
ape	analyses of phylogenetics	mm	millimeter
	and evolution	mM	millimolar
ARCH	archaea specific primers	MRPP	multiple-response
AXIOME	automation, extension,		permutation procedure
	and integration of	n	sample size
	microbial ecology	N	normal
bp	basepair	NA	not applicable
BSA	bovine serum albumin	NaOH	sodium hydroxide
CCA	constrained (canonical)	NCBI	National Center for
	correspondence analysis		Biotechnology
cm	centimeter		Information
COX1	cytochrome oxidase	ng	nanogram
	subunit I gene	nM	nanomolar
CRHBA	Conostogo River	nRF	normalized Robinson-
	Horseback Adventures		Foulds score
C-section	caesarean section	NTC	no template control
DGGE	denaturing gradient gel	ORE	Office of Research Ethics
	electrophoresis	OTU	operational taxonomic
DNA	deoxyribonucleic acid		unit
dNTP	deoxynucleotide	p value	probability value
	triphosphate	PANDAseq	paired-end assembler for
dsDNA	double stranded		DNA sequences
	deoxyribonucleic acid	PCoA	principle coordinates
EDTA	ethylenediaminetetraacetic		analysis
	acid	PCR	polymerase chain reaction
et al	and others	PERMANOVA	permutational multivariate
-F	forward primer		analysis of variance
FAPROTAX	Functional Annotation of	pH	power of hydrogen
	Prokaryotic Taxa	PhiX	bacteriophage genome
g	gram	pM	picomolar
ggplot	grammar of graphics plot	Pro341Fi	universal prokaryotic
$gyrB$	DNA gyrase subunit B		forward Illumina primer-
h	hour		position 341
H_2O	water	Pro805Ri	universal prokaryotic
			reverse Illumina primer-
			position 805

ppb	parts per billion
PyNAST	python nearest alignment space termination tool
QIIME	quantitative insights into microbial ecology
qPCR	quantitative polymerase chain reaction
-R	reverse primer
RDP	ribosomal data project
RISA	ribosomal internal spacer analysis
rRNA	ribosomal ribonucleic acid
s	second
SDS	sodium dodecyl sulfate
sp	singular species
spp	multiple species
SRA	sequence read archive
SYBR	Synergy Brands, Inc.
TAE	tris-acetate EDTA
Taq	*Thermus aquaticus*
U	unit
UV	ultraviolet light
V3	16S rRNA gene variable region 3
V4	16S rRNA gene variable region 4
vs	versus
w/v	weight/volume
x	times

Chapter 1
The vertebrate skin microbiome

1.1 Introduction

The microbiome is the collection of genetic material belonging to the microorganisms inhabiting a specific environment, whereas the microbiota defines the community composition of the microorganisms that are present. To date, the microbiota of various body regions of animals have been explored including the gut, oral, respiratory tract, vagina, and skin. In comparison to the skin microbiome, the connections between the gut microbiome and host health are much better established. The microbial consortia of the host gut can influence digestion (1), provide vitamins and amino acids that the host are unable to synthesize alone, and impact neurological function through the gut-brain axis. Links have been elucidated between dysbiosis of the gut microbiome and conditions, such as allergies (2), obesity (3), *Clostridium difficile* infection (4), autism (5), and irritable bowel syndrome (6). The associations between gut-host interactions are longstanding. Indeed, because of vertical transmission, largely by maternal inheritance, microbial communities associated with mammalian hosts reflect the phylogeny of mammalian species (7). Despite immense progress in understanding the human microbiome, the skin microbiome of humans and other animal species have received relatively little research attention.

Skin microbiome research seeks to better understand the largest organ of the body by providing information on the processes by which a host organism evolves in association with its microbial symbionts (8), characterizing the immune system and diagnose illnesses (9, 10), and exploring the origin and etiology of disease (11–13). The advent of high-throughput sequencing has greatly expanded knowledge of the skin microbiome and its implications for health. For example, it is now recognized that humans are uniquely colonized by skin microbial

communities that are linked to diet (14), age (15, 16), and the specific body region sampled (17, 18). These baseline data are important for understanding how skin microbiota contribute to skin health and disease.

The majority of skin microbiome studies have focused on humans and amphibians. Fish and birds have received substantially less attention, and many existing studies are cultivation-based. Few studies have explored the skin microbiome of reptiles (Table 1) (8). Although most skin microbiome research has focused on the human microbiome, the aim of this chapter is to summarize understanding of skin microorganisms that associate among members of classes within the subphylum *Vertebrata*. This subphylum has been chosen instead of solely focussing on Class *Mammalia* because there have been few high-throughput sequencing studies to date that have collected non-human mammalian skin samples. Analyzing the literature from multiple animal classes allows for a more detailed dissemination of current skin microbiome knowledge. Specifically, links will be explored between the skin microbiome and vertebrate characteristics, including geographic location, biological sex, animal interactions, diet, captivity, maternal transfer, and disease states.

Table 1: Molecular studies investigating the non-human vertebrate skin microbiome. Only studies that used culture-independent methods were included. Studies within a vertebrate clade are listed in alphabetical order according to first author.

Study	Species	Common names and sample size	Body region	PCR primers and sequencing platform	# sequences obtained (raw unless stated)	Dominant taxa	Geographic location	Biological sex
Mammals								
(Apprill *et al.*, 2011)	*Megaptera novaeangliae*	19 humpback whales (including 3 health compromised)	Biopsy of dorsal region or sloughed skin from tags/ water skimmings	27F-B/1492R 16S rRNA gene ABI 3730XL	Not reported	*Bacteroidetes Tenacibaculum*	North Pacific near Hawaiian islands	12 males, 4 females, 4 unknown
(Apprill *et al.*, 2014)	*Megaptera novaeangliae*	56 humpback whales	Biopsy of upper flank near dorsal fin	27FB/ 519R 16S rRNA gene 454 pyrosequencing	754,593	*Flavobacteria Tenacibaculum Gammaproteobacteria Psychrobacter*	North Atlantic, North Pacific and South Pacific oceans – free-swimming	Not stated- no difference between sex observed
(Avena *et al.*, 2016)	*Myotis lucifugus, Perimyotis subflavus, Myotis septentrionalis, Myotis sodalis, Myotis volans, Myotis ciliolabrum, Myotis yumanensis, Eptesicus fuscus, Myotis evotis, Lasiurus cinereus, Corynorhinus townsendii*	70 little brown bats, 5 tricolored bats, 2 northern long-eared bats, 12 Indiana bats, 24 long-legged myotis, 3 western small-footed bats, 23 Yuma myotis, 19 big brown bats, 2 western long-eared bats, 2 hoary bats, 2 Townsend's big-eared bats	Forearm and muzzle (same swab)	515F/806R 16S rRNA gene Illumina HiSeq (eastern US) and MiSeq (Colorado)	Rarefied to 9,800 sequences per sample	*Proteobacteria Bacteroidetes Thermoleophilia*	Virginia, New York, and Colorado, USA	Recorded
(Cheng *et al.*, 2015)	*Sarcophilus harrisii*	23 Tasmanian devils	Chest-abdomen and pouch	27F-519R 16S rRNA gene 454 pyrosequencing	1,223,550	*Firmicutes: Clostridia Bacilli* *Gamma-Proteobacteria*	Four locations in Tasmania	Number male/female not stated
(Council *et al.*, 2015)	*Pan troglodytes, Gorilla gorilla, Papio, Macaca mulatta*	7 chimpanzees, 5 gorillas, 11 baboons, 2 rhesus macaques	Axillae	515F/806R 16S rRNA gene 454 pyrosequencing	798,818	*Firmicutes Actinobacteria Proteobacteria Bacteroidetes*	North Carolina zoo, USA. *Rm* = semi-free ranging in Puerto Rico	Unknown
(Hoffman *et al.*, 2014)	*Canis lupus familiaris*	12 healthy and 6 allergic dogs	12 skin sites (healthy) 4 skin sites (allergic)	28F/519R 16S rRNA gene FLX-titanium amplicon pyrosequencing (bTEFAP)	779,812	*Proteobacteria Oxalobacteriaceae*	Companion animals – Texas, USA	6 males, 6 females healthy; 4 males, 2 females allergic
(Klitgaard *et al.*, 2008)	*Bos taurus*	32 beef and dairy cattle	Lower limbs from slaughterhouse	10FX/1509R 16S rRNA gene Trep-46F/1509R ABI 3130 genetic analyzer	Not reported	*Treponema spp. Fusobacterium necrophorum Steptococcus dysgalactiae Pasteurella spp. Klebsiella oxytoca*	Denmark	Unknown

Reference	Species	Sample size	Sampling sites	Method/Primers	Sequences	Dominant taxa	Location	Sex
(Meason-Smith *et al.*, 2017)	*Felis catus*	11 healthy and 9 allergic cats	12 skin sites (healthy) 6 skin sites (allergic)	ITS1F/ITS2R ITS1 region Illumina MiSeq	9,770,840	*Cladosporium Alternaria*	Companion animals – Texas, USA	5 males, 6 females healthy; 4 males, 5 females allergic
(Zinicola *et al.*, 2015)	*Bos taurus*	89 Holstein dairy cows	Punch biopsies of healthy and lesioned hooves	515F/806R 16S rRNA gene Illumina MiSeq	21,345,584 quality filtered sequences	*Firmicutes Spirochaetes Bacteroidetes Actinobacteria Proteobacteria Tenericutes*	Three dairy farms in New York, USA	89 females
Birds								
(Denesvre *et al.*, 2015)	*Gallus gallus domesticus*	15 chickens	Side of body protected by feathers	bacteriophage phi29 polymerase using random primers Illumina HiSeq 2000	2.4×10^8	herpesviruses from the *Mardivirus* genus	France – captive	Unknown
(Kulkarni and Heeb, 2007)	*Taeniopygia guttata*	48 zebra finches	Wing feathers, cloaca, beak	Amplicon sequencing of kerA gene	NA	Only *Bacillus licheniformis* was amplified	Switzerland - caged	24 males, 24 females
(Lucas *et al.*, 2005)	*Sturnus vulgaris*	42 European starlings	Chest feathers were removed	16S-23S intergenic spacer region S-D-Bact-1522-b-S-20/L-D-Bact-132- a-A-18	NA	RISA provides information on bacterial densities	University of Lausanne, Switzerland	15 males, 27 females
(Roggenbuck *et al.*, 2014)	*Coragyps atratus, Cathartes aura*	26 black vultures, 24 turkey vultures	Facial skin	341F/806R 16S rRNA gene 454-Roche-FLX Titanium	650,697 trimmed	*Clostridia* and *Fusobacteria*	Nashville, USA	Not stated- no difference between sex
Reptiles								
(Stöhr *et al.*, 2013)	*Anolis sagrei, Dopasia gracilis, Anolis carolinensis, Iguana iguana, Pogona vitticeps*	3 brown anoles, 1 Asian glass lizard, 1 green anole, 1 green iguana, 1 central bearded dragon	Skin biopsies from affected regions, mixed organs, small intestine	Ol T1/ Ol T2R of the major capsid protein (MCP) gene AdVs and IIV were also tested	Presence/absence of viruses reported	*Ranavirus Reovirus Iridovirus Adenovirus*	Germany	Various ratios in case studies
Amphibians								
(Bataille *et al.*, 2016)	*Bombina orientalis*	11 wild and 18 captive fire-bellied toads	Ventral area, thigh, toe webbing (1st swab). Back (2nd swab)	ITS1-3 Chytr 5.8S Chytr GAIIx Illumina sequencer	1,026,343	*Proteobacteria Actinobacteria Bacteroidetes*	Gangwon Province, South Korea	Measured
(Becker *et al.*, 2014)	*Atelopus zeteki*	27 wild, 10 captive Panamanian golden frogs	Ventral and dorsal surfaces, thighs, and feet	515F/806R 16S rRNA gene Illumina MiSeq	Rarefied to 19,500	*Proteobacteria Actinobacteria Bacteroidetes*	Wild frogs obtained from Río Mata Ahogado, Panamá; captive from Washingtin,DC,USA	Unknown
(Belden *et al.*, 2015)	*Agalychnis callidryas, Dendropsophus ebraccatus, Craugastor fitzingeri, Anaxyrus americanus, Lithobates catesbeianus, Pseudacris crucifer*	62 red-eyed treefrogs, 53 pantless treefrogs, 21 Fitzinger's robber frogs, 9 American toads, 35 American bullfrogs, 34 spring peepers	Ventral surface, thigh, hindfoot (one swab)	515F/806R 16S rRNA gene Illumina	Rarefied to 7000 sequences per sample	*Proteobacteria Actinobacteria Bacteroidetes Firmicutes*	Four sites in Panama and seven site in USA	Unknown
(Costa *et al.*, 2016)	*Pelophylax perezi*	31 Perez's frogs	Ventral and dorsal region, head, lateral region, surface of thigh, foot (1 swab)	27F/1525R 16S rRNA gene DGGE	NA	*Actinobacteria Alphaproteobacteria*	Five sites in Portugal	Unknown
(Federici *et al*, 2015)	*Rana italica*	6 Italian steam frogs: 3 infected, 3 uninfected	Lateral, ventral, and dorsal surfaces of the body, thighs, and feet	783F/1027R 16S rRNA gene Illumina HiSeq 1000	612,786-1,016,572 reads per sample	*Comamonadaceae Moraxellaceae Pseudomonadaceae*	Nestore Valley, Italy	5 males, 1 female

4

(Fitzpatrick and Allson, 2014)	*Plethodon jordani*	29 red-cheeked salamanders	Trunk (2 swabs each)	515F/806R 16S rRNA gene Hi-Seq 2000	1 965 175 after processing	*Gammaproteobacteria Acidobacteria*	North Carolina, USA	Unknown
(Hernández-Gómez *et al*., 2016)	*Cryptobranchus alleganiensis bishopi, Cryptobranchus alleganiensis alleganiensis*	Two subspecies of hellbender giant salamander – 6 Ozark, 5 eastern	Dorsal region, plantar surface of foot, wounds	27F/338R 16S rRNA gene Illumina HiSeq 2500	15,590,201 filtered sequences	*Proteobacteria, Bacteroidetes, Cyanobacteria, Fusobacteria*	Missouri, USA	Unknown
(Jani and Briggs, 2014)	*Rana sierrae*	8-10 per population of Sierra Nevada yellow-legged frogs	Skin (region unspecified)	8F/338R 16S rRNA gene Roche/454 GS FLX	393,119 (experimental) and 159,126 (field) quality filtered sequences	*Actinobacteria Betaproteobacteria Gammaproteobacteria*	Four sites in California, USA	Unknown
(Kueneman *et al*., 2014)	*Anaxyrus boreas, Pseudacris regilla, Taricha torosa, Lithobates catesbeianus, Rana cascadae*	47 western toads, 30 Pacific tree frogs, 16 California newts, 11 American bullfrogs, 91 cascades frogs	Adults = ventral surface and limbs. Larvae = entire body	515F/806R 16S rRNA gene Illumina HiSeq	33.4 million	*Bacteroidetes Gammaproteobacteria Alphaproteobacteria Firmicutes Sphingobacteria Actinobacteria*	California's Central Valley and the Trinity Alps in Northern California, USA	Unknown
(Lauer *et al*., 2008)	*Hemidactylium scutatum*	87 four-toed salamanders	Ventral and lateral sides	357F/907R 16S rRNA gene	Not reported	*Bacteroidetes Firmicutes Actinobacteria Proteobacteria*	Three ponds in Virginia, USA	87 females
(Longo *et al*., 2015)	*Lithobates yavapaiensis, Eleutherodactylus coqui*	37 lowland leopard frog, 52 common coqui	Standardized number of swab strokes per frog and body region	16S-23S intergenic spacer region 1406F/23S-125R 515F/806R 16S rRNA gene Illumina MiSeq	1 179 178	*Proteobacteria Actinobacteria Bacteroidetes*	Arizona, USA and Puerto Rico	Unknown
(Loudon *et al*., 2014)	*Plethodon cinereus*	65 red-backed salamanders	Ventral surface	515F/806R 16S rRNA gene Illumina HiSeq 2000	23.3 million	5/8 core OTUs were *Pseudomonadaceae*	Virginia, USA	Unknown
(McKenzie *et al*., 2012)	*Lithobates pipiens, Pseudacris triseriata, Ambystoma tigrinum*	7 northern leopard frogs, 14 western chorus frogs, 12 tiger salamanders (all larval stage)	Entire body	27F/388R 16S rRNA gene 454 pyrosequencing	Average of 1220 sequences per sample (range 780–1510)	*Acidobacteria Actinobacteria Bacteroidetes Cyanobacteria Firmicutes Proteobacteria*	Four pond habitats in Colorado, USA	Unknown
Fish								
(Larsen *et al*., 2013)	*Mugil cephalus, Lutjanus campechanus, Cynoscion nebulosus, Cynoscion arenarius, Micropogonias undulatus, Lagodon rhomboides*	15 striped mullet, 8 red snapper, 11 spotted seatrout, 24 sand seatrout, 27 Atlantic croaker, 17 pinfish	Dorsal fin tissue	RISA Bact-8F/UNI534R 16S rRNA gene ABI 3730xl sequencer	Rarefied to 69 sequences per sample	*Proteobacteria Firmicutes Actinobacteria Aeribacillus* observed in all species	Coastal waters of Alabama and Mississippi, USA Offshore of Louisiana, USA	Unknown
(Schmidt *et al*., 2015)	*Poecilia sphenops*	30 Black Molly fish	Entire fish, including gut	967F/1064R 16S rRNA gene Illumina HiSeq	9.2×10^5 avg. reads per sample post processing	*Gammaproteobacteria Fusobacteria*	Purchased from supplier in USA	Unknown

1.2 Vertebrate skin composition

Skin represents the interface between vertebrates and their corresponding terrestrial, aquatic, and atmospheric environments. Each vertebrate class has distinct physiological skin characteristics that have the potential to influence their skin microbiomes. The discussion below will focus on differences between the skin structure for each class, such as gland distribution, the types of cell layers present, and microbial density estimates.

The vast majority of skin microbiome research to date has focused on human subjects because of their clinical and economic importance to humans. Humans possess 1.5-2.0 m^2 of skin with a depth of 2-3 mm (17). Within this tri-layered structure is a system of glands and hair follicles that interact with the nervous, circulatory, and immune systems (19). The outermost layer is the epidermis, which is frequently studied in part due to its non-invasive sampling protocols and direct contact with the surrounding environment. It is here that commensal organisms protect the body from transient microorganisms (20) with the potential to cause disease by either producing inhibitory compounds (21) or outcompeting for resources (22). The epidermis is a constantly shedding and hostile environment compared to the gut or mouth because of its lower temperature, pH, and moisture levels, coupled with high concentrations of salt and antimicrobials (23). Molecular cartography maps of the human skin surface have determined that it has a diverse composition (17). The microbiota are not only present on the outermost layer, but extend below the epidermis into the subcutaneous region (24). Estimates suggest that a range of 10^6 to 10^9 microorganisms/cm^2 are present on skin (25, 26). This difference of several orders of magnitude can be attributed to sampling different body locations instead of the skin layer depth of the sample. Swabs remove only the outermost layer of

6

sloughing skin, whereas a scrape with a sterile surgical scalpel removes the entire epidermal layer. A biopsy is a more invasive technique that cuts into the lower dermis and potentially the hypodermis layers. Although the more invasive techniques collect a higher number of bacteria/cm^2 than the skin swabs, there are no consistent depth-dependent differences in the detected microbial communities (26). Indeed, these techniques have been shown to share 97.2% of sequence reads when the same location and individual is sampled with all three methods (26).

The Class *Mammalia* includes the closest evolutionary relatives of humans. The placental mammals originate from the Late Jurassic period, circa 160 million years ago (27). Non-human mammals typically possess denser fur over a larger proportion of their bodies. There are two types of sweat glands present on mammals that may impact microbial diversity differently: apocrine and eccrine. The large and spongy apocrine glands are associated with fur and hair (28). They do not play a significant role in thermal cooling for humans and other primates because they are situated only in the axilla (armpit), pubic, and anal regions. Apocrine glands produce oily viscous exudates and are the only effective glands in hoofed mammals. In contrast, the eccrine glands, which are small and associated with pores, produce a dilute secretion compared to apocrine glands. Primates possess varying proportions of these glands. Humans possess mostly eccrine glands, whereas gorillas and chimpanzees have approximately 66% eccrine and 33% apocrine (28). The proportion of apocrine glands increases to 50% in gibbons, orangutans, and old world monkeys. New world monkeys and old world simians are almost entirely composed of apocrine glands.

The skin of avian reptiles (Class *Aves*, herein referred to as "birds" for clarity) has distinct physiological features from mammals. Although the most striking difference between birds and mammals is the presence of plumage, birds also have a thinner epidermis, no

sebaceous glands, and a higher proportion of lipids in the transitional layer of the epidermis (29). Birds are descended from reptiles. Their feathers are considered modified scales and a component of the integument, which is the outer protective organ system that includes the layers of skin, glands, hair, and nails in vertebrates (30). Moreover, birds possess avian scales on their feet and only a single gland type (31). The uropygial glands exude an oily secretion that is used to coat feathers.

Non-avian reptiles (Class *Reptilia*, herein referred to as "reptiles" for clarity) include crocodiles, turtles, snakes, and lizards. This class of amniotes ("membrane surrounding the fetus") represent the first animals to transition to land, which resulted in accompanying shifts to their integument. Reptiles were also the first organisms to evolve a stratum corneum (i.e., horny outer skin layer) with multiple layers and a programmed cell death, coupled with additional lipids to prevent water loss on land (32). A terrestrial lifestyle also led to the loss of gas exchange and mucous, which occurred approximately 340 million years ago (32). The pleated-sheet beta-keratin polypeptides involved in creating sauropsid feathers, scales, and claws are distinct from the helical alpha-keratin polypeptides that form hair (31).

Amphibians are anamniote tetrapods. They possess a thin and persistently moist layer of skin that is water permeable and able to undergo gas exchange (33). Unlike the other vertebrate classes, their skin contributes to respiration and osmoregulation. These tetrapods were the first vertebrates to evolve corneous cells (32), which form a protective external envelope around the organism and aid in terrestrial survival. An absence of protective integument layers, namely feathers or fur makes them particularly susceptible to skin ailments (13). Additionally, their skin is covered in a sugar rich mucosal layer that can serve as a growth substrate for pathogenic bacteria and fungi. Consequently, many amphibian microbiome studies have focused on

elucidating the differences between infected and uninfected animals in an attempt to create conservation strategies to save species from becoming extinct. As a result, the amphibian skin microbiome is better characterized than several of the other vertebrate classes.

Fish constitute a paraphyletic group of taxa that are defined as aquatic craniates with gills that lack limbs with digits (34). This diverse group includes jawless, cartilaginous, armoured, and bony fish, all of which do not possess a corneous cell envelope. Like amphibians, fish possess a layer of mucous that surrounds the epidermis (35) and represents an additional critical barrier between the animal and its aquatic environment. Their scales are formed in the mesoderm layer and do not contain keratin, in contrast to keratinized reptilian scales that are formed in the epidermis (36). The mucous is a complex viscous mixture of immunogenic compounds, such as mucins, immunoglobins, lysozyme, antimicrobial peptides, and defensins (9, 37). Despite these bactericidal compounds, the mucous also possesses numerous sugars and amino acids suitable for bacterial growth; the commensal biota must be sampled immediately after death to avoid falsely characterizing the rapid increase in bacterial growth that occurs post mortem (38). The mucosal layer is responsible for contributions to both innate and adaptive immunity in fish (9). Cultivation studies have enumerated a range of 10^2-10^7 culturable microorganisms/cm^2 skin (39). This wide range has been attributed to variations between capture technique. Trawling leads to larger microbial loads than capture with a baited line, due to sediment contamination from contact with the seabed and release of fish gastrointestinal contents (40). Additionally, salmon (*Salmo salar* L.) have higher microbial loads in their spawning grounds than their marine habitat due to varying numbers of bacteria in the water (41). The true microbial density is likely several orders of magnitude higher because culturing techniques only enumerate a fraction of the total microbial community.

1.3 Microbial taxonomic diversity

High-throughput sequencing efforts focused on characterizing human skin reveal that it is inhabited by mostly neutral or beneficial microorganisms (18, 25, 42). The majority of skin studies have focused on bacteria by targeting the bacterial 16S rRNA gene; however, archaea (43), fungi (44), protozoans, and microscopic arthropods are also present. The three phyla *Actinobacteria, Firmicutes,* and *Proteobacteria* dominate; *Bacteroidetes* and *Cyanobacteria* are the other bacterial phyla that are frequently present at approximately 1% abundance (20, 45). All other bacterial phyla are typically below 0.5% abundance. Although low diversity is observed at high bacterial taxonomic ranks, there is high diversity at the species level (11). Core human OTUs include *Staphylococcus epidermidis*, *Corynebacterium*, and *Propionibacterium* (46). The proportions of these organisms vary between different body locations, moisture levels, and individuals (42). For example, *Propionibacterium* is more abundant in oily regions, such as the face (20). The microbial community structure is influenced by additional factors, for example biological sex (45), hygiene products (17), and ethnicity (47).

Despite the importance of the mammalian microbiome, only a few studies have been conducted on mammalian skin (Table 1). Initial studies of dogs and cats observed minimal skin bacterial diversity (48), yet this may be attributed to limitations of culturing. A culture-based study of squirrels, raccoons, cattle, pigs, sheep, and dogs cultured 98 strains of *Micrococcus* and 221 strains of *Staphylococcus* (49). Another cultivation-based survey of *Staphylococcus* observed members of this genus on 100% of pigs and cows, 90% of humans and horses, 77% of laboratory mice, and 40% of dogs (50). Overall, the most dominant phylum and family on dogs were *Proteobacteria* and *Oxalobacteriaceae*, respectively (51). More variation was observed among body locations at the class taxonomic level. The most abundant genus overall was

10

Ralstonia, whose relative abundance was low in the lip commissure (5%) but high in the conjunctiva (35%). Skin biopsies and sloughed skin from 56 humpback whales (*Megaptera novaeangliae*) from the North Pacific, South Pacific, and North Atlantic oceans demonstrated that highly abundant core genera *Psychrobacter* and *Tenacibaculum* were present on free-swimming whales (52). Recently, the axillae of 63 primates across five species have been sampled and 16S rRNA genes sequenced (53). Human axillae were associated with distinct microbial communities, with lower overall diversity.

Many amphibian species have had their skin microbiota sampled in an attempt to understand factors influencing declining amphibian populations due to skin fungal infections (Table 1) (54). Wild tiger salamanders (*Ambystoma tigrinum*), western chorus frogs (*Pseudacris triseriata*), and northern leopard frogs (*Lithobates pipiens)* harbour a similar level of diversity as human skin (55). Of the 18 bacterial phyla observed on amphibian skin, *Acidobacteria, Actinobacteria, Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, and *Proteobacteria* were the most abundant (55). Red-backed salamanders (*Plethodon cinereus*) had eight core OTUs, including *Pseudomonas* (56), present on >90% of specimens. Salamanders with less diverse communities had greater proportions of core OTUs. Italian steam frogs (*Rana italica*) were characterized by 16 distinct phyla (57). A fifth of all OTUs were present in all subjects. A culture-based study of Cascade frogs (*Rana cascadae*) enumerated 20 higher order taxa and 31 genera (58).

The fish skin microbiota of numerous species have been analyzed due to the economic importance of their health to the fishing and aquaculture industries. Early culturing work on North Sea cod showed that fish can undergo seasonal variations in skin bacterial numbers (59). The predominant cultured isolates were *Pseudomonas, Achromobacter, Corynebacterium, Flavobacterium*, and *Vibrio*. The phyla *Proteobacteria, Firmicutes,* and *Actinobacteria* were

dominant in multiple species of fish, which are the three dominant phyla in human studies, although in different abundances. The core OTU *Aeribacillus* was observed in all species, whereas other OTUs reflected species-specific distributions, such as *Microbacterium* in the northern red snapper and *Neorickettsia* in the flathead grey mullet (60).

Very few high-throughput studies have been conducted on birds and reptiles (Table 1). The skin microbiota of two species of new world vultures (*Coragyps atratus* and *Cathartes aura*) exceeds the diversity of their gut microbiota (61) (528 versus 72 OTUs, respectively), which has also been observed in a previous study of humans in body locations with higher diversity levels (18). The scavenger lifestyle of vultures, resulting in frequent contact with carcasses, may explain this increase in skin microbial diversity. *Clostridia* and *Fusobacteria* were genera associated with dominant vulture skin OTUs, which are not present in facial skin studies of humans (20), frogs, and salamanders (55). Sequencing additional non-scavenger avians will elucidate if a typical skin microbiome is more similar to humans and amphibians. A culture-independent skin microbiome study of non-avian reptiles focused on the lizard virome (62). Multiple viruses were associated with lethal skin lesions, including *Ranavirus*, *Adenovirus*, and *Reovirus*.

Few skin microbiome studies to date have focused on non-bacterial microorganisms. To date, the domain Archaea has rarely been studied on skin. One study focusing on archaea enumerated a relative abundance of up to 4.2% of the human skin microbiome, which consisted primarily of thaumarcheotes that may be involved in ammonia oxidation from human secretions (43). Recently, it has been postulated that there may be a correlation between the archaeal abundance and the age of the human individual, according to a study that sampled the torso of 51 human participants (63). *Methanibrevibacter* has been detected in human navels (64). This

methanogen is one of the dominant gut archaea and therefore may not be a permanent skin commensal. Halophilic archaea were associated with a single participant who had not bathed in several years (64). Archaea have recently been detected in the axillae of primates, albeit in low abundance (53). Only 15 of 5309 unique OTUs were affiliated with archaeal taxa.

The virome is the collection of nucleic acid sequences from the virus community in a habitat. A high-throughput study of 15 chickens (*Gallus gallus domesticus*) determined that chicken skin is predominately inhabited by *herpesvirus* from the *Mardivirus* group (65). The authors postulated that the viruses arose from vaccination or an asymptomatic infection. The skin virome of healthy chickens is significantly different from that of humans. Notably, chicken skin was absent of papillomaviruses and polyomaviruses that are typically detected on human skin (42, 66). The human skin virome has numerous double stranded DNA viruses, the largest proportion of which are bacteriophages belonging to the order *Caudovirales* that infect common skin bacteria, for instance *Propionibacterium* and *Corynebacterium* (67). A major challenge to sequencing the virome is the lack of a conserved sequence in viruses. It is postulated that an even more complex community of commensal viruses is likely present on skin.

Eukaryotic microscopic fungi are an additional group of organisms within the skin microbiome (i.e., the "mycobiome"). These organisms are less abundant than bacteria according to metagenomic analysis (42), and are estimated to represent <1% of the microbiota for the majority of the human body locations excluding facial regions, such as the forehead and ears. The human mycobiome is less diverse than the human bacteria microbiome, with the genus *Malasezzia* present at >90% relative abundance (68). The feet possess the highest levels of fungal diversity because they are in contact with built environment surfaces and soil. These samples contained elevated levels of *Aspergillus*, *Cryptococcus*, *Epicoccum*, and *Rhodotorula*

(68). In contrast, the cat mycobiome is significantly more diverse and is not dominated by

Malassezzia, but instead has high relevant abundances of *Cladosporium* and *Alternaria* (69). The

pre-aural space was the most diverse and exceeded fungi levels on feline feet. The authors

suggest that the majority of feline fungi were likely acquired from the environment and

represented transient species.

1.4 Predictors of Microbial Community Diversity

Sampling animals from each of the vertebrate clades have elucidated host and

environmental factors that have significant impacts on skin microbial communities (Table 1).

Body location, biological sex, age, geographic location, species, diet, living in the wild versus

captivity, proximity to other animals, maternal transfer, and disease states are all important

influences on microbial community structure.

1.4.1 Body location

Skin microbial diversity varies among body locations of sampled individuals. Moist, oily,

and dry regions in humans associate with distinct levels of microbial species diversity (26);

diversity is lowest in regions containing sebaceous glands. Metagenomics also supports the

finding that microbial communities are strongly predicted by individual and body region (42).

This individuality is so pronounced that the microbial community on hands can be linked to the

organisms deposited on a keyboard after contact (70), which may have forensic applications.

Furthermore, a built environment study determined that homes can be linked to the families that

inhabit them (71). After the inhabitants had moved, the new home rapidly became colonized with

their unique microbial signature.

Similar to human skin studies, the ability to use high-throughput sequencing has

expanded our understanding of mammalian skin diversity. A 16S rRNA gene study of 18 healthy

and allergic dogs observed higher species richness and diversity on haired skin than mucosal surfaces (51). Differences exist among the body regions of fire-bellied toads (*Bombina orientalis*), such that the dorsal sides of wild toads associates with higher diversity and richness than ventral sides, whereas captive toads exhibit the opposite result (54). These studies demonstrate that future microbiome studies should sample multiple body locations per animal to accurately capture the skin community. Differences in microbial communities among multiple body locations is particularly important because many non-human vertebrate studies use a single swab to sample all body locations, and may overlook intrabody variations in microbial communities.

1.4.2 Biological sex

Intrinsic factors of the individual, for example biological sex, are correlated with differences in microbial skin communities on certain vertebrate species. Female humans have higher microbial diversity on their hands (45) and significantly different communities within the stratum corneum layers of the upper buttocks (72) and also on the forehead (73). European starlings (*Sturnus vulgaris*) and bluebirds (*Sialia sialis)* also have significantly different diversity levels between both sexes on their plumage (74, 75). These variations may be attributed to physiological variations between the sexes, such as pH (76). In contrast, multiple studies have determined no statistically significant difference between males and females in dogs and vultures (51, 52, 61). At times there are no visible non-invasive differences between males and females within a species, resulting in an inability to document this factor. Lack of sex documentation is especially prevalent in amphibian and fish studies (Table 1). Recording the biological sex, when possible, of each specimen will better characterize the effect biological sex has on skin microbial communities.

1.4.3 Age

Specimen age is an additional intrinsic host factor that affects multiple vertebrate classes. Humans skin has pronounced differences in microbial loads and community composition throughout their lifespan (15, 77). Infant skin becomes rapidly colonized in the initial days after birth and is dominated by *Staphylococcus* (16). Puberty results in an increase in the number of microorganisms (15), which stabilizes throughout adulthood and eventually decreases in seniors (73). One study involving canine skin observed that demodicosis, a disease caused by parasitic mites, was most abundant in puppies and senior dogs (78). Additionally, tadpoles are associated with distinct microbial skin communities before they undergo metamorphosis (79). The common coqui (*Eleutherodactylus coqui*) has community variations between its juvenile and adult forms (80). Influences on bird microbial communities have been observed to include nest location and age (74).

1.4.4 Geographic location

Many studies have consistently demonstrated skin microbiome shifts based on geographic location. A study of 11 species of North American bats concluded that geographical location and site were important predictors of microbial community composition (81). Notably, researchers have determined that the whale skin microbiome varies geographically (82). Salmon have varying bacterial loads based on whether they are in marine or freshwater environments (41). Denaturing gradient gel electrophoresis (DGGE) analysis of female four-toed salamanders (*Hemidactylium scutatum*) determined that only 25% of the bacterial community being shared amongst populations in varying locations and produced less variable results than a culture-based analysis of the same samples (83). A large study on five different amphibian species (i.e., *Anaxyrus boreas, Pseudacris regilla, Taricha torosa, Lithobates catesbeianus*, and *Rana*

cascadae) in the United States determined wetland site was the largest predictor within each species (79).

Variations in microbial communities based on the geographic location the host inhabits can be partially explained by the microorganisms collected from local abiotic environments. A study of red-cheeked salamanders (*Plethodon jordani)* demonstrated that the salamanders shared their most abundant bacterial taxa with the moist forest floor debris (84). In contrast, skin swab samples of redback salamanders (*Plethodon cinereus*), eastern newts (*Notophthalamus viridescens*), and larval bullfrogs (*Rana catesbieana*) were distinct from the water they inhabited (85). This is in accordance with a humpback whale study that identified skin microbiota that were distinct from the surrounding seawater (82). It is possible that terrestrial animals' skin retains a higher bacterial load from soil than aquatic animals do from the surrounding water. The skin community may also be affected by the contaminant levels in the surrounding environment. A study on the Perez's frog (*Pelophylax perezi)* demonstrated that frogs living in a metal-rich contaminated site had distinct profiles from frogs in uncontaminated environments (86). All frogs had bacteria predominately from the *Actinobacteria* and *Alphaproteobacteria* taxonomic groups, whereas those from contaminated sites had more OTUs that were associated with acid-metal contaminated effluent tolerance. Testing the surrounding soil or water for both biotic and abiotic composition may therefore add more insight into what factors influence skin microbial community composition.

Seasonal variation of fish skin microbial communities, which at times is coupled with geographic location, have been supported by analyzing other species of fish, including lemon sole and skate (87). The study suggested that variations may be due to the timing of plankton blooms and changes in the microbial community from the surrounding water. Other factors that

may influence the fish skin microbiome include pH, dissolved oxygen concentration, and temperature (39). Fish located in warmer waters have higher proportions of mesophiles (59), whereas those near coast lines possess higher proportions of halotolerant bacteria (39). Manipulating salinity resulted in a reproducible shift in the microbial community that is significantly different from that of surrounding water in the enclosure (88). As with other vertebrates, geographical location also influenced the bacterial community of six fish species significantly (60). Seasonal variation has also been observed in the lowland leopard frog (*Lithobate yavapaiensis)*, which may be linked to disease incidence because frogs are at an increased risk of *Batrachochytrium dendrobatidis* infection during this season (80). The authors postulated that changes in frog secretion levels in adaptation to temperature changes contributed to the temporal bacterial community changes (89).

1.4.5 Species

Host species is a significant predictor of the skin microbial community that will be present on a given body location. An early mammalian study determined that non-human animals had distinct dominant *Staphylococcus* from humans (50). A more recent sequence based study of 11 species of North American bats observed that host species was an important predictor of microbial community composition (81). Amphibian host species was the most important predictor of community composition in a study of five species that included toads, frogs, and newts (79). This trend in amphibians is further supported by a study on Panamian frogs, which determined that there were key differences between hosts at bacterial taxonomic levels below the phylum level (90). A study of 102 fish from six species inhabiting the Gulf of Mexico (*Mugil cephalus, Lutjanus campechanus, Cynoscion nebulosus, Cynoscion arenarius, Micropogonias undulatus,* and *Lagodon rhomboides*) determined that each species had a distinct

18

skin community, based on ribosomal internal spacer analysis (RISA) and 16S rRNA gene amplification (60). Studies that sample multiple species in the same geographic location will allow for more detailed comparisons between species and classes of vertebrates.

1.4.6 Diet

Diet has been linked to the composition of the gut microbiome relating to obesity in humans (3, 91) and healthy non-human mammals, including carnivores, omnivores, and herbivores (7). Although the link between diet and microbial skin communities has rarely been studied, diet has been shown to impact numerous skin diseases (14) and thus may shift microbial community structure due to changes in immune function (92), secretion levels (93), and available vitamins, for example B_{12} (94). The B_{12} pathway of *Propionibacterium* is downregulated in humans with acne. The nutritional status of the host affects this pathway, because vitamin supplementation resulted in downregulation. This finding supports clinical observations that taking this vitamin supplement results in acne. In non-human mammals, significant differences in the number of *Tenacibaculum* and *Psychrobacter* spp. between humpback whales undergoing anabolic and catabolic metabolic states have been observed (52). Providing captive red-eyed tree frogs (*Agalychnis callidryas*) with a carotenoid rich diet increased skin bacterial richness and abundance, which may protect against fungal diseases (95). The skin microbiota can also change based on the metabolic status of the host. Salmon that are deprived of food have significant differences in both bacterial and fungal community composition and density, which was postulated to be a result of a decrease in the number of mucosal cells (93). Diet is also postulated to affect the skin because carnivores and herbivores have different microbial gut communities (7), which will be present on skin if fecal contact or coprophagy occurs, especially in enclosed environments associated with captivation (96). Future studies that experimentally alter an animal

diets and analyze accompanying skin microbial community shifts have the potential to determine the cause of diet-linked dermatological diseases, such as rosacea (97), atopic eczema (98), and acne (99).

1.4.7 Captivity

Animals in captivity have lower levels of microbial diversity on their skin than the same species in the wild. Wild amphibians have higher bacterial diversity levels on their skin than the same species in captivity. Wild frogs in Panama had different bacterial communities from frogs within the US, although these differences were insignificant at the phylum level (90). Additionally, wild red-eyed tree frogs (*Agalychnis callidryas*) had over twice the number of bacterial OTUs on their skin as their captive counterparts, demonstrating that captive animals have a significant decrease in diversity (95). The Panamanian golden frog (*Atelopus zeteki*) shares approximately 70% of bacterial OTUs on their skin between wild and captive specimens, although significant differences in richness, community structure, and phylogenies still existed (100). Overall, wild fire-bellied toads had higher diversity, which varied based on the presence of a *B*. *dendrobatidis* infection (54). Within the mammalian class, Tasmanian devils had significant differences in skin microbial communities between wild and captive specimens, although larger differences were observed between gut microbiota (101). Captive devils had elevated levels of *Mycobacterium*, a common cause of skin infections in captive facilities. Studies that aim to provide evidence to improve the conservation status of wild animals affected by skin diseases should therefore be sampling the wild skin microbiota for the most accurate microbial skin community information about the wild host species of interest.

The majority of human microbiome studies cannot adequately analyze the effects of time spent outdoors, because modernized humans in North America spend 93% of their time in an

enclosed vehicle or building (102). However, there are a few remaining cultures who spend higher proportions of time outdoors. For instance, a group of Venezuelan Amerindians with no known antibiotic use or prior contact with other cultures had the highest fecal and skin microbial diversity of any sampled human to date (103). Members from this isolated Yanomami hunter-gatherer village possessed bacteria with antibiotic resistance genes, indicating these genes are present in the human microbiome in the absence of antibiotic exposure. Their skin microbiota did not exhibit dominance by a single OTU in contrast to the high levels of *Staphylococcus*, *Propionibacterium*, and *Corynebacterium* in westernized individuals. Several OTUs that are typically not associated with the skin microbiome, such as *Solibacteriaceae* and *Knoellia*, were present at elevated levels (103).

1.4.8 Proximity to other animals

Humans shed $>10^6$ biological particles/hour (104), which changes the built environment surfaces they touch and the rooms they inhabit (105). Personal objects, including phones, shoes, and fabrics can be linked to their owners (106, 107), indicating a strong individual microbial signature that is shared with the surrounding environment. It has been recently shown that companion animals transfer microbes to their owners and, in turn, shift the human skin microbiome (108). These studies demonstrate that the shedding of the skin microbiome impacts both the microbial community composition of inanimate objects and living macroorganisms alike. Vertebrates that are therefore living in the same enclosed habitat, such as companion animals in a barn, or zoo animals in a cage, are likely altering each other's respective microbiomes. However, cohabitating in the same pond was not a significant factor influencing community structure of amphibians (55). This demonstrates that the sampled microorganisms

comprise a skin-associated microbiome, instead of only transient organisms that are sourced from the surrounding environment.

Bacterial communities have been shown to be influenced by the social structure of animals. The community nests of the four-toed salamander (*Hemidactylium scutatum*) possessed cutaneous bacteria that were inhibitory to the lethal fungus *Mariannea* (109). These nests had higher survivability rates than solitary nests with lower amounts of antifungal bacteria in their skin communities. In turn, the bacterial communities may influence the behavior of their hosts. Odour cues produced by bacteria are involved in numerous behaviors, including mating, marking territory, and recognition (110). Determining the distribution of organisms on vertebrate skin has the potential to answer several questions about animal behavior that were raised previously by Archie and Theis (111), such as how animals recognize individuals and kin, assess mate quality, and social relationships.

Birds are social animals whose sexual and social constructs have been shown to aid in bacterial transmission (12). A study of caged zebra finches (*Taeniopygia guttata*) concluded that feathers infected with *Bacillus licheniformis* resulted in an oral-fecal-genital route of transmission. Preening resulted in autoinfection, which progressed to a sexual infection whose transmission rates varied by biological sex (12). European starlings (*Sturnus vulgaris*) with larger brood sizes have higher levels of bacteria on their feathers (74). Manipulating their brood size resulted in significantly different bacterial community composition on plumage, but not richness or feather degradation. Additionally, bluebirds (*Sialis sialis)* sharing the same nest transmit plumage bacteria, based on results from culturing techniques (75). This has implications on the distribution of feather-degrading bacteria that are associated with body condition and feather coloration.

1.4.9 Maternal transfer

The transmission of microbiota from mother to offspring influences the developing microbiome of the gut (112), mouth (113), and skin (114). Maternal transmission has been noted in several vertebrate species (114) including humans, domesticated chickens (115), ray-finned fish (116), and turtles (117). In humans, the sebaceous skin surrounding the breast transfer core skin commensals, including *Corynebacterium*, *Staphylococcus*, and *Propionibacterium* (46, 118). Infants that are born by C-section have their communities dominated by skin microorganisms, whereas those born by vaginal delivery have characteristic vaginal microbiota, such as *Lactobacillus* and *Prevotella* (119).

There are currently few studies that have determined the skin organisms that are transferred maternally to nonhuman vertebrates. The pouch of the Tasmanian devil (*Sarcophilus harrisii*), where the marsupial protects its underdeveloped offspring, has been shown to have an overall similar microbial community composition to the skin in terms of phylotype richness and the number of OTUs present (101). Significant differences were observed, with an increase *Clostridia* abundance and decrease in *Bacilli*. Despite these differences, the pouch samples grouped with skin samples instead of mouth and gut samples. This study demonstrates the microorganisms marsupial neonates would be coming into contact with while they are developing in the maternal pouch. In amphibians, transmission of skin bacteria to four-toed salamander embryos has been observed (109). These salamanders at times use communal nests with eggs from a minimum of two females, which leads to higher rates of offspring survival. Communal nests were more likely to contain skin bacteria that inhibit *Mariannaea*, a fungus that is lethal to four-toed salamanders. Only 27% of females have these beneficial skin bacteria;

having multiple females in contact with a nest resulted in higher proportions of surviving embryos (109).

1.4.10 Disease/Dysbiosis

Microbial diversity is defined as the number of distinct types of microorganisms present in a region as well as their abundances (47). Significant differences in the microbial diversity between healthy and diseased humans have been documented for the gut and vagina. Lower gut microbial diversity associates with obesity (91). It should be noted that lower diversity does not always result in a disease state. For example, bacterial vaginosis occurs when the vaginal microbial community is more diverse (120). Microbial communities are typically more diverse on healthy skin, and there is evidence that microbial community composition affects several human skin conditions. Dysbiosis, defined as a shift from a normal microbiome, is associated with numerous skin diseases (11), such as acne vulgaris, rosacea, psoriasis, and atopic dermatitis (22, 121, 122). Microbial shifts have been associated with bovine digital dermatitis (123), demodectic mange (78), *B. dendrobatidis* infections (80), and camel dermatophilosis (124) in other vertebrates.

Multiple studies have been conducted on mammals to determine the relationship between the skin microbiome and diseases. Allergic dogs have lower bacterial richness on their skin than their healthy counterparts (51), whereas allergic cats had higher levels of the fungi *Agaricomycetes* and *Sordariomycetes* and reduced *Epicoccum* compared to controls (69). Digital dermatitis affects the hooves of cattle and results in lameness, which results in major economic losses to the agricultural industry (125). Animals with digital dermatitis have higher bacterial diversity characterized by increases in *Bacteroidetes*, *Proteobacteria*, and *Spirochaetes*. In particular, *Treponema* spp. (126) are abundant in deep lesions and likely originate from the gut

reservoir (123). Sheep footrot is a similar infectious disease that results in lameness in herds of sheep (127). *Dichelobacter nodosus* has been shown to initiate the disease, whereas *Fusobacterium necrophorum* has a secondary role in infection (127). The above two foot diseases are examples of polymicrobial diseases, where shifts in several OTUs of the skin microbiome are required before clinical symptoms are evident. Recently, a metagenomics study of psoriasis patients suggested that variations at the strain level may contribute to the disease (128). Strain-level analysis has the potential to elucidate more information about how shifts in microbial communities are related to disease states, compared to relying solely on diversity metrics of 16S rRNA gene profiles that are frequently used to characterize dysbiosis.

Amphibian skin has been well studied in an attempt to prevent infections, for instance *Ranavirus*, mycotic dermatitis, and chytridiomycosis that impact wild populations [16,112]. This vertebrate class is currently the most threatened, with 32.5% of the 1856 identified global amphibian species being classified as threatened with impending extinction (130). Greater understanding of the skin microbiome is required to create effective conservation management programs for animals with declining populations due to skin diseases. A variety of fungi have been cultivated from the skin of injured hellbender salamanders (*Cryptobranchus alleganiensis bishopi*), including *Acremonium, Cladosporium, Curvularia, Fusarium, Streptomycetes*, and *Penicillium* (131). Isolated opportunistic bacterial pathogens included *Aerococcus viridans, Aeromonas hydrophila, Gordonai terrae, Granulicetella adiacens, Stenotrophomonas maltophilia*, and *Streptococcus pneumoniae*. Several of these bacterial species are able to form biofilms. The cutaneous microbiome of two giant salamander subspecies (*Cryptobranchus alleganiensis)* were studied to explain the reason the Ozark hellbender subspecies is affected by

chronic wounds, whereas the eastern subspecies is not (132). Salamanders with wounds had higher OTU abundances than those without wounds.

Batrachochytrium dendrobatidis is a fungal pathogen that causes chytridiomycosis and has been responsible for the population decline of amphibians. This lethal fungus has been linked to an altered skin microbiome (133). Skin bacteria are known to produce antifungal secondary metabolites that inhibit this pathogen (21). The four bacterial genera *Bacillus, Chitinophaga, Janthinobacterium*, and *Pseudomonas* were isolated from red-backed salamanders (*Plethodon cinereus*) and assayed to determine their ability to inhibit *B. dendrobatidis*. These microorganisms acted synergistically to prevent infection; a co-culture of *Bacillus* and *Chitinophaga* was the most effective with the metabolite tryptophol (21). A reduced cutaneous community on redback salamanders likewise has been shown to lead to more symptoms of the disease, namely weight loss and limb lifting (134). Two closely related frog species (*Rana sierra* and *Rana muscosa*) were observed to have differential responses to *B. dendrobatidis* infections based on differences in their skin microbiota. The majority of *R. sierra* individuals had anti- *B. dendrobatidis* bacteria and were able to persist with *B. dendrobatidis* for six years, whereas *R. muscosa* had lower proportions of these OTUs and went extinct within a year (135), indicating that herd immunity may protect frogs from *B. dendrobatidis* infections. The genera *Janthinobacterium, Pseudomonas*, and *Flavobacterium* were present in higher abundances in uninfected Italian Steam frogs (57).

The composition of the fish skin microbiota and related skin pathogens have also been studied to prevent large economic losses affecting the fishing and aquaculture industries. A study of the pathogen *Vibrio anguillarum* demonstrated that colonization via rainbow trout (*Oncorhynchus mykiss*) skin is an important step for disease to progress to other body regions

26

(136). Fish have also been shown to possess beneficial skin bacteria that help to prevent infections. For example, rainbow trout have commensal lactic acid bacteria on their skin that prevent *Lactococcus garvieae* from colonizing by producing inhibitory compounds and outcompeting for nutrients (137).

Reptiles are prone to infection by a variety of predominately Gram-negative commensal bacteria, including *Aeromonas, Klebsiella, Proteus, Pseudomonas,* and *Salmonella* (138). Other skin infections have been caused by viruses, fungi, and parasites. Fungal dermatitis in the United States has affected numerous reptilian species, including dusky pigmy rattlesnakes (*Sistrurus miliarius barbouri*), garter (*Thamnophis sirtalis*), and ribbon (*Thamnophis sauritis*) snakes (139). Herpesvirus is currently infecting both wild and captive turtles and tortoises resulting in necrotizing lesions (138). Examples of affected species include Argentine tortoises, Mediterranean tortoises, Pacific pond turtles, and painted turtles. Fibropapillomatosis is decimating wild populations marine turtles, especially the green, loggerhead, and olive ridley sea turtles (138). This viral infection has spread globally and has no current protocol to prevent transmission in wild populations. Other skin associated infections, for example inclusion body disease (IBD), are prevalent on multiple continents (138). IBD has primarily been reported on boid snakes, including Burmese pythons and Boa constrictors in Africa, Australia, Europe, and North America. Reptilian skin has been shown to harbour several viruses that lead to lesions and premature death (62). Baseline high-throughput sequencing data of healthy and diseased skin states is required to implement conservation measures, such as probiotic treatments.

1.5 Probiotics: an application of skin microbiome research seeks to reduce disease incidence

Now that critical baseline data is becoming available for several vertebrate species, this knowledge can be applied to improve the health of humans, in addition to domesticated and wild

animals. Probiotics involve introducing a microorganism into a host to induce a measured and reproducible positive effect on host health. Currently, several research groups are working to identify effective probiotic treatments. For example, a probiotic study of Panamanian golden frogs (*Atelopus zeteki*) determined that the initial skin bacterial composition and resulting skin metabolites were greater predictors of recovery from *B. dendrobatidis* infection than a treatment of probiotic *B. dendrobatidis* -inhibitory bacteria (140). However, other experiments have been able to successfully use probiotics to limit *B. dendrobatidis* infections by considering the interactions between the probiotic and *B. dendrobatidis*, the host, resident skin community, and the environment, as reviewed by Bletz et al (141). Non-invasive assays have been developed to test probiotics, and have concluded that the "mucosome" can be altered successfully to prevent fungal infection, whereas temperature may also influence infection rates (10). In a study of Cascade frogs, only 5 of 43 isolates were able to directly inhibit *B. dendrobatidis,* indicating either a small proportion of OTUs inhibited *B. dendrobatidis*, or that bacterial commensals may work synergistically in ways that are not apparent for individual isolates (58). Additionally, skin products, such as soaps and sprays, with live bacterial cultures are now being formulated for human use. More studies are needed to test the efficacy of these products.

1.6 Future directions

Although the animal subphylum *Vertebrata* possesses a highly diverse range of animal species with varying skin physiology, social constructs, and skin conditions, several common trends are apparent. The habitat and geographic location of an animal, maternal effects, and disease status are factors affect the vertebrate microbial skin community [77, 126]. Additionally, biological sex, age, species, and disease state have been shown to affect a wide range of vertebrates (11, 75, 79, 90).

It is crucial to sample a wide range of animals to have meaningful baseline data for conservation management programs. Numerous skin diseases have been linked to population declines and threaten the extinction of a variety of animals. More information is needed on the disease transmission mechanisms of both wild and captive animals. Deeper sequencing should therefore be completed on a wider variety of species and include bacteria, archaea, fungi, viruses, and microscopic eukaryotes. As stated earlier, there are currently few reptilian skin microbiome studies. Class *Reptilia* is a large clade that is associated with numerous skin diseases. Sequencing a large range of animals from all four reptilian orders including turtles, snakes, lizards, and crocodiles will provide crucial baseline data for conservation efforts, in addition to the pet industry.

Information on how microbial community composition and functions change among different species and locations is crucial for elucidating the impact of microorganisms and their products on the skin of pets, livestock, and captive animals. Progressing to metagenomic sequencing on vertebrate skin will provide information on microbial biogeochemical pathways that may impact host skin, including the production of volatile compounds and vitamins. Furthermore, there is currently no knowledge on the coevolution of skin microbiota and their hosts, as has been completed with gut studies (143). Determining which microorganisms have undergone coevolution has enormous implications for understanding evolutionary history and managing human health. The hologenome theory of evolution postulates that the genetic diversity of microorganisms significantly influences their plant and animal hosts enough to result in adaptations and evolution (144). Coevolution studies promise to elucidate the effect the skin microbiome has had on hosts over millions of years.

1.7 Research Description

1.7.1 Research Overview

Skin is the largest organ of the body and provides a critical barrier between a host and its external environment (20). Studying the skin microbiome of mammals has implications for animal husbandry, veterinary practises, and improving our understanding of mammalian evolution. These communities of microorganisms can prevent disease from transient, invading organisms and interact with their host (145). A shift from a healthy skin microbiome has been associated with skin conditions, such as rosacea (97). It is therefore crucial to characterize the organisms present on mammalian skin and how they change between species, biological sex, and geographic location.

Previous skin microbiome research has predominately focused on human skin because of its importance to human health and its potential to create effective treatments for skin conditions, including psoriasis, rosacea, and atopic dermatitis (20, 26, 42, 121). Additionally, many studies have focused on the skin microbiome of amphibians in an attempt to prevent the spread of organisms, for example *B. dendrobatidis,* that have been decimating amphibian populations (133, 135, 140, 141). In contrast, the non-human mammalian skin microbiome has been relatively uncharacterized. The majority of studies have focused on only a few species of mammals, such as pets, primates, and marine mammals (51–53, 69), which does not allow for broad evolutionary analyses across Class Mammalia. Characterizing the distribution of microorganisms across many species and orders creates a baseline dataset by which future studies can begin to identify specific skin microorganisms that have co-evolved with their hosts.

1.7.2 Objectives and hypotheses

The advent of high-throughput sequencing has led to rapid advances in our understanding of the skin microbiome and how it impacts host health, particularly with humans. However, many intriguing questions remain to be answered. Although it is known that body location and individuality influence the humans skin microbiome, little is known about other influences such as the effect of living intimately with a partner. Daily interaction with a shared environment has the potential for cohabiting couples to share a skin microbial community, which has health implications. Moreover, although human and amphibian subjects have been relatively well characterized, very few studies have analyzed the skin microbiome of mammals. Sampling a wide range of mammals has implications for conservation, informing veterinarian practises, and evolutionary history between mammalian hosts and their microbial symbionts.

The first objective of my research was to determine if cohabitation leads to a detectable impact on the microbiome of partnered sexually active cohabiting individuals, compared to the influences of factors related to individuality and lifestyle. It was hypothesized that cohabitation would have a significant impact that would lead to the ability to correctly classify couples based on random forest modelling. This influence can be attributed to humans shedding skin and its associated microbiota (105), which would in turn be picked up from the built environment by their partner, as well as sharing microorganisms through direct contact.

The second objective of this research was to characterize the distribution of bacteria and archaea on mammalian skin. It was hypothesized that humans would possess lower microbial community diversity than animals in the wild, on farms, or in zoos. This would be measured by human skin having a lower Shannon index, fewer OTUs, and several indicator species. I investigated whether the influences from the extrinsic factors, including habitat and location,

influence microbial communities more than intrinsic host factors, namely those related to species-specific diet and physiology. It was hypothesized that the species of the mammalian host would significantly influence the microbial community, as measured by the PERMANOVA F statistic. Diet was also expected to partially play a role because carnivores and herbivores have different microbial gut communities (7), which will be present on skin if fecal contamination occurs.

Chapter 2
The Skin Microbiome of Cohabiting Couples

2.1 Introduction

Skin is the largest organ of the body, forming a critical protective barrier between an organism and its environment. The average human is covered by 1.5 to 2.0 m^2 of skin, varying from 2 to 3 mm in depth (17). Skin is divided into three tissue layers: the epidermis, the dermis, and the hypodermis. The epidermis is of particular interest because it is the exposed layer that contains a diverse microbial community of largely beneficial and benign microorganisms (20), while protecting the body from transient microorganisms with the potential to cause disease. This outer layer is relatively hostile and constantly shedding, associated with antimicrobial compounds and low in moisture, yet with high acidity, hydrophobicity, and salinity (23). Despite these relatively harsh conditions, between one million and one billion microorganisms inhabit each cm^2 of skin (25, 26).

Microbial surveys demonstrate that human skin is inhabited by a diverse community of bacteria, archaea, fungi, protozoans, and arthropods (18, 25, 42, 146). In general, four bacterial phyla dominate the skin: *Proteobacteria, Actinobacteria, Firmicutes,* and *Bacteroidetes* (20, 45). In one survey of skin microbiota, *Propionibacteria*, *Corynebacteria*, and *Staphylococcus* spp. comprised over 62% of sequences detected across 20 body sites (46). Moist, oily, and dry regions were each associated with different levels of microbial species diversity, which was lowest in oily regions that were associated with the sebaceous glands (46).

Human skin microbiota are strongly defined by body region and individuality (42). The main factors affecting the skin microbiota are skin location, biological sex, geographical location, ethnicity, skin depth, antibiotics, cosmetics, age, and health (147). Hygiene practices also influence the skin microbiome, including the use of lotions, antibiotics, soaps, and cosmetics

(148, 149). Three dimensional molecular cartography maps of human skin demonstrated that the molecular composition of human skin is defined by the microorganisms present, molecules from hygiene products, and local skin anatomical structure (17).

Although cohabitation may impact an individual's skin microbiome, no study has yet explored the relationship between the skin microbial communities from a wide range of skin locations of intimate cohabiting couples. A previous human study determined that the palms of hands, oral cavity, and gut microbiomes were more similar within families (150). Moreover, humans and animals share a microbial community that is affected even by a short absence of several days (71). A study on the effects of antibiotic use on cohabiting individuals detected household-specific microbial communities, although there was no significant difference between the number of shared taxa (151). To the best of our knowledge there are no other studies on cohabitation that determined which skin regions are the most related between partners.

The objective of this investigation was to characterize the distribution of bacteria and archaea on the skin of intimate cohabiting heterosexual couples and determine whether cohabitation leads to a detectable impact on the microbiome of partnered individuals, compared to the influences of factors related to individuality and lifestyle.

2.2 Materials and Methods

2.2.1 Ethics

The study has been approved by the Office of Research Ethics at the University of Waterloo (ORE #20993). The following procedures were conducted in accordance with the approved documentation. Written consent was received from all individuals and cannot be linked to completed surveys or samples. All participants remain anonymous.

2.2.2 Sample collection

A total of 17 body regions of 10 cohabiting and sexually active couples living in South Western Ontario were sampled to determine the distribution of their microbial communities (Figure 1). Participants all reported as being healthy heterosexual adults between the ages of 20-49 years and had lived together ranging from four months to fourteen years. For consistency, the majority of subjects reported Caucasian heritage (18), although East Asian (1) and Asian (1) participants were also included. The upper eyelids, outer nostrils, inner nostrils, armpits, torso, back, navel, inner thighs, bottom of feet, and palms of hands were sampled by the participants themselves using sterile foam swabs (Figure 1). While applying moderate pressure, skin was swabbed five times in a forwards and backwards motion. The swab was then rotated and repeated in adjacent areas at the same body site for a total of 20 strokes per swab. Sample swabs were returned to their initial plastic storage container and frozen at -20ºC until DNA extraction. All participants provided comprehensive metadata for analysis. These data were collected to test whether lifestyle choices also affected the skin microbiome and to determine if any the participants had any confounding factors that may impact the results (Appendix). Although all categories were analyzed to determine if any lifestyle choices had an effect, only significant factors are discussed.
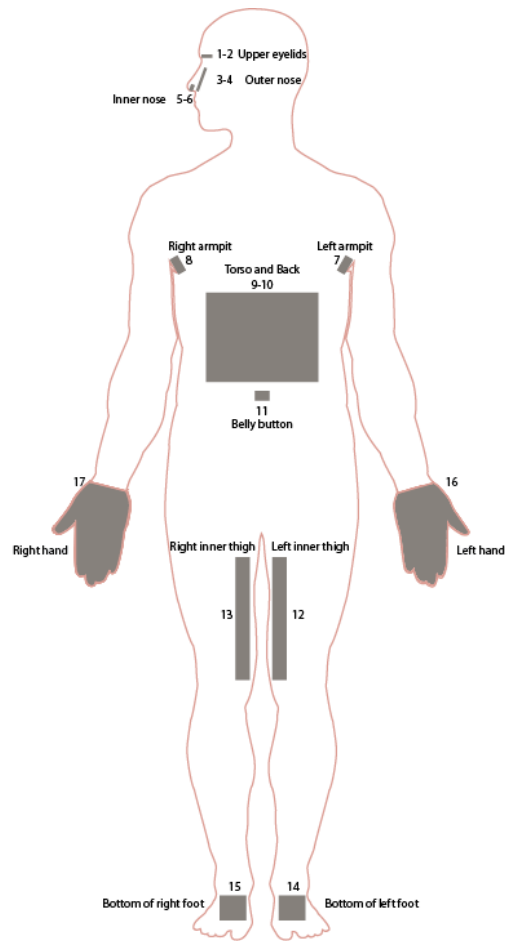
Figure 1: Body locations sampled for this study.

2.2.3 DNA extraction and amplification

Genomic DNA was extracted using the PowerSoil-htp 96 Well DNA Isolation Kit (MO BIO Laboratories) according to a previously published protocol (152). The beadbeating manufacturer's protocol was used at speed 20 for 10 min on a Mixer Mill MM400 plate shaker (Retsch) with a plate adapter set (MO BIO Laboratories). A final 75 μl volume of DNA was eluted and stored at -20°C in the kit-supplied EDTA-free elution solution.

A 464-bp fragment of the V3-V4 region of the 16S rRNA gene was amplified by using the universal prokaryotic primers Pro341Fi (5'-CCTACGGGNBGCASCAG-3') and Pro805Ri (5'-GACTACNVGGGTATCTAATCC-3') (153). The V3-V4 region has been used by multiple

skin microbiome studies (61, 72, 105, 154) and has been experimentally shown to produce the

closest sequence agreement to known low-diversity mock community samples (155). However,

previous studies have commented on the potential for bias between this region and V1-V3,

particularly with *Propionibacterium* and *Staphylococcus* (156–158). The primers were modified

to include adapters for binding the flow cell (Illumina), a 6-base barcode for multiplexing, and

complementary forward and reverse regions required for Illumina primers, as described

previously (159). Two rounds of PCR were conducted targeting the same 16S rRNA gene region.

The first round was conducted for 25 cycles without the Illumina adapters, followed by a second

round of 15 cycles in order to attach the adapters. I determined that this procedure improves

yield and sensitivity for low biomass samples from a wide range of skin and surface swab DNA

extracts. The first PCR amplification mix contained 2.5 μl of 10x ThermoPol *Taq* buffer (New

England Biolabs), 1.5 μl of 10 mg mL^{-1} BSA, 0.05 μl of 100 mM dNTPs (New England

Biolabs), 0.05 μl of each 100 μM forward and reverse primer (Integrated DNA Technologies),

0.125 μl of 5 U μl^{-1} *Taq* polymerase (New England Biolabs), 1-10 ng of template DNA (3 μl for

the majority of samples), and nuclease-free PCR-grade H$_2$O to a 25 μl total reaction volume. The

reaction was run on either the T100 Thermal Cyler (BioRad) or the C1000 Thermal Cycler

(BioRad) at 95°C for 30 s (initial denaturation) and 25 cycles of 95°C for 15 s (denaturation),

55°C for 30 s (annealing), 68°C for 60 s (extension), and a final extension of 68°C for 10 min.

The second PCR amplification mix contained 2.5 μl of 10x ThermoPol *Taq* buffer, 1.5 μl

of 10 mg mL^{-1} BSA, 0.05 μl of 100 mM dNTPs, 0.05 μl of 100 μM forward primer, 1 μl of 5 μM

reverse primer, 0.125 μl of 5 U μl^{-1} *Taq* polymerase, 1 μl of product from the first PCR reaction,

and 18.8 μl nuclease-free PCR-grade H$_2$O to a 25 μl total reaction volume. The reaction was run

on the T100 Thermal Cyler and the CFX96 Real-Time System at 95°C for 30 s and 15 cycles of

95°C for 15 s, 55°C for 30 s, 68°C for 60 s, and a final extension of 68°C for 10 min. One sterile swab control per extraction kit and one negative template control per 96 well plate were included to monitor potential contamination. Each reaction was performed in triplicate to eliminate any potential PCR bias from low biomass samples (160). All PCR amplifications were prepared in a PCR hood that was UV treated for 15 min. All triplicate PCR amplifications were pooled and stored at -20°C until further use.

PCR products were visualized on 1% (w/v) agarose gels (BioBasic; run through a 1x TAE buffer) using gel electrophoresis after staining with 1 μg mL^{-1} ethidium bromide (Calbiochem). AlphaView software (Protein Simple) was used to image gels on an AlphaImager HP (Alpha Innotech). 50 ng of the 1 Kb Plus DNA reference ladder (Invitrogen) was loaded to verify fragment size. Gels were analyzed to check for amplicon size, nonspecific binding, contamination, and amplification confirmation. Samples with little to no amplification were re-amplified with an increased template volume in an attempt to obtain PCR products. All triplicate PCR reactions were subsequently pooled and stored at -20°C until further use.

2.2.4 Illumina library preparation

Pooled samples were quantified using the AlphaView band analysis tool (Protein Simple). Relative concentrations of each product were determined, and each 96 well plate was pooled using an equal quantity of PCR product. The pools were purified using the manufacturer's protocol for the Wizard SV Gel and PCR Clean-up System (Promega) and stored in nuclease-free H$_2$O at -20°C until library quantification.

The purified pools were quantified using the Qubit 2.0 Fluorometer (Life Technologies) with the Qubit dsDNA HS Assay Kit (Invitrogen) and diluted to 6 nM. The 6 nM pools were merged into a single 100 μl pool, ensuring each sample had an equivalent amount of PCR

product in the final pool. The correct DNA concentration was determined using Qubit, the qPCR PerfeCTa NGS Library Quantification Kit for Illumina Sequencing Platforms (Quanta Biosciences), and gel quantification. The qPCR kit was specifically designed to quantify Illumina libraries. The kit contained the Illumina forward (P5; 5'–AATGATACGGCGACCACCGA–3') and reverse (P7; 5'–CAAGCAGAAGACGGCATACGA–3') primers and a premade mastermix called SYBR Green SuperMix (Quanta Biosciences). The 20 μL reactions were prepared by making the standard curve and NTC according to the manufacturer's protocol. The library dilutions were modified to 1:20 and 1:2000 dilutions to conserve reagents. Reactions were performed in duplicate and run on the C1000 Thermal Cycler using a CFX96 optical module. The qPCR cycler was programmed as follows: 95°C for 1 min (initial denaturation), and 35 cycles of 95°C for 15 s (denaturation), 63°C for 45 s (annealing and extension), a plate read after each cycle, and a melt curve analysis that ranged from 65–95°C that increased by 0.5°C increments every 2 s. The concentrations calculated from all quantification methods were compared to ensure consistent concentration readings and were subsequently stored at -20°C until sequencing occurred. The library was quantified the day of sequencing using a Qubit fluorometer to ensure freezing had not altered the DNA concentration.

2.2.5 Illumina sequencing

High-throughput sequencing on a MiSeq (Illumina) was used for analyzing the amplified V3-V4 region of the 16S rRNA gene. The MiSeq Reagent v2 kit-500 cycles (Illumina) was used according to the manufacturer's protocol to prepare the final quantified pool for sequencing. A 10% PhiX control was included to increase sample diversity. In brief, the library and the PhiX control were denatured with 0.2 N NaOH and diluted to 8 pM, before being merged 10:1. The pooled library/PhiX solution was loaded onto the MiSeq Reagent v2 cartridge (Illumina) and

sequenced. Sequencing was conducted using the following software: Illumina Experiment

Manager v. 1.6.0, MiSeq Test Software 1.0.4.0, and Fluidics Test Software 1.1.2.0. Clusters of

the V3-V4 amplicon were formed monoclonally and paired ends were sequenced. The 6bp

barcode ensured the samples could be mapped and sorted. After sequencing was complete the

quality was analyzed using the MiSeq Control Software v. 2.5.0.5. Analyses included image

analysis, base calling, Phred quality calculations, and barcode sorting or demulitplexing.

2.2.6 Assembly of sequence data

The following bioinformatics pipeline was composed entirely of open source software

and was managed by Automation, eXtension, and Integration Of Microbial Ecology: v. 1.5

(Figure 2) (161). The generated paired-end sequences were assembled with PANDAseq v. 2.8

(162) using the default parameters of a 0.9 quality threshold, a minimum sequence overlap of

one bp and a minimum read length of 32 bp. PANDAseq used the Illumina barcodes to sort the

raw data and searched for sequence overlap. Poor quality sequences with mismatches and low

quality scores were discarded. The assembled sequences were analyzed by Quantitative Insights

Into Microbial Ecology v. 1.9.0 (QIIME) (163). The sequences were then clustered *de* novo with

UPARSE (164) at 97% and 99% sequence identity, which also removed chimeras (improperly

merged sequences from two different OTUs) and sequences that only appeared once in the

dataset (164). The clustered sequences were then aligned with PyNAST v. 1.2.2 (165). RDP v.

8.1 was used to assign all taxonomy with a minimum confidence of 80% using the most recent

Greengenes database v. 13.8 (166).
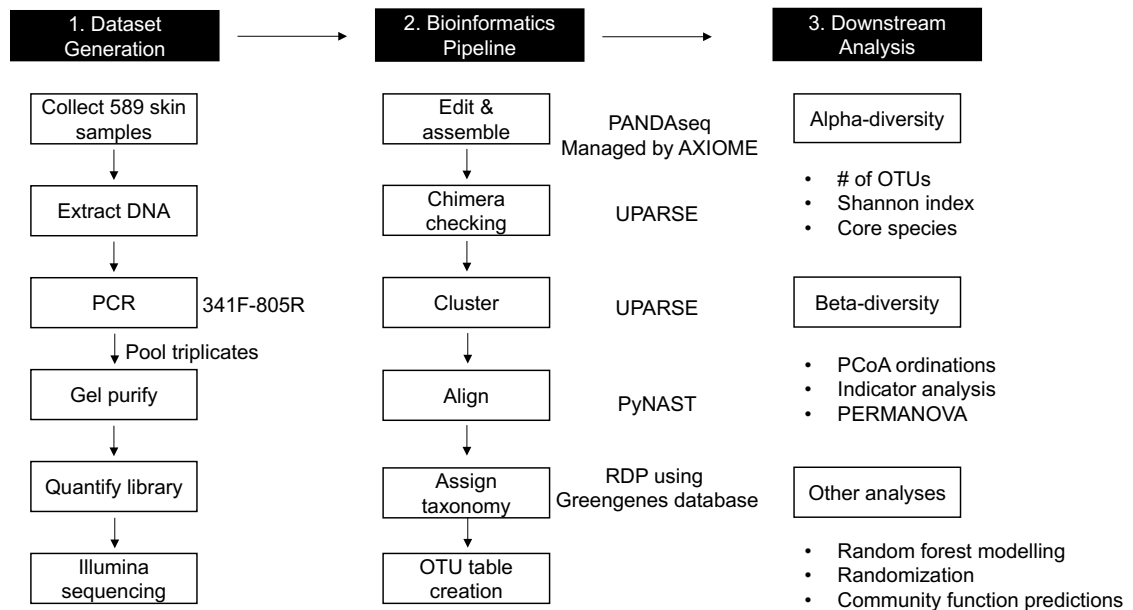
Figure 2: Flowchart illustrating the dataset generation bioinformatics pipeline, and downstream analysis steps.

Samples with fewer than 5293 reads were removed. These samples did not have a visible band on the agarose gel, but had been included in case there was sufficient DNA for downstream analyses. All of the NTCs and sterile swab controls contained less than half the number of reads as this cutoff. This resulted in the removal of the following 10 samples in the human dataset: three armpit and feet samples, as well as one sample each from back, thigh, hand, and torso. Rarefication was used to control for uneven sequencing depth by equalizing the number of reads per sample.

2.2.7 Statistical analyses

Sample alpha diversity was measured using the Shannon index (167) and the total number of observed OTUs. Non-parametric two sample t-tests were calculated to determine significance of each of the 79 metadata categories using the multiple_rarefaction.py, alpha_diversity.py, collate_alpha.py, and compare_alpha_diversity.py commands in QIIME v.

1.9.0. Multiple rarefactions were conducted with a minimum size of 100 reads, maximum of 5290 reads, increasing in intervals of 100 sequences after creating 10 rarefactions for a total of 530 rarefactions. A Bonferroni correction was used to avoid false significance from a dataset of 330 samples.

Sample beta diversity was measured using ordinations and PERmutational ANalysis Of VAriance (PERMANOVA). PERMANOVA calculates the percent variation explained by each metadata category and was run using the adonis function from the vegan package v. 2.4-0 (168) in RStudio (169) with 1000 permutations. Heatmaps were created in R studio using the Analyses of Phylogenetics and Evolution v. 4.1(ape) (170) and RColorBrewer (171) packages. All analyses were generated using a Bray-Curtis distance matrix when applicable. This matrix was calculated by adding the number of shared OTUs from two samples and dividing this sum by the total number of OTUs from the samples. Principle coordinate analysis (PCoA) ordinations were used to visualize the microbial variation between the samples. Ordinations were created with the phyloseq v. 1.14.0 (172) and ggplot2 v. 2.1.0 (173) packages in RStudio (169).

Indicator species were classified to determine which organisms were highly abundant across various metadata categories. Core species, classified as OTUs that have at least one sequence per sample in a particular category, were also determined to identify organisms that were ubiquitous on all skin samples. The Dufrêne-Legendre indicator species analysis was conducted to determine if specific OTUs were indicative of metadata categories (174). An indicator was defined as having a significant p value (<0.05) and an indicator value threshold of 0.7 with a minimum mean abundance of 10 reads.

Random forest modeling was used to determine the accuracy with which samples can be correctly classified to couples. The supervised_learning.py command in QIIME v. 1.9.0 was

used with the options of creating 1000 trees and 10 fold cross validation. All possible incorrect couple pairings, where all couples were matched with a non-partner participant of the opposite sex, were generated using the module itertools.permutations in a custom script in Python.

2.3 Results and Discussion

2.3.1 Moisture level and individuality strongly influence microbial diversity

I analyzed the diversity of bacteria and archaea on 330 skin samples obtained from 17 skin regions of 10 sexually active cohabiting couples, which yielded 8,753,153 reads. The two-step PCR A total of 4,639 unique operational taxonomic units (OTUs) were obtained from 1,746,690 sequences, rarefied to 5,293 reads per sample. Five no-template PCR controls and the sterile swab sample all contained fewer reads than the rarefied sequence count and produced no visible bands on an agarose gel following PCR amplification. Across all samples, the most abundant phyla were *Actinobacteria*, *Firmicutes*, and *Proteobacteria*, which is consistent with findings from previous human skin microbiome studies (20, 26, 45). These three phyla constituted 94.9±5.2% of all reads (Figure 3) whereas the remaining sequences were affiliated with 38 phyla. *Bacteroidetes* was the only other phylum present above 1% relative abundance. Archaea comprised only 66 of the 16S rRNA genes (0.004%), yet this may be an underrepresentation of their actual abundance because of known primer mismatches to archaeal 16S rRNA genes (175). The most abundant OTU was the common skin bacterium *Staphylococcus epidermidis* (176)*,* which constituted 14.5±15.4% of all sequences. There were 11 OTUs that were present above 1% relative abundance across the complete data set, representing 54.3±23.1% of all sequences.
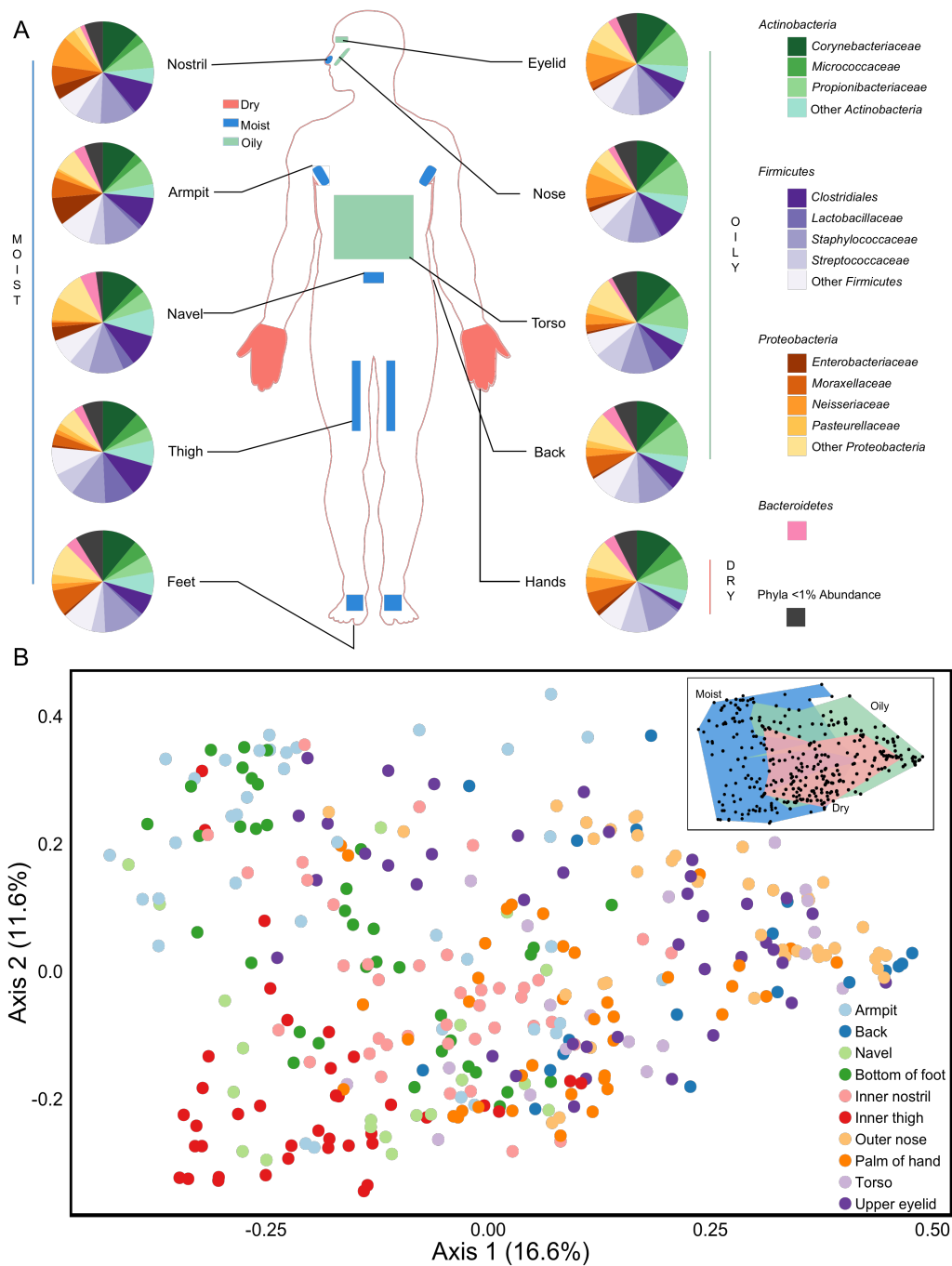
Figure 3: Microbial diversity of the 10 body locations sampled. A) Pie charts illustrating the relative abundance of microbial families present >1% and the phyla to which they belong, organized by each of the 10 body locations sampled. B) PCoA ordination calculated using the Bray-Curtis dissimilarity metric. The 330 samples from all body locations are included and are denoted by body location.

Known moisture levels of each body location were strongly associated with the observed variation in skin microbial communities (Figure 3). Skin regions are typically categorized as moist, oily, or dry based on previous research that determined the density of sebaceous glands and corresponding moisture and sebum levels (20, 177, 178). Grouping of samples from these three moisture levels was statistically significant (PERMANOVA; $F_{2,327}$ = 18.8, $p < 0.001$). Significant variations in the abundance of *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* were observed among moisture levels (Figure 3) as were alpha diversity differences (Figure 4). Similar shifts in abundance levels, such as high levels of *Propionibacterium* on facial skin sites, have been noted previously (18, 46). The Shannon index demonstrated that dry palm samples were significantly more diverse (non-parametric two samples t-test; $p = 0.003$), in comparison to both moist and oily regions, which had similar average Shannon diversity. Palms and feet were the most diverse sites sampled and had significantly higher numbers of OTUs, likely due to their frequent contact with microbiota on floors and objects.
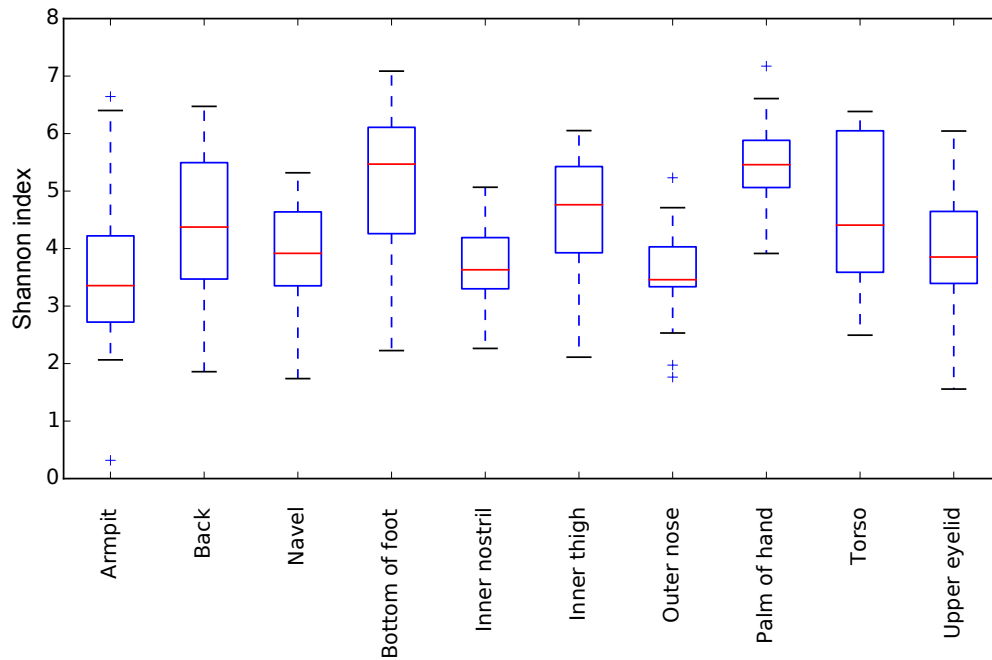
Figure 4: Boxplots of diversity by 10 body locations. Both the Shannon index and number of OTUs were plotted for all 330 samples.

Strong individuality was reflected by 16S rRNA gene profiles , and supports previous research that suggested individuality shapes the skin microbiome (42). When each body location was analyzed separately, the left and right replicates of each body location from an individual was associated with highly similar microbial profiles in the majority of cases (Figure 5). When comparing Bray-Curtis distances, a sample had the lowest distance to another sample from the same participant 57.9% of the time. Within-individual closest matches ("individuality") were highest for the thigh (89.7%) and eyelid (77.5%) sites (Figure 6A), and were statistically significant (PERMANOVA; $F_{19,310} = 3.65$, $p < 0.001$) when all samples were analyzed. Furthermore, when focusing on individual body locations, with a minimum of two samples per participant, the F ratio increased (PERMANOVA; $F_{19,20} = 3.12\text{-}13.69$, $p < 0.001$) and was the metadata category that explained the most variation for each body location, except for the outer

nose (Figure 7). The weakest and strongest individuality was exhibited by the outer nose and thigh, respectively. The thigh may have exhibited high individuality because it is a skin region that is not frequently in contact with the external environment, compared to other regions, such as the hands. Furthermore, the left and right thighs are in more frequent contact with each other than other body regions, such as the left and right outer nose. Areas of the face with stronger individuality, such as the eyelids, may experience fewer perturbations from hand contact than the nose, especially with participants who wore glasses. Participants who did not wear glasses had modestly more community similarity to their hands than those with glasses, according to Bray-Curtis distances (0.64 vs 0.59, $p = 0.12$), however this was not statistically significant given the small sample size. The outer nose may also express the lowest individuality due to the low level of diversity observed, which was lower than any other body region. This low diversity may impact the ability to discern an individual due to fewer OTUs that are unique. Furthermore, a previous study of the face microbiome determined that microbial communities of this body region are strongly influenced by measured sebum and moisture levels in specific locations (179). The most significant predictors of microbial community composition were cheek sebum and forehead moisture. Increased sebum levels resulted in corresponding increases in *Propionibacterium* and decreases in overall diversity. These variations in sebum and moisture could result in similar communities between different individuals with similar secretion levels, which were not measured in this current study.
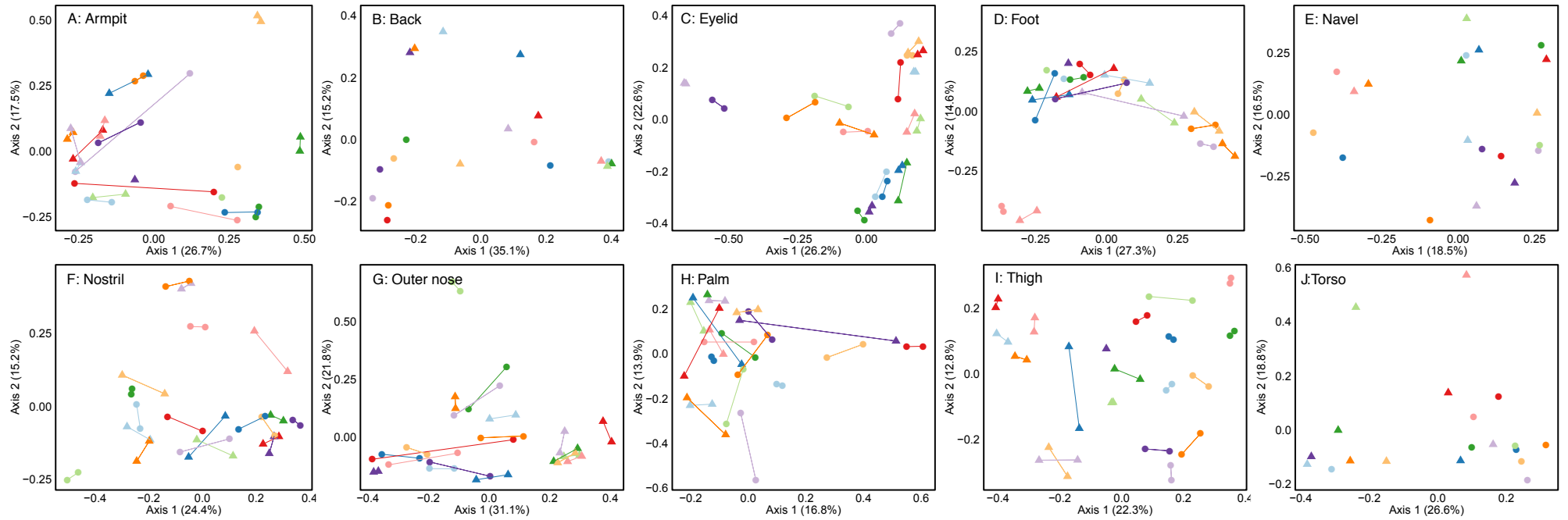
Figure 5: Ordinations (PCoA) generated by using the Bray-Curtis dissimilarity metric for each of the 10 body locations sampled. Lines connect samples from a participant. Female samples are denoted by circular points, whereas male partners are represented by triangles. Where a single sample per person was collected for specific body locations (i.e., back, navel, torso) no lines connect the participant samples. Samples are colored according to partner.
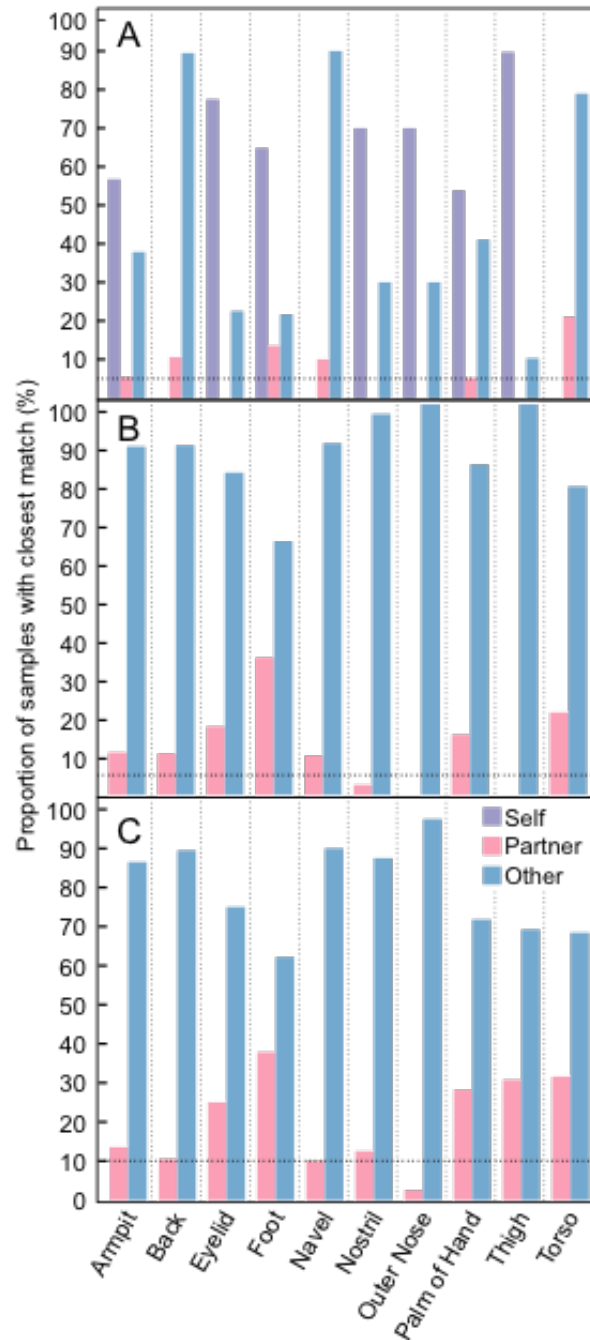
Figure 6: Samples were matched with another sample in the dataset that possessed the most similar microbial community. Matches were analyzed to determine the percent of samples belonging to self, partner, or another participant. A) Proportion of samples that had the lowest Bray Curtis distance with either another sample from within an individual, from within a cohabiting couple, or to any of the other participants. B) Proportion of samples that had the lowest Bray Curtis distance with non-self samples. C) Proportion of samples that had the lowest Bray Curtis distance with non-self, opposite-sex samples. The dotted line represents the threshold that would be expected by random chance from the 20 participants.
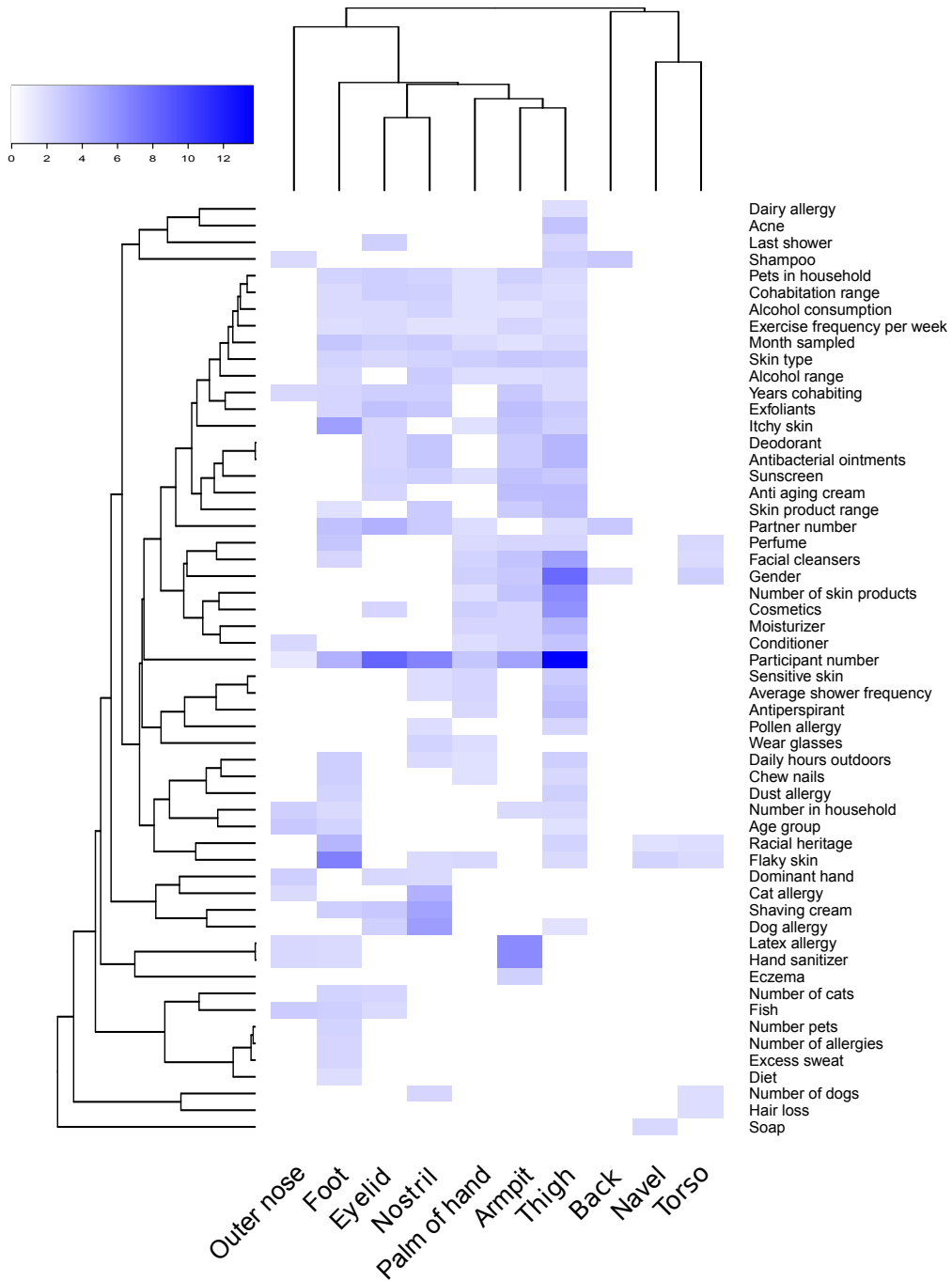
Figure 7: Heatmap summarizing the significant metadata factors that were collected from a participant survey. Categories with higher PERMANOVA F statistics have higher variation in community dissimilarity within 10 body locations. White regions of the heatmap represent nonsignificant results. Body locations and metadata categories were arranged into dendrograms using the Bray-Curtis dissimilarity metric.

2.3.2 Biological sex can be determined from thigh skin microbiome samples

Female skin microbial communities were significantly more diverse, according to the Shannon index (4.59 versus 3.98, $p < 0.001$), which is consistent with a previous study (45), and may be due to physiological differences, for instance lower pH (76). Using samples from all body locations, biological sex could be determined 80.0% of the time, which is only 2.5x greater than expected by chance. However, when biological sex was classified for each body location, the thigh could be correctly classified 100% of the time. One can therefore consistently determine biological sex based on the thigh region, but cannot classify sex with the same certainty using any other skin region. This finding was corroborated by ordinations based on Bray Curtis distances (Figure 8, Figure 9), was statistically significant (PERMANOVA; $F_{1,328} =$ 8.0, $p < 0.001$), and was further supported by using indicator species analysis because the thigh region had the most indicator OTUs.
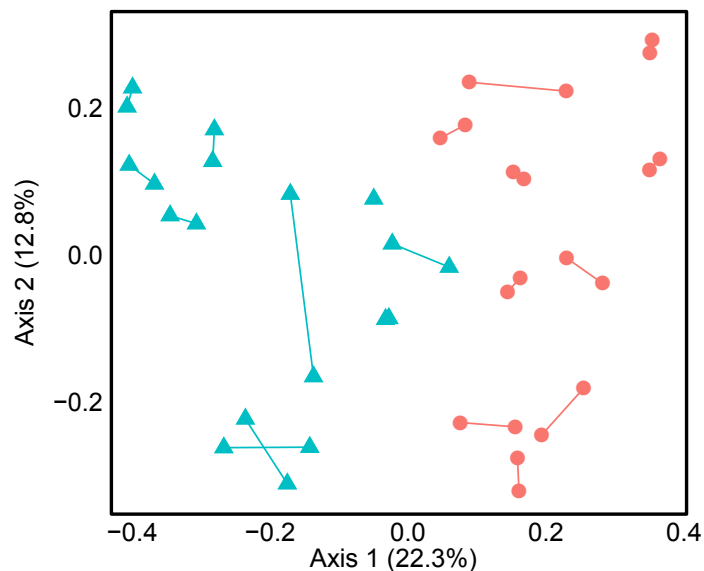
Figure 8: PCoA ordination of thigh samples calculated using the Bray-Curtis dissimilarity metric. The left and right samples from an individual are connected. Blue triangles represent male samples, whereas females are represented by coral circles.
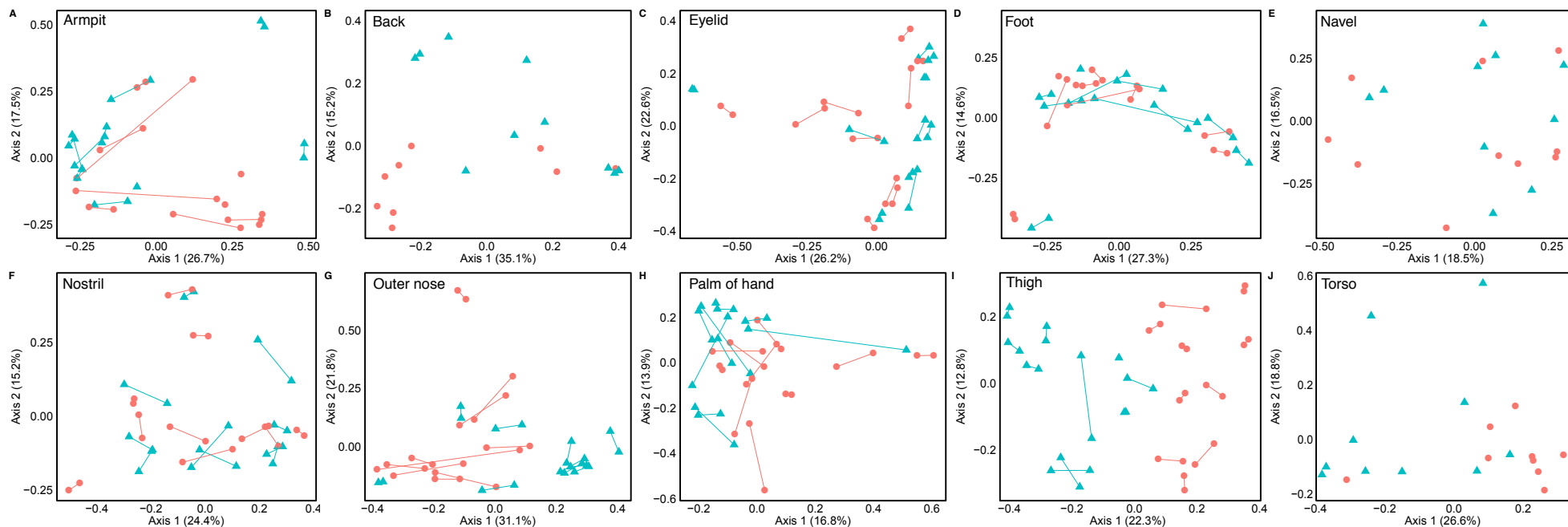
Figure 9: PCoA ordinations calculated using the Bray-Curtis dissimilarity metric for each of the 10 body locations sampled. The left and right samples from an individual are connected. A single sample per person was collected on body locations (back, navel, torso) where no lines connect the samples. Blue triangles represent male samples, whereas females are represented by coral circles.

Male participants had a significantly larger proportion of sequences affiliated with the

Actinobacteria phylum (50.8±25.5% versus 38.2±20.9, *p* < 0.001), whereas female participants

had significantly more *Proteobacteria* (18.1±15.9 versus 11.7±17.3%, *p*=0.001) detected on their

skin (Figure 10). Several OTUs were more abundant on either male or female skin (Table 1). The

majority of these bacteria are human skin commensals, whereas *Alloiococcus* in males is

associated with the ear canal and associated infections (180, 181). In contrast, women had 11.1-

fold more *Lactobacillus*, which is an organism that dominates the vaginal microbiome (182).

There were no core OTUs present in one sex that were not core in the other.

Table 2: Microorganisms disproportionately abundant on each biological sex. Shown are OTUs
that were present on a minimum of 30% of samples of that sex.

More abundant on men		More abundant on women	
OTU taxonomic affiliation	Increase in abundance (%)	OTU taxonomic affiliation	Increase in abundance (%)
Alloiococcus	344.0	*Lactobacillus*	1111.4
Dermabacter	271.2	*Lactobacillus iners*	440.0
Brevibacterium	164.5	*Lautropia*	408.8
Moraxella	149.9	*Streptococcus luteciae*	332.7
Faecalibacterium prausnitzii	128.7	*Caulobacteraceae*	273.3
Abiotrophia	112.4	*Planococcaceae*	273.3
Neisseria	109.1	*Sphingomonas*	191.3
Escherichia coli	73.1	*Cloacibacterium*	180.9
Veillonella dispar	73.0	*Paracoccus*	174.4
Corynebacterium	70.5	*Streptococcus*	169.8
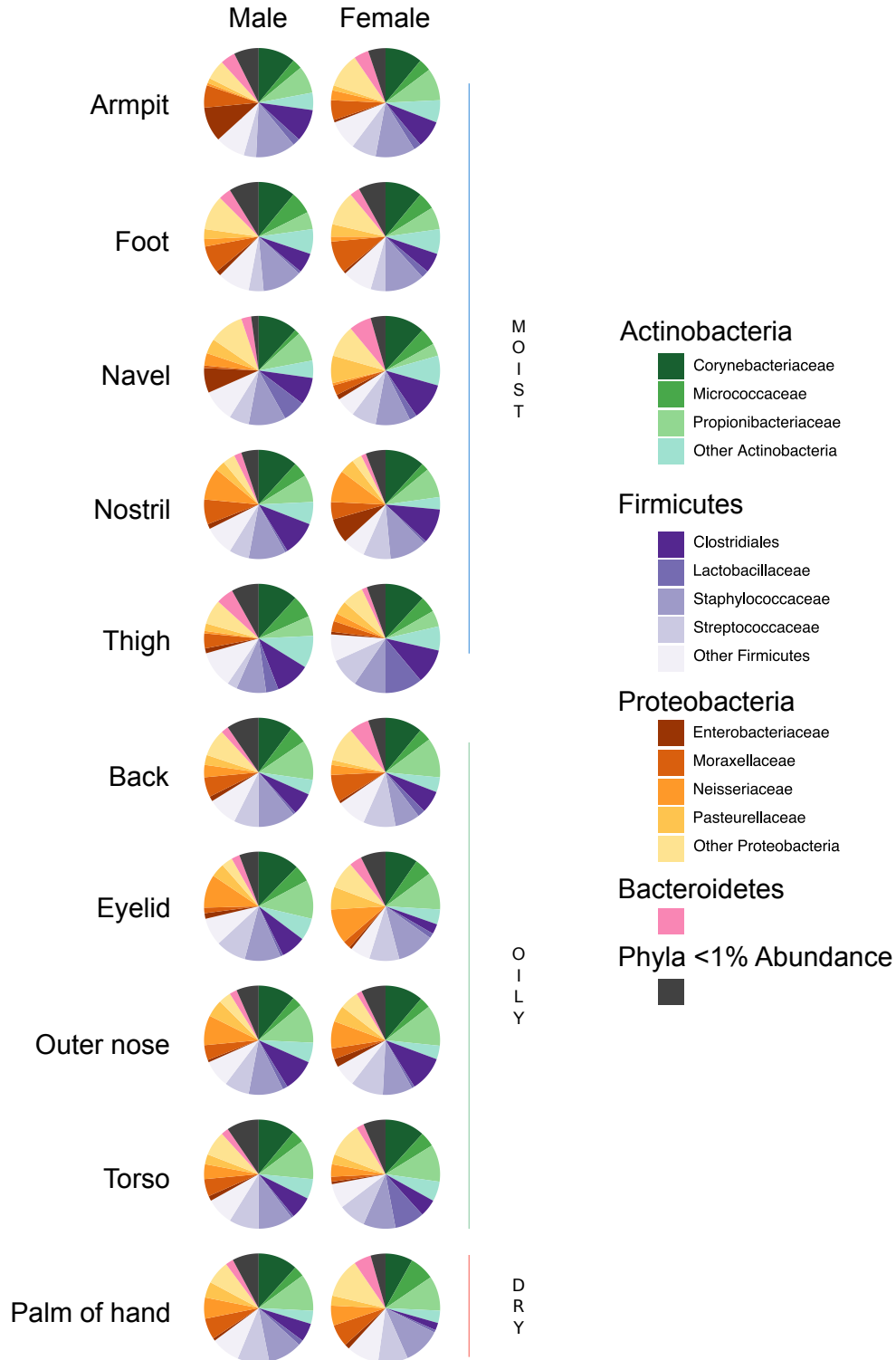
Figure 10: Pie charts illustrating the relative abundance of microbial families present >1% and the phyla to which they belong, organized by each of the 10 body locations sampled and by biological sex.

2.3.3 Co-habiting partners can be predicted based on skin microbiome profiles

By analyzing all samples together, random forest modelling identified that couples' samples could be matched correctly 86±6.1% of the time, which is 6.5-fold greater than chance and always had a lower error rate than the 1000 randomized groupings of samples from couple participants ($p < 0.001$; Figure 11A). Couple H05 had distinct microbial communities with more indicator organisms than the other couples. When the 34 samples from couple H05 were removed from the random forest modelling, there was no significant difference in the model's ability to classify the remaining nine couples ($p = 0.68$). This indicates that outliers are not significantly influencing the analysis. When the same 1000 randomized groupings of samples from all couple participants were analyzed using PERMANOVA, the incorrect partner F statistics were significantly lower than the correct couple pairs ($p = 0.006$; Figure 11B). When the PERMANOVA analysis was divided by body location, partners possessed the most similar microbial communities on their feet, eyelids, and back compared to incorrect couple pairings (Figure 12).
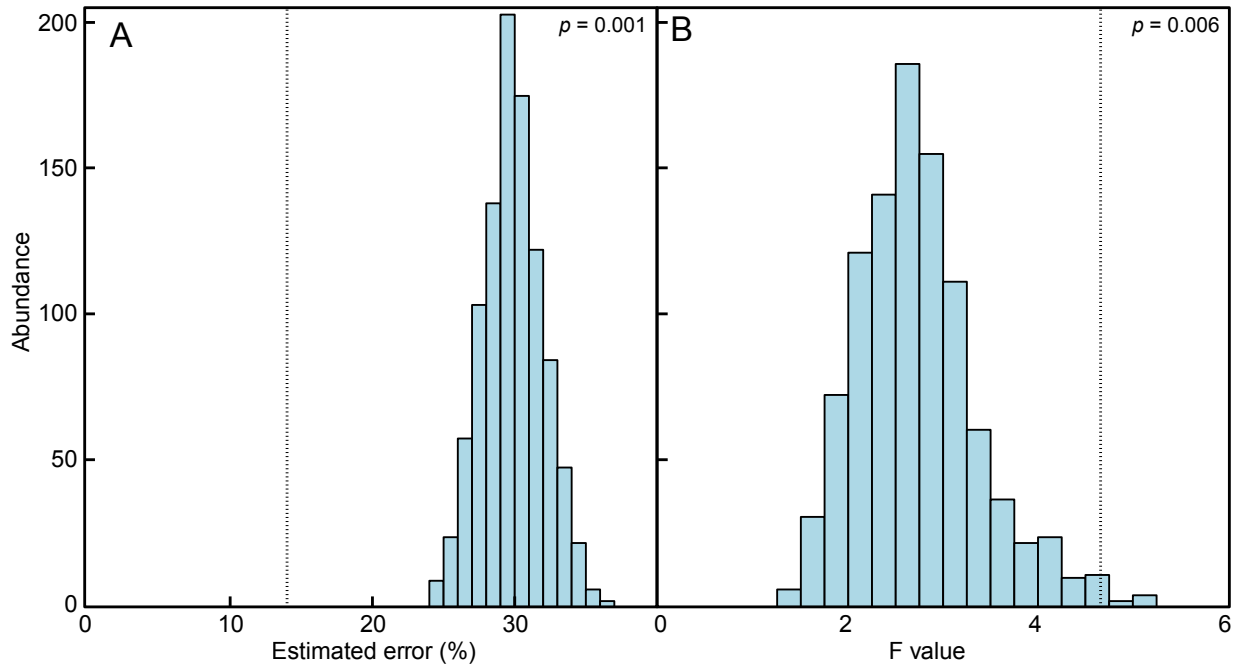
Figure 11: Barcharts of the dataset with the correct couple composition compared to randomly assorted incorrect pairings. Distribution of the A) estimated supervised learning error rates and B) PERMANOVA F statistics of 1000 unique artificially shuffled partner pairings. The dotted line represents the position of the result from the correctly matched couples' dataset ($p < 0.001$ and $p = 0.006$).
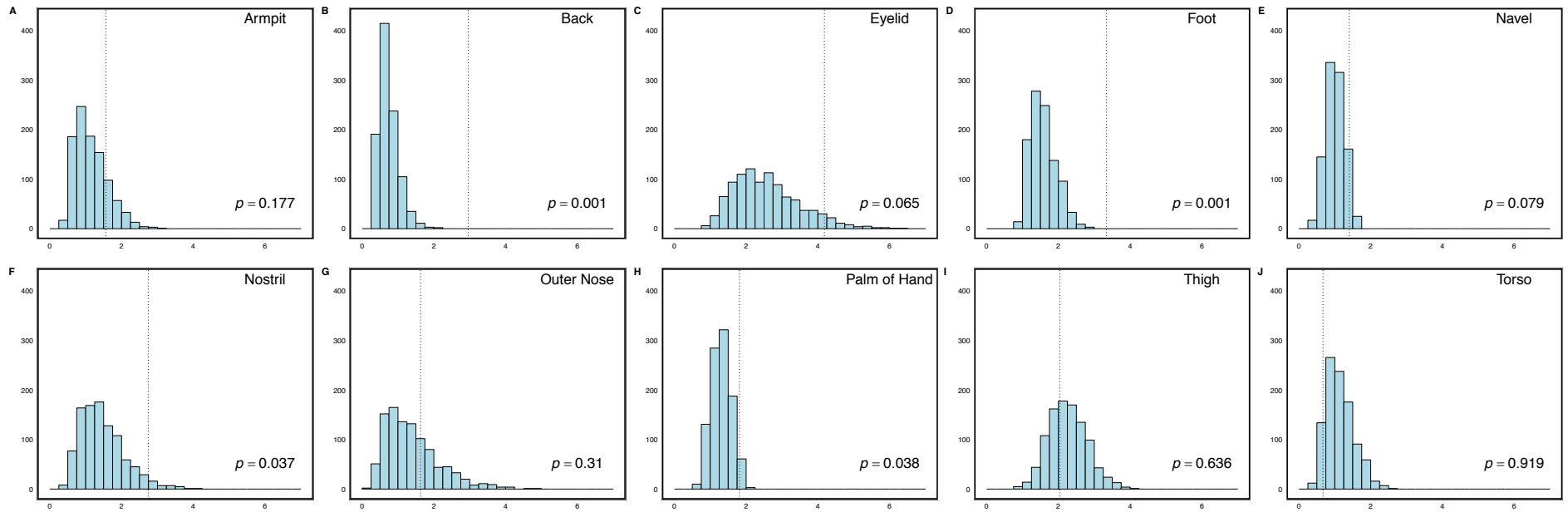
Figure 12: Barplots of the distribution of the PERMANOVA F statistics of 1000 unique artificially shuffled partner pairings for each body location (A-J). The dotted line represents the position of the result from the correctly matched couples' dataset.

To further assess the similarity between couples' samples, a Bray-Curtis distance matrix was created to determine the number of times the sample with the lowest distance, and therefore the most similar bacterial community, belonged to the same individual, their partner, or another participant (Figure 6). When only the closest non-self samples were tested, several body locations had microbial communities that were closest to their partner. For example, 35% of couples' feet samples were closest to those of their partner, which is ~7-fold higher than expected by chance (Figure 6B). The torso (21%), navel (20%), and eyelid (17.5%) were also closer to their partner more frequently than expected by chance.

The closest non-self, opposite sex matches were also analyzed to correct for the influence of biological sex. Although there was little difference in the majority of body locations, the thigh and torso samples matched partners correctly more frequently when only considering opposite sex individuals (Figure 6C). Although the closest non-self samples matched to their partner more often than expected by chance, the majority of the samples matched to one of the other 18 participants in the study. It is postulated that this may be partially attributed to shared factors between these participants, such as biological sex. The majority of non-self samples that did not match to a partner matched with a participant of the same sex (62.4%, $p = 0.005$). Partners can therefore be correctly paired with random forest modelling better than using the closest matching sample method in part because random forest modelling is restricted to different sex pairs.

Investigating associations of samples with other samples from self, partners, and others was also performed at a 99% sequence identity threshold to determine if a dataset with higher species resolution was better able to match self or partners. The 99% identity dataset showed a significant improvement in linking samples by closest match to sampled individuals using Bray-Curtis distances (78% vs 69%, $p = 0.003$), but only modestly increased average partner

identification, albeit insignificantly (23.1% vs. 20.3%, $p = 0.09$). This was corroborated by random forest modelling. The models were worse at classifying partners (80% +/- 5% versus the original 86% +/- 6%; Figure 13), which was partially due to an increased inability to classify couple 10, which had been living together for the least amount of time compared to the other participants. However, although random forest modelling was worse at classifying the true partners, it was also worse at classifying the incorrectly paired partners (Figure 13), indicating a difference in overall classification abilities.
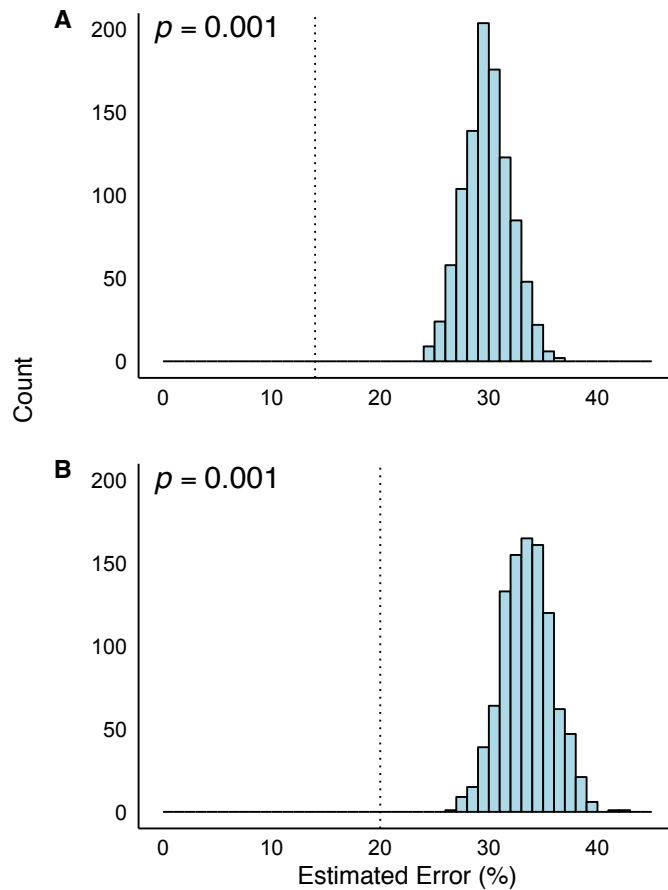
Figure 13: Barplots of the dataset with the correct couple composition compared to randomly assorted incorrect pairings. Distribution of the A) estimated supervised learning error rates at 97% sequence identity and B) 99% sequence identity of 1000 unique artificially shuffled partner pairings. The dotted line represents the position of the result from the correctly matched couples' dataset ($p < 0.001$ for both analyses).

An indicator species analysis was also performed to link couples based on highly abundant organisms that were unique to them. Only three of the 10 couples had at least one indicator organism. One of these couples had seven indicator species. This couple had several unique factors that were not observed in any of the other participants. This couple reported more time outdoors, a higher exercise frequency, flaky skin, and were a different racial heritage than the other participants. Future skin microbiome studies would need to be conducted to determine the extent of the effects of race, exercise frequency, and time spent outdoors. The couple did not have a clinical diagnosis of any skin ailments that may cause flaky skin, for example psoriasis. Specific body locations had a higher number of indicators per couple. Additionally, analyzing indicators by each individual body location resulted in a higher number of indicators for each couple.

Microbiota transmission between partners is likely a combination of the built environment and direct contact. Humans shed over one million biological aerosols per hour (104), changing the composition of the surfaces they touch and the rooms they occupy (105, 183). Indeed, microbiome individuality enabled hand microbial communities to be linked to computer keyboards based on physical contact (70). Forensic studies have shown that humans can be correctly matched, based on microbiome profiles, to the fabrics they grasp (107) and personal objects, including cell phones and shoes (106). It is therefore plausible that partner shedding and direct contact affects the other inhabitants of an individual's primary residence. Sharing of microbiota was most apparent by sampling feet, which can be explained by the feet being in direct contact with home surfaces. Indeed, homes have been shown to have microbiota that are signatures of their inhabitants (71, 150). Daily direct contact between cohabiting individuals would further increase microbiota sharing.

Future skin microbiome studies are encouraged to include same sex couples to answer intriguing questions about how intimately living with a member of the same sex affects the microbiome. For example, skin regions that are heavily influenced by biological sex, such as the thigh (Fig. S2) or hands (45), may be more effective at classifying same sex couples because biological sex would no longer be a confounding factor. I predict that homosexual couples would overall be matched more successfully than heterosexual couples because they would share more similar skin microbiota both from sharing a location and biological sex.

2.3.4 Hygiene, pets, and allergies correlate with the microbiota of body sites

Lifestyle choices for each participant, such as time spent outdoors, pet ownership, and alcohol consumption were analyzed to determine if these factors exhibited any significant effect on skin microbiota. Although the sample size was small (17 body locations; 20 participants), several factors were nonetheless significant after using the Bonferroni correction. The following lifestyle choices may warrant future experimentation with a larger sample size to further elucidate their impact on skin communities.

Participants who consumed multiple servings of alcohol per day had significantly less diverse communities, based on the Shannon index, than those who consumed one serving per month. This result was corroborated by PERMANOVA data, which identified alcohol consumption as a metadata category contributing the highest explained variation (Figure 7). An indicator of the highest consumption rate of alcohol was *Brevibacterium* (indicator value = 0.70; mean = 129, versus a mean of 2 in the lower consumption groups). This organism possesses an alcohol dehydrogenase (184) and, given that ethanol is secreted through the sweat glands (185), it is possible that higher rates of alcohol consumption could feasibly impact members of the skin microbiota. However, it should be noted that many organisms possess this enzyme and additional

metagenomic studies are needed to properly determine the effect alcohol has on skin microbiota. Although one study showed that psoriasis was more prevalent in those who consumed more alcohol (186), further research is needed to identify and characterize links between increased alcohol consumption, human skin health, and skin microbiota.

Spending more time outdoors and owning pets were associated with higher levels of microbial skin community diversity. Based on Shannon indices, participants who spent over four hours per day outside had more diverse communities than people who were outside for less than an hour per day. Pet ownership also had a significant increase on the diversity levels of thighs and nostril samples, which is in accordance with previous research that demonstrated that homes with dogs had higher levels of diversity (150, 187). These studies have stated that diversity is increased both by dog associated taxa and their need to be outdoors daily. The nostrils are postulated to have experienced a shift in microbiota due to inhaling biological particles shed from the animals.

A high number of skin products was correlated with higher diversity, but this may be linked to other biological sex-associated factors. In particular, facial regions, such as the eyelids had higher diversity levels on participants who applied facial cleansers, moisturizers, or cosmetics. Females had higher rates of skin product use. Body regions whose microbiota varied by biological sex, for example the thighs due to the influence from the vaginal microbiome, therefore also appeared to be strongly correlated with sex specific hygiene products, such as facial cleansers, although these products would not have an effect on the microbial community of body regions they do not directly contact. A study that mapped the 3D molecular cartography of human skin noted that skin composition was influenced by hygiene product application (17). An additional explanation for higher skin microbial diversity is that skin products alter moisture

62

levels by increasing skin capacitance and water barrier function (188). Indeed, moist skin regions are typically more diverse than oily regions (20). Our study further indicates that this lasting impact on the skin environment may increase the diversity level of facial skin.

The inner nostril was included as a skin region because it contains sebaceous and sweat glands, as well as a keratinized epidermal layer that is more similar to skin than the nasal cavity (189). Participants with higher numbers of allergies had significantly more diverse nostril and outer nose communities. Those with dairy, wheat, and strawberry allergies exhibited higher diversity, whereas those with dust allergies had lower diversity. Asthma sufferers have been reported to have higher microbial diversity of the lung microbiome (2, 190, 191). It is therefore possible that increased diversity at the entrance to the respiratory tract may influence other ailments, such as allergies. Allergies did not appear to have an effect on any other body locations.

There were several additional observations that should be interpreted with caution. Participant H04B did not wear deodorant, yet had significantly more diverse armpit communities (6.2±0.1 versus 3.3±1.2). Couple H04 had an elevated level of gastrointestinal related organisms, including *E. coli*, *Enterobacteriaceae*, and *Shigella* on their armpits, nostril, and outer nose. This couple had an infant in diapers and also reported experiencing a gastrointestinal illness a few weeks prior to sampling. The elevated gut-associated microorganisms on the skin may be due to either frequent diaper changes and/or persisting microorganisms (or their DNA) from previous illness. Future studies might compare the skin of multiple couples with infants to couples with older children to explore the effect on skin from habiting with children.

2.3.5 Limitations

A limitation of the study was a limited sample size of twenty individuals. The goal of the current study was to sample from a wide range of body locations in order to identify the regions that are most useful for linking couples via their skin microbial profiles. This was conducted instead of sampling a low number of sites from many individuals to avoid missing key trends in the microbiota across the body. For example, if I had only sampled the thighs of many couples, I would not have been able to elucidate any cohabitation effect on the skin microbiome. Now that trends from each body location have been observed, future studies can focus on sampling a broader range of participants while reducing the number of samples required per participant, especially because individuality was pronounced. Moreover, only skin samples were included in this study. Including more distinct microbiome regions such as gut, respiratory, oral, and groin/vaginal regions may improve the ability to classify partners based on shared microbial profiles.

Additionally, this study sequenced the V3-V4 region of the 16S rRNA gene using a two-step PCR protocol. Two rounds of amplification did not result in a difference in community composition according to a preliminary subset of four samples that were sequenced using both protocols with one and two rounds of amplification (Figure 14). Research in this area has been progressing rapidly, and has resulted in numerous research articles and reviews discussing the inherent biases in each primer region (155–158). It is possible that relative abundances of certain organisms, such as *Staphylococcus* and *Propionibacterium*, would be altered if a different region had been used for this study, for example the V1-V3 region (158). Indeed, mismatches of only a few basepairs can result in poor amplification of taxa that are abundant on skin, as was the case with Propionibacterium in a previous study (192). It has therefore been suggested by Zeeuwen and colleagues (2016) that a skin microbiome specific primer set should be designed that is

64

optimized for bacteria present on human skin, which does not necessarily need to exclude the V4

primer region (193). Further validation would include sequencing a defined mock community

with staggered concentrations to monitor any potential biases that may occur within the selected

primer region or two-step amplification protocol.
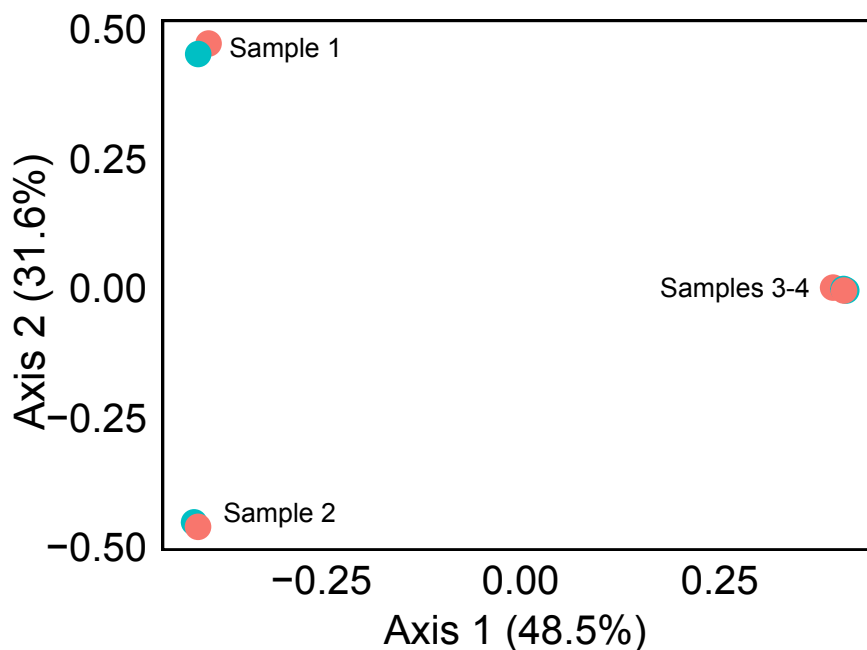
Figure 14: PCoA ordination calculated using the Bray-Curtis dissimilarity metric for four preliminary samples that underwent both one-step and two-step PCR protocols. Coral and turquoise datapoints denote replicates of the sample that underwent one and two rounds of PCR.

A potential limitation of using machine learning tools is overlearning, which occurs when

the model learns background noise and random error. This imbalanced classification problem

can occur when a complex model has too many parameters for the number of observations.

Overlearning typically affects datasets where one dominant group has many more samples than

the others (194). In this study, each couple had an identical number of samples, except for minor

variations from the 3% of samples that were removed due to no amplification. Therefore, it is not

expected that overlearning significantly affected this study due to the sampling design.

This study sampled from very limited ethnic diversity; nearly all participants were of Caucasian heritage. The cohabiting couple of non-Caucasian descent had more indicators than the other couples. It is unknown the degree to which racial heritage attributed to this change in microbial diversity, compared to other factors, namely reported skin conditions, time spent outdoors, and activity levels. Additional studies should include a higher diversity of participants to determine the effects that racial heritage and related social changes may have on the skin microbiome. Indeed, other microbiome studies have concluded that race was a significant factor governing the microbiome of the vagina (182, 195) and gut (47).

2.4 Conclusion

This study was the first to analyze the distribution of microorganisms on cohabiting couples by sampling a wide range of skin regions. Although body location and individuality were the most substantial influences on the skin microbiome of sampled cohabiting couples, machine learning approaches were able to classify samples from cohabiting couples in >86% of test cases. Couples were most similar based on feet microbiota, likely reflecting the collection and distribution of dust from floors to all occupants of a home. In contrast, the inner thigh region was the best indicator of individuality and biological sex. Possessing pets, consuming lower amounts of alcohol, and increases in exercise were all associated with higher levels of microbial diversity.

2.5 Data Availability

The sequence data associated with this chapter are available in the Sequence Read Archive (SRA) under BioProject ID PRJNA345497.

Chapter 3
The Mammalian Skin Microbiome

3.1 Introduction

Skin constitutes the primary physical barrier between mammals and their external environment. Characterization of the microbiota on skin is essential for diagnosing skin conditions (10), understanding how an animal coevolves with its microbiota (8), and studying the interactions between the microbiome and the host immune system (9). Skin microorganisms also produce compounds that influence animal behavior, such as intra-specific behavior modifying pheromones (196) and volatile organic compounds resulting in body odour (110, 197, 198). Cultivated human skin microbiota have been linked to the rates at which hosts are bitten by mosquitos (199, 200), with implications for disease transmission.

High-throughput sequencing has provided crucial information about the factors that influence the skin microbiota and how these microbial communities impact health and skin conditions. Humans are uniquely colonized by skin microbial communities that vary between body regions (17, 18, 20), individuals (47), age (15, 16), and diet (94). Skin conditions, for example atopic dermatitis, can occur when the resident skin microbial community undergoes dysbiosis (77, 98). The composition of human skin microbial communities can also be linked to host hygiene. Previous studies have shown that skin microbial communities are affected by deodorants, soaps, and cosmetics (148, 149, 201). Indeed, three dimensional maps of human skin have shown that the residues from skin products are detectable and can influence the skin microbiome (17). A microbiome study of a tribe of Amerindians concluded that these individuals had the highest microbial diversity of any sampled humans to date. This study demonstrated that

the microbiome of humans can vary significantly based on lifestyle choices. These individuals spent longer periods of time outdoors and had no known previous contact with modern humans, antibiotic exposure, or commercial skin care products (103). Despite having no known exposure to antibiotics, bacteria obtained from these individuals had antibiotic resistance genes.

Although many studies have characterized the human microbiome, far less is known about the skin microbiome of non-human mammals, especially from studies that employed high-throughput sequencing techniques. The skin of companion animals, such as cats (48, 69, 202) and dogs (51) have been sequenced. These studies demonstrated that cats with allergies have higher levels of the fungi *Agaricomycetes* and *Sordariomycetes* (69) and bacterial communities that are unique to individual felines (202), whereas allergic dogs exhibited lower bacterial species richness. Moreover, bovine skin afflicted with bovine digital dermatitis possesses a distinct microbiome from healthy skin (123). A baseline dataset of what constitutes a healthy skin microbiome for a variety of species is crucial for determining the cause of skin ailments.

Multiple studies have been conducted on both wild and captive animals to elucidate the role host species, geographic location, body region, and captivity status exhibit on the skin microbiome. Analyzing the skin microbiota of 63 individuals from five primate species revealed that human axillae were associated with distinct microbial communities, with lower overall diversity (53). The authors suggested that differences were due both to shifts in human hygiene routines as well as host-microbe evolution. Skin biopsies and sloughed skin from 56 free-swimming humpback whales (*Megaptera novaeangliae*) from the North Pacific, South Pacific, and North Atlantic oceans demonstrated that core genera were present despite large geographical distances (52). However, skin microbial communities also exhibited shifts between geography locations and whale satiation states throughout their migration. A large study of bats determined

that the host species, geographic region, and site were significant factors influencing skin communities (81). Microbial diversity of skin and pouch samples from Tasmanian devils demonstrated a strong influence of geographic location and revealed significant differences between wild and captive populations (101). Together, these previous studies indicate that both phylogeny and habitat can impact skin microbial communities. However, studies that focus on a wide range of species and body location are important for better elucidating factors that influence skin microbial communities and generating a more clear understanding of microbiome-host coevolution, especially considering that species sampled to date represent only a small proportion of known mammals.

The objective of this research was to create a skin microbiome baseline for the class Mammalia in order to identify correlations of skin microbiota with species, geographical location, hygiene, and body region. A total of 589 back, torso, and inner thigh skin samples from 38 mammalian species were analyzed by high-throughput sequencing of the 16S rRNA gene. The results provide evidence for the uniqueness of human skin microbiota, the importance of host and geographic location in shaping skin microbial communities, and phylosymbiosis of hosts and their corresponding skin microbiota. To our knowledge, this represents the largest mammalian skin microbiome study to date, exclusive of human-only studies. Animals were sourced from a variety of locations, such as households, farms, zoos, and the wild, which represents a spectrum of environments and hygiene regiments. A baseline understanding of the mammalian skin microbiome is crucial for making informed decisions related to veterinarian research and conservation strategies, and provides implications for mammalian evolutionary history.

3.2 Materials and Methods

3.2.1 Ethics

The study has been approved by the Office of Research Ethics at the University of Waterloo (A-15-06). The following non-invasive procedures were conducted in accordance with the approved documentation and no animals were harmed throughout this study.

3.2.2 Sample collection

Species from 10 orders of the class Mammalia were sampled to characterize the distribution of microorganisms on skin (Additional File 1). Both males and females were included for each species, when available, to account for variations in hormone levels and secretions that are known to affect microbial communities (45). Only healthy and sexually mature animals that were not exposed to antibiotics in the previous six months were included when possible. Due to the opportunistic nature of sample collection, 25 animals were sampled that had been exposed to antibiotics within this timeframe: one alpaca, two cats, two indian rhinoceri, three dogs, four olive baboons, six bovine, and seven horses. Of these 25 animals, only seven had received antibiotics in the previous two months. Furthermore, there was no significant difference in the diversity levels between animals who were exposed to antibiotics and those who were not across all samples and within each affected species. These findings have led us to conclude that the microbial communities of the 25 animals exposed to antibiotics in the previous six months were not significantly influenced by the antibiotic exposure, and were therefore included in subsequent analyses. A spectrum of habitats and hygiene practices were also included, ranging from frequent grooming of pets and farm animals, to animals in captivity and

70

the wild. Complete information on the biological sex, age, diet, location, health history, grooming, and exposure to antibiotics were collected (Additional File 1).

Additional File 1: Metadata table containing all survey responses, PCR setup information, and sample codes. Animal samples names are coded as follows: A = Animal, # = the animal subject number, B = Back, I = Inner thigh, T = Torso. AL= African Lion, AP=Alpaca, Aoudad Sheep=AS, Arctic Wolf = AW, Asian Elephant = AE, Bactrian Camel = BC, Beaver = BE, Bovine = BV, Cape Eland = CE, Cat = C, Cheetah = CH, Dog = D, Donkey = DK, Giant Panda = GP, Goat = G, Groundhog = GH, Horse = H, Indian Flying Fox = IFF, Indian Rhinoceros = IR, Olive Baboon = OB, Pony = P, Przewalski's Horse = PH, Rabbit = RB, Red Kangaroo = RK, Reindeer = R, River Otter = RO, Rothschild Giraffe = RG, Sable Antelope = SA, Sheep = S, Spotted Hyena = SH, Squirrel = SQ, Straw Coloured Fruit Bat = FB, Sumatran Orangutan = SO, Swamp Wallaby = SW, Two-Toed Sloth = TS, White Lion = WL, White Rhinoceros = WR. Human sample code names are coded as follows: H01-H10 signify each couple, whereas A-B differentiates individuals within a couple, 09 = torso, 10 = back, 12 = left inner thigh, 13 = right inner thigh.

Animals were sampled from multiple locations in Southern Ontario from November 2015 to September 2016: The African Lion Safari, Conestogo River Horseback Adventures, Donkey Sanctuary of Canada, Kitchener-Waterloo Humane Society (KWHS), Toronto Zoo, pet owners, and from local farms sourced from the University of Guelph. Animals from the Toronto Zoo and African Lion Safari were sampled when they were brought in for regular husbandry practices. Additional companion animals were obtained from volunteers who were recruited by word of mouth. The KWHS supplied wild animals that were collected by KWHS staff within 24 hours of death; the specimens were stored in plastic bags in a -20ºC freezer until sampled.

The back, torso, and inner thigh regions of 177 non-human mammals were collected using sterile foam swabs (Puritan). In addition, data was included from 77 equivalent samples from 20 human participants from a previous study (203) in the analysis for comparison purposes, for a total of 589 samples. These regions were chosen to capture both moist and oily regions and avoid sensitive areas that may cause distress. Skin was swabbed by moving aside hair or fur with

gloved hands to expose the skin. While applying moderate pressure, the skin was swabbed 10 times in a forwards and backwards motion. The swab was rotated and repeated in adjacent areas for a total of 40 strokes per swab. When the area was complete, the swabs were returned to their initial plastic storage container and frozen at -20 ºC until further use. All volunteers and veterinary technicians were trained with a detailed protocol to ensure sample collection consistency.

3.2.3 Sample preparation

All DNA extractions, PCR protocols, and Illumina sequencing were conducted according to a previously published protocol (203) to enable comparisons between the human and non-human samples (Figure 2). In brief, DNA was extracted using the PowerSoil-htp 96 Well DNA Isolation Kit (MO BIO Laboratories) and stored at -20°C until further use. The V3-V4 region of the 16S rRNA gene was amplified using the Pro 341Fi (5'-CCTACGGGNBGCASCAG-3') and Pro 805Ri (5'-GACTACNVGGGTATCTAATCC-3') primers (153). Each amplification was performed in triplicate to minimize potential PCR bias from low biomass samples (160), and was conducted in a PCR hood that was UV treated for 30 min after having undergone a treatment with UltraClean Lab Cleaner (MoBio) to remove DNA, RNA, DNase, and RNAses (204).

In addition to control extractions with sterile swabs, no-template negative controls were included for each DNA extraction plate and PCR plate. All samples were sequenced on three different MiSeq (Illumina) lanes using the MiSeq Reagent v2 kit-500 cycles (Illumina) according to the manufacturer's protocol. The first lane's library was diluted to 8 pM with a 10% PhiX control and included human samples from a previously published study (203) in addition to 37 mammalian samples. The second library was diluted to 4.5 pM with a 10% PhiX control, and contained 266 unique mammalian samples and corresponding controls. The third library was

diluted to 6 pM, with a 30% PhiX control, and contained 209 unique mammalian samples and corresponding controls. Varying library and loading concentrations were used in an effort to optimize cluster density. Six "run control" samples consisting of human, zoo, pet, and wild animal samples were included in each of the three runs, confirming the absence of detectable run bias (Figure 15). The low diversity observed in human samples (Figure 19) was not due to variations in Illumina run sequencing because the 37 non-human animal samples included in the first lane possessed the same diversity levels as samples from the same species that were sequenced in other lanes.
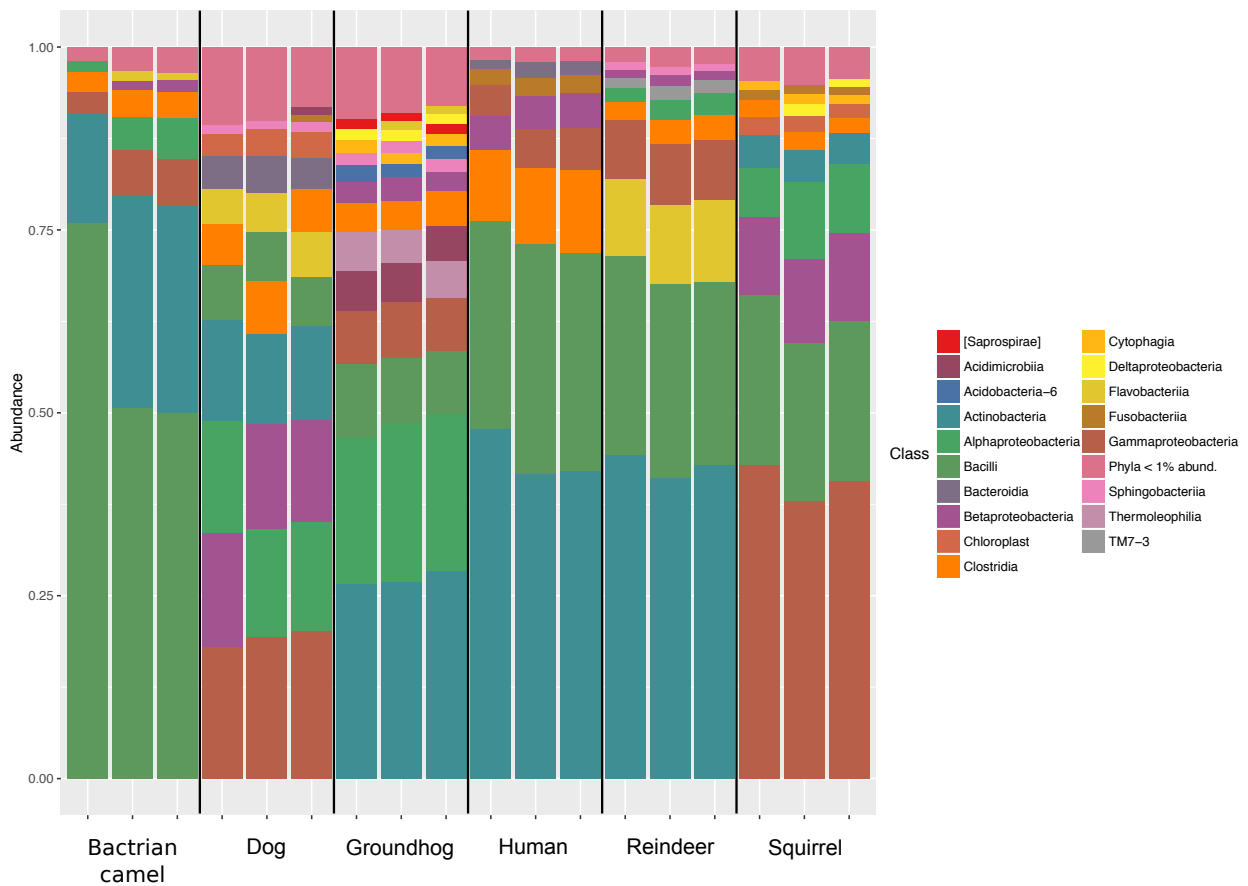
Figure 15: Taxaplot of six "run control" samples included in each of the three MiSeq runs. OTUs present >1% relative abundance were visualized. Genus names and OTU ID numbers are overlaid over bars.

3.2.4 Processing of sequence data

Raw DNA sequence reads were processed using the same open source bioinformatics pipeline described previously (203) that was managed by Automation, eXtension, and Integration Of Microbial Ecology: v. 1.5 (161). PANDAseq v. 2.8 (162) generated paired-end sequences using the default parameters of a 0.9 quality threshold, a minimum sequence overlap of 10 bases, and a minimum read length of 300 bases. Quantitative Insights Into Microbial Ecology v. 1.9.0 (QIIME) (163) was used to analyze sequence data, which underwent *de novo* clustering and chimera/singleton removal at both 97% and 99% cluster identity using UPARSE (164). PyNAST v. 1.2.2 (165) was used to align 16S rRNA gene sequences. Subsequently, RDP v. 8.1 (205) assigned prokaryotic taxonomy using Greengenes database v. 13.8 (166). Samples were rarefied to 1654 sequences in the dataset that contained all samples. Analyses such as PERMANOVA underwent 1000 iterations of rarefication to avoid underrepresenting diversity.

3.2.5 Negative control analysis

The no-template, DNA extraction kit, and sterile swab controls were analyzed for contaminants after sequence processing. A total of 3 of 4 kits controls, 4 out of 5 sterile swabs, and 67 out of 69 no template PCR controls contained fewer reads than all other samples. The sterile swab and kit control that contained a higher number of sequences were processed with different kits, implying that contamination from an adjacent well may have impacted this kit control (206), instead of an inherent contamination within the DNA extraction reagents (the contaminated kit control was processed in a plate with a clean sterile swab, and vice versa). The most abundant kit control contaminant was related to the *Neisseriaceae,* at 48.7% abundance in the control sample. This OTU was present in ~27% of samples in this run, the majority of which were cats. Indeed, cat #136 had a very high number of *Neisseriaceae* sequences (~42,000), and

was located adjacent to the kit control well. It is therefore postulated that this particular kit control's high contamination was from an adjacent well via cross-contamination instead of from a source that would impact all samples, such as kit reagents, implying that there was no significant impact on all samples. Additionally, one of the contaminated no template PCR controls was dominated by an OTU affiliated with *Rhodocytophaga* (36.2%), which had only a single read in one animal sample included in the study. To reduce the known impact from well to well cross-contamination (204), all samples were randomized. This ensured that samples from the same animal were distributed across multiple plates and MiSeq runs, as were samples from within a mammalian species or order. Observed influences, including host taxonomy and geography, cannot be due to these groups of samples being situated proximally within the same extraction or PCR plate.

The following 19 animal swabs were removed in the mammal dataset due to failure to amplify: eight cats, two beavers, and one each of river otter, cape eland, white rhinoceros, cheetah, horse, dog, Indian flying fox, and reindeer samples. These unamplified samples represent 3.6% of total mammalian samples. There was a disproportionate number of cat samples requiring removal, which may be due to several factors, such as pet owners sampling more lightly on cats resulting in insufficient sample collection. If the swabs were not pressed firmly against the animal's skin, it is possible that only a small number of microorganisms were collected that were below the sequencing detection limit. Alternatively, cats may possess lower overall skin microbial abundances.

3.2.6 Statistical analyses

The majority of statistical analyses were conducted using the same programs and software version numbers as described previously (203). In brief, alpha-diversity of all samples

was measured with the following QIIME commands: multiple_rarefaction.py,

alpha_diversity.py, collate_alpha.py, and compare_alpha_diversity.py. Subsequently, the 42

metadata categories were compared using the Bonferroni correction to avoid false positives due

to testing a high number of hypotheses.

Beta diversity was visualized using ordinations generated with the Bray-Curtis distance

metric. These figures were created in RStudio (169) with the phyloseq (v. 1.14.0) and ggplot2 (v.

2.1.0) packages. Beta diversity was measured using PERmutational Analysis Of Variance

(PERMANOVA) with the adonis function from the vegan package (v. 2.4-0) in R. Using 1000

permutations, the percent variation explained by each metadata category was calculated and

visualized in a heatmap using ggplot2, vegan, Heatplus 2.16.0 (207), and RcolorBrewer v. 1.1.2

(171).

The functions from the prokaryotic clades were predicted using Functional Annotation of

Prokaryotic Taxa v.1.0 (FAPROTAX) (208). This conservative algorithm currently matches 80

functions, such as fermentation and methanogenesis, against 7600 functional annotations of 4600

prokaryotic taxa.

3.2.7 Phylosymbiosis analysis

The phylosymbiosis analysis of skin microbiota profiles and host phylogeny was adapted

from a previously described protocol (209). The mammalian phylogenetic trees were constructed

using the mitochondrial cytochrome oxidase subunit I gene (COX1) for each species that was

obtained from NCBI. Downloaded sequences were aligned with Muscle v. 3.8.31(210). The

resulting initial alignment was edited by removing gap positions and 5'/3' end overhangs with

Jalview v. 2.9 (211). The final edited alignment was created using RaxML online Blackbox

server v. 8.2 (212). The DNA sequences were aligned using the CAT model of heterogeneity,

without an outgroup, constraints, a binary backbone, or partitioned model. Sequences were aligned to create a maximum-likelihood consensus tree. All mammalian host trees were verified to be in concordance with well-established mammalian phylogenies (213–217).

Microbiota dendrograms were constructed using QIIME v. 1.9.0. Initially, the OTUs from within each species' samples were summed using a custom R script to create a single sample with all sequences per species. The resulting table was processed using the jackknifed_beta_diversity.py command. Species were rarefied to the highest possible sequence count that included all species within the specific taxonomic ranking. This resulted in a rarefication of 1900 sequences for all mammals, 9,100 for Artiodactyla, 25,7000 for Carnivora, and 37,500 for Perissodactyla. Rarefication was conducted 1000 times and a consensus tree built to correct for the low number of rarefied sequences. Each of the above mammalian clades had bacterial consensus dendrograms created at 97% and 99% OTU identity threshold using Bray-Curtis, weighted and unweighted UniFrac distance metrics.

Congruencies between host phylogenies and microbial dendrograms were determined using the ape R package (170). Normalized Robinson-Foulds scores, which measure the number of differences between the host and bacterial phylogenies, divided by the total number of possible differences, were calculated to quantify congruence (218). The significance of this score was determined by constructing 100,000 randomized trees with identical leaf nodes to the bacterial dendrograms and comparing each to the host phylogeny to calculate the number of stochastic dendrograms with equivalent or better Robinson-Foulds scores.

3.3 Results and Discussion

The diversity of bacteria and archaea were analyzed from 589 mammalian skin samples, which yielded 8,416,281 reads. A total of 22,728 unique operational taxonomic units (OTUs)

were obtained that corresponded to 44 prokaryotic phyla. The following general taxonomic distributions of the mammalian skin microbiome exclude the human samples that were published previously as part of a broader human cohabitation study (203). There were six phyla present above 1% abundance that constituted 96.0±4.0% of all reads: *Firmicutes* (33.6±20.4%), *Proteobacteria* (28.5±19.1%), *Actinobacteria* (23.6±16.1%), *Bacteroidetes* (7.6±4.9%), *Cyanobacteria* (1.5±2.6%), and *Chloroflexi* (1.1±1.8%). These abundances represent a significant decrease of 33.2% in the abundance of the phyla *Actinobacteria* ($p < 0.001$), and significant increases in the abundance of *Bacteroidetes* ($p < 0.001$), *Chloroflexi* ($p < 0.001$), *Cyanobacteria* ($p = 0.01$), and *Proteobacteria* ($p < 0.001$) compared to human skin samples of the same body regions (203). There was no significant difference in the abundance of the phylum *Firmicutes* among human and non-human mammalian groups of samples. The remaining sequences were affiliated with 42 additional phyla. The most abundant OTU was *Macrococcus,* which constituted 2.9±10.9% of all sequences. The large standard deviation is due to only 65.2% of samples possessing this OTU, whereas it was the most abundant OTU in several host species, in particular Przewalski's horses, donkeys, and dogs. *Staphylococcus equorum*, two strains of *Corynebacterium*, and *Sphingomonas* were the only four other OTUs that were present above 1% relative abundance across the entire non-human dataset, representing 8.1±14.0% of all sequences. The most abundant OTU varied significantly among species (Table 3).

Table 3: Summary of each mammalian species sampled with data on the most abundant OTU.

Order	Common name	Sampled individuals	Most abundant OTU	Abundance of most abundant OTU (%)	OTUs >1% abundance	# unique OTUs
Artiodactyla	Alpaca	3	*Macrococcus*	11.4	18	1136
	Aoudad Sheep	9	*Staphylococcus*	5.0	13	2088
	Bactrian Camel	15	*Planomicrobium*	16.5	19	1372
	Bovine	45	*Staphylococcus*	7.4	15	4182
	Cape Eland	11	*Ruminococcaceae*	4.2	12	1840
	Goat	6	*Staphylococcus*	14.2	10	1539
	Reindeer	18	*Alkanindiges*	12.9	18	1295
	Rothschild Giraffe	9	*Corynebacterium*	5.5	20	1395
	Sable Antelope	3	*Oligella*	2.7	17	1086
	Sheep	3	*Corynebacterium*	8.1	14	988
Carnivora	African Lion	9	*Psychrobacter sanguinis*	6.9	17	1481
	Arctic Wolf	9	*Weeksellaceae*	5.9	11	2021
	Cat	48	*Neisseriaceae*	6.7	12	3399
	Cheetah	20	*Enhydrobacter*	11.9	12	2277
	Dog	35	*Macrococcus*	2.4	7	4356
	Giant Panda	6	*Clostridium*	27.6	15	946
	River Otter	2	*Rhodococcus*	9.2	19	417
	Spotted Hyena	3	*Actinobacillus*	7.3	23	436
	White Lion	6	*Psychrobacter*	22.1	14	823
Chiroptera	Indian Flying Fox	18	*Streptococcus*	16.1	21	927
	Straw Coloured Fruit Bat	9	*Clostridium butyricum*	21.2	14	871
Diprotodontia	Red Kangaroo	18	*Sharpea*	6.0	10	2115
	Swamp Wallaby	3	*Flavobacteriaceae*	7.5	15	958
Lagomorpha	Rabbit	7	*Staphylococcus succinus*	22.1	15	997
Perissodactyla	Donkey	21	*Macrococcus*	6.1	11	5036
	Horse	68	*Corynebacterium*	9.1	8	5645
	Indian Rhinoceros	6	*Actinomycetales*	5.4	25	893
	Pony	3	*Gemellaceae*	15.0	13	916
	Przewalski's Horse	15	*Macrococcus*	34.5	6	2153
	White Rhinoceros	14	*Corynebacterium*	18.0	19	1314
Primates	Human	77	*Propionibacterium acnes*	16.5	15	1628
	Olive Baboon	15	*Lactobacillus*	4.9	14	1890
	Sumatran Orangutan	9	*Neisseriaceae*	15.4	14	1219
Proboscidea	Asian Elephant	15	*Micrococcus*	8.3	17	1224
Rodentia	Beaver	1	*Moraxellaceae*	7.7	15	319
	Groundhog	6	*Macrococcus*	3.3	12	1955
	Squirrel	21	*Escherichia coli*	5.5	11	2906
Xenarthra	Two Toed Sloth	3	*Kocuria*	7.6	13	922

3.3.1 Humans have a distinct microbial community from the majority of animals

An indicator species analysis determined that all human samples have elevated levels of *S. epidermidis*, *Corynebacterium*, and *Propionibacterium acnes* (Table 4), which is in accordance with previous literature (20, 26, 45). In contrast, animals were associated with soil-related organisms, such as *Arthrobacter* and *Sphingomonas*, albeit at lower average abundance than human indicators. This finding was corroborated by a core OTU analysis (Figure 16). A core OTU was defined as one that was present in a minimum of 90% of non-rarefied samples. All mammalian clades shared six core OTUs including *Arthrobacter*, *Sphingomonas*, and *Agrobacterium*. Five mammalian orders were analyzed further that contained multiple host species and were not composed of pets/humans. Each of the orders except Perissodactyla had core OTUs that were not shared with any of the other mammalian orders. These core OTUs represent microbiota that are persist despite different varying geographical locations and enclosures. The presence of a large proportion of soil organisms (Figure 17) may be explained by frequent contact of the skin with the external environment. Although the sampling of terrestrial mammals did not included a step to rinse off environmental microbiota, as has been completed in amphibian microbiome studies (85), future studies might test alternative sampling protocols to access the mammalian skin microbiome in order to minimize sampling of allochthonous microorganisms.

Table 4: Indicator analysis of human and non-human animals. Indicator OTUs were defined as having an indicator value threshold of >0.7 and $p < 0.05$. Reported averages correspond to the number of sequences per sample, rarefied to 1654 reads per sample total. Multiple OTUs with the same genus are different strains.

Indicator source	Indicator value	Animal average	Human average	Consensus lineage
	0.82	15	1	*Arthrobacter*
	0.81	20	2	*Sphingomonas*
Animal	0.77	6	1	*Microbacteriaceae*
	0.74	9	1	*Agrobacterium*
	0.73	12	1	*Phycicoccus*
	0.70	8	0	*Methylobacterium adhaesivum*
	0.70	7	0	*Sphingomonas*
	0.98	4	220	*Corynebacterium*
	0.92	7	273	*Propionibacterium acnes*
	0.90	2	75	*Corynebacterium*
	0.89	1	34	*Finegoldia*
Human	0.85	3	43	*Streptococcus*
	0.85	1	26	*Corynebacterium*
	0.84	1	25	*Corynebacterium*
	0.81	37	163	*Staphylococcus epidermidis*
	0.77	0	7	*Anaerococcus*
	0.76	1	30	*Corynebacterium*
	0.75	1	14	*Peptoniphilus*
	0.71	0	15	*Propionibacterium acnes*
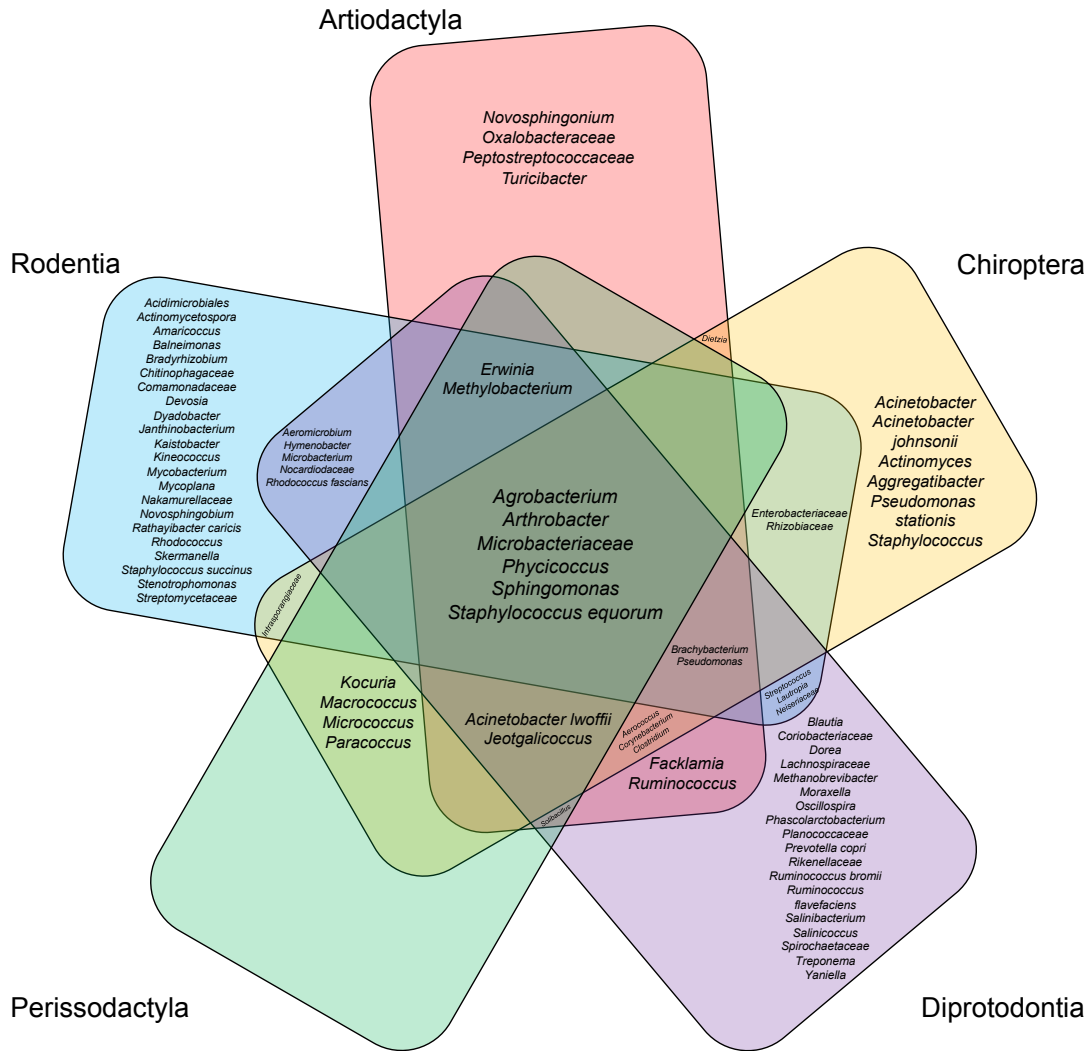
Figure 16: Venn diagram of core OTU analysis. A core OTU was defined as being present in >90% of samples in a designated category. The five mammalian orders were included that had multiple species, and did not have animals that typically inhabit indoors, such as humans, cats, and dogs. The most resolved taxonomic ranking for each OTU was included.
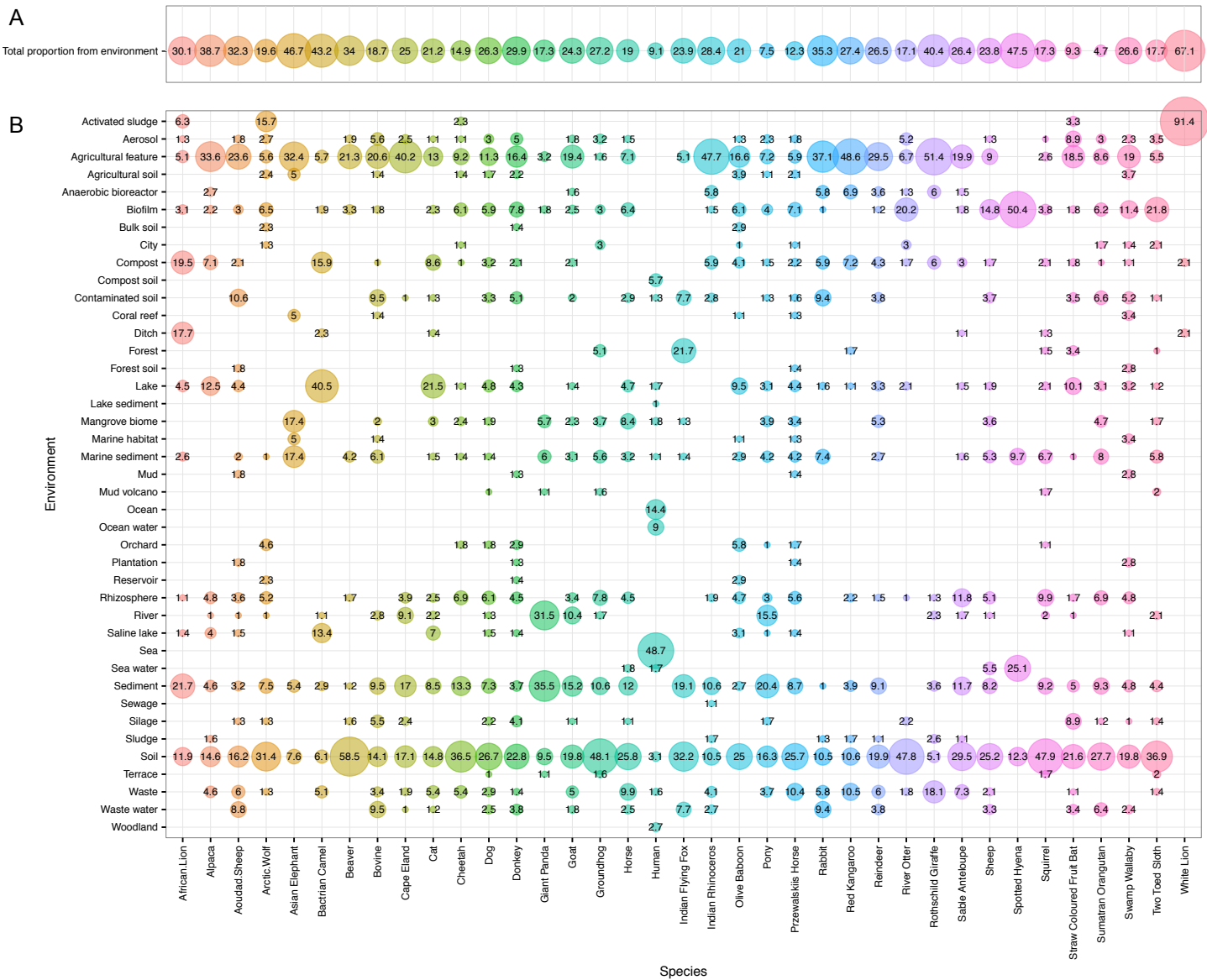
Figure 17: Bubbleplot of the proportion of OTUs associated with a non-skin environment for each mammalian species, according to a SeqEnv analysis. A. Proportion of total sequences that were not associated with skin. B. Distribution of non-skin associated sequences across environmental habitats. Only environments present >1% relative abundance are shown.

Human samples possessed a unique microbial community from all other non-human mammals, except for several pets from the order Carnivora (Figure 18). In addition, human skin was significantly less diverse than all other mammalian orders according to both Shannon indices (6.54 vs 3.96, $p < 0.001$; Figure 19) and the number of distinct OTUs, which supports the findings of a study on five primate species that determined humans have lower diversity than

other primates (53). Other orders whose microbial communities grouped tightly together include Diprotodontia (kangaroos), Chiroptera (bats), Rodentia (squirrels), and non-human Primates. A subsequent PERMANOVA analysis demonstrated that the factor most strongly associated with community variation for all samples was whether the host was a human ($F_{1,587} = 37.8$, $p < 0.001$; Figure 20).
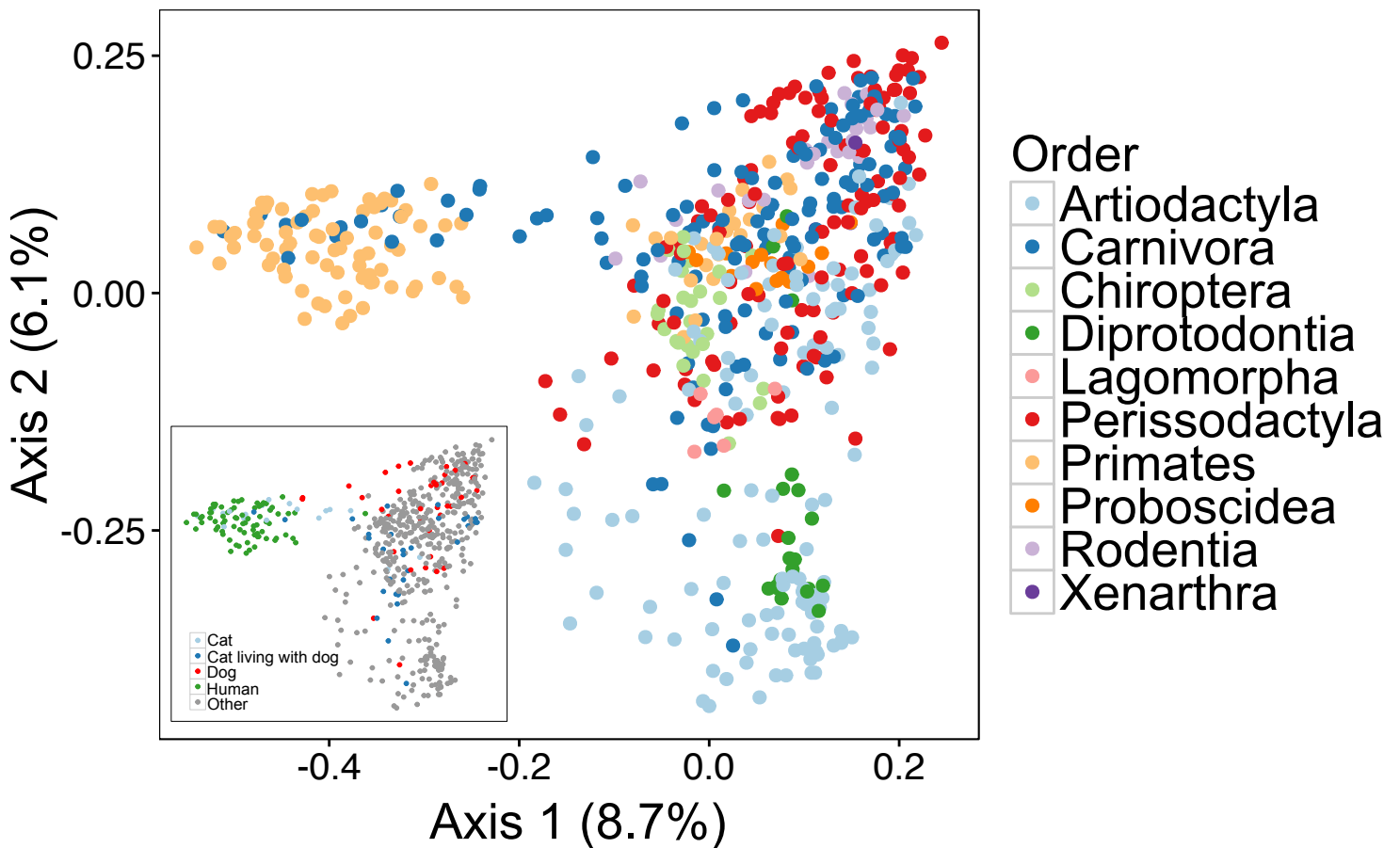
Figure 18: Ordinations (PCoA) generated by using the Bray-Curtis dissimilarity metric for each of the three body locations sampled. Samples are colored according to mammalian order. Inset: Ordination coloured according to human and pet samples.
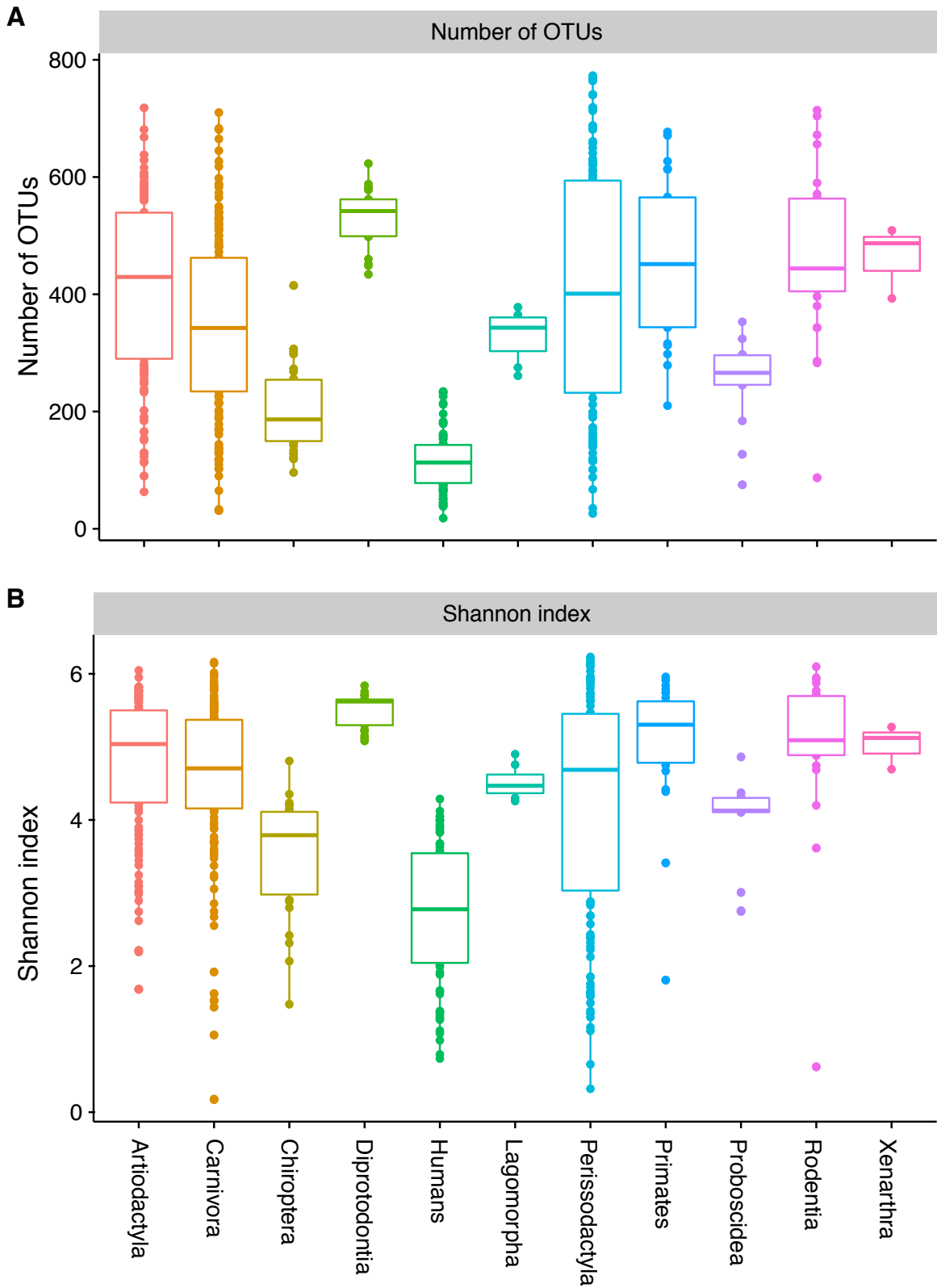
Figure 19: Boxplot of Shannon indices for 10 mammalian orders and humans.
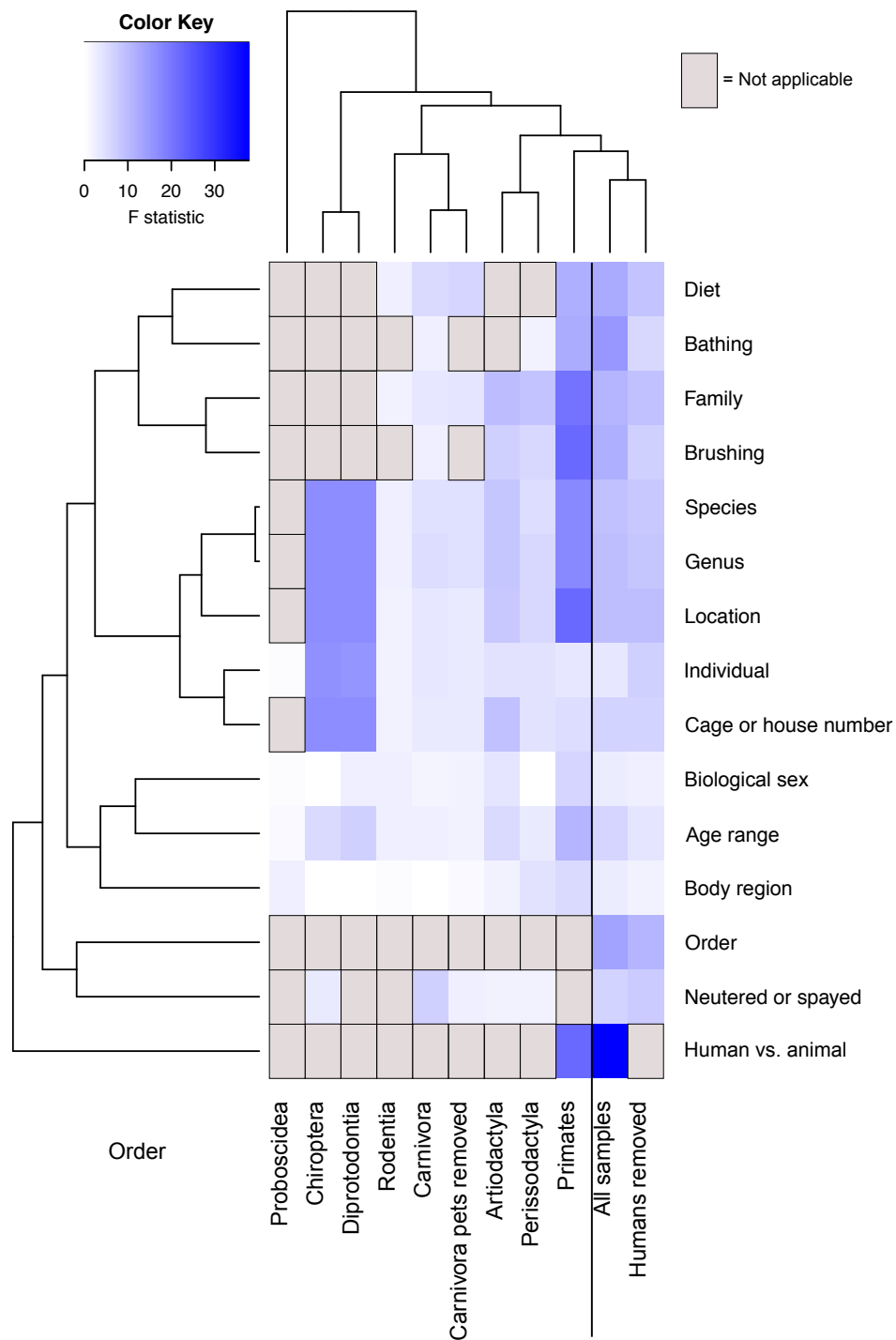
Figure 20: Heatmap summarizing the significant metadata factors correlating with the observed skin microbiota for sampled individuals from mammalian orders. Categories with higher PERMANOVA *F* statistics have higher variation in community dissimilarity. Grey regions of the heatmap represent categories that do not apply. Samples and categories are clustered according to Bray-Curtis distances.

By analyzing all samples together, random forest modelling identified that human and animal samples could be distinguished correctly 98.5±1.2% of the time. The OTUs that contributed most to the model include *Corynebacterium* (2.0%), *P. acnes* (1.2%), *Moraxellaceae* (1.2%), and *Macrococcus* (0.8%). These organisms were all within the top 10 most abundant OTUs in a dataset of all samples. A single female human back was grouped with the majority of the animal samples because of elevated abundances of *Luteimonas, Planomicrobium,* and *Planococcaceae*. The animals that were incorrectly classified were housepets, which had elevated levels of *Corynebacterium* and *P. acnes*. When all pets were removed from the dataset, humans could be distinguished from animals 99.8% of the time, which is 78.2-fold better than expected by chance. Because humans have undergone recent evolutionary divergence from other non-human primates, these results suggest that modern human practices, such as spending the majority of time indoors, frequent bathing, and wearing clothing have strongly impacted the diversity and composition of measured skin microbiota.

Studies to optimize skin sampling methodology may also be conducted to determine if there is a more optimal protocol to accurately sample the mammalian skin microbiome. Mammals were associated with OTUs that are traditionally associated with environmental environments, for example soil (Figure 17), indicating that a washing step to remove external transient organisms may result in more accurate sample collection of the true skin microbial community. To date, the protocol for terrestrial mammals has generally not involved a washing step to remove external debris, although it was used previously in an axilla culturing study that was conducted in the 1950s (197). Amphibian skin microbiome studies have demonstrated that the microbial community in rinse water differed significantly from the community on the rinsed skin (85), and is widely adopted in amphibian research (54, 57, 79, 83, 85, 86, 95, 109).

Although the current study observed that the external surface of a human differs significantly

from all other mammals, more methodological testing needs to be done to determine if a wash

step would provide a more accurate representation of the mammalian skin microbiome.

3.3.2 Taxonomic order is the most important influence on the mammalian skin microbiome,
followed by the host geographic location

The effects of mammalian taxonomy, body region, and location were analyzed to

elucidate whether these factors contribute to influencing the skin microbiome. Mammalian order

had the strongest association with the observed variation among animal skin communities

(PERMANOVA; $F_{9,502}$ = 11.3, p < 0.001; Figure 20), which was followed by the geographic

location (PERMANOVA; $F_{4,507}$ = 9.6, p < 0.001; Figure 20). Random forest modelling was also

conducted on a dataset of only animals to determine how well intrinsic factors, such as host

taxonomy and extrinsic factors, such as location could be classified. Animals could be classified

best according to their taxonomic order. This model was correct at classifying animals into their

corresponding order 87.8±5.0% of the time, which is 5.9-fold better than expected by chance.

Seven OTUs contributed a minimum of 0.5% of the model's accuracy. The most important

OTUs used to distinguish host orders include the family *Clostridiaceae* (1.0% contribution to

accuracy), *Peptostreptococcaceae* (0.9%), *Turicibacter* (0.6%), *Pasteurellaceae* (0.6%),

Ruminococcaceae (0.5%), *Gemellaceae* (0.5%), and *Enhydrobacter* (0.5%).

Peptostreptococcaceae and *Turicibacter* are core to only hosts within the order Artiodactyla

(Figure 16). *Enhydrobacter* dominated certain carnivores, for example the cheetah (Figure 16)

whereas *Gemellaceae* was abundant in Perissodactyla (pony). Lower taxonomic orders, such as

family (86.1±3.9%), genus (84.4±4.7%) and the species (83.4±6.7%), were progressively

classified less accurately. This weaker classification ability may be in part due to smaller number of samples per group for training the model.

The random forest model was more accurate at classifying the order (87.8±5.0%) than the host's geographic location (81.9±2.7%) or its specific cage/house (80.4±12.4%), however this difference was not significant due to the inability to classify any samples from the single specimen within the order Xenarthra. The Toronto Zoo, African Lion Safari, and one of the farms that supplied a single species (bovine) all had classification errors lower than 5%. The accuracy of the classifier was compared to the sample size of each category, because machine learning has been shown to overlearn, which might influence datasets where certain categories contain more samples than others (194). There was only a weak trend between the number of samples collected from each location or order and the ability to classify ($R^2 = 0.33$-0.49; Figure 21), indicating that even locations with a relatively low number of samples can be successfully classified if the location only had a single corresponding species sampled. Four OTUs contributed a minimum of 0.4% to the model's accuracy. The most important OTUs used to distinguish host order include *Jeotgalicoccus* (0.5%), *Staphylococcaceae* (0.5%), *Gemellaceae* (0.5%), and *Corynebacterium* (0.4%). The majority of these OTUs are abundant on animals from farms and households with pets, whereas *Corynebacterium* was most abundant on certain zoo animals (e.g., giraffes and white rhinoceros) as well as farms (e.g., sheep and horses), despite them being strongly associated with human skin (Table 4).
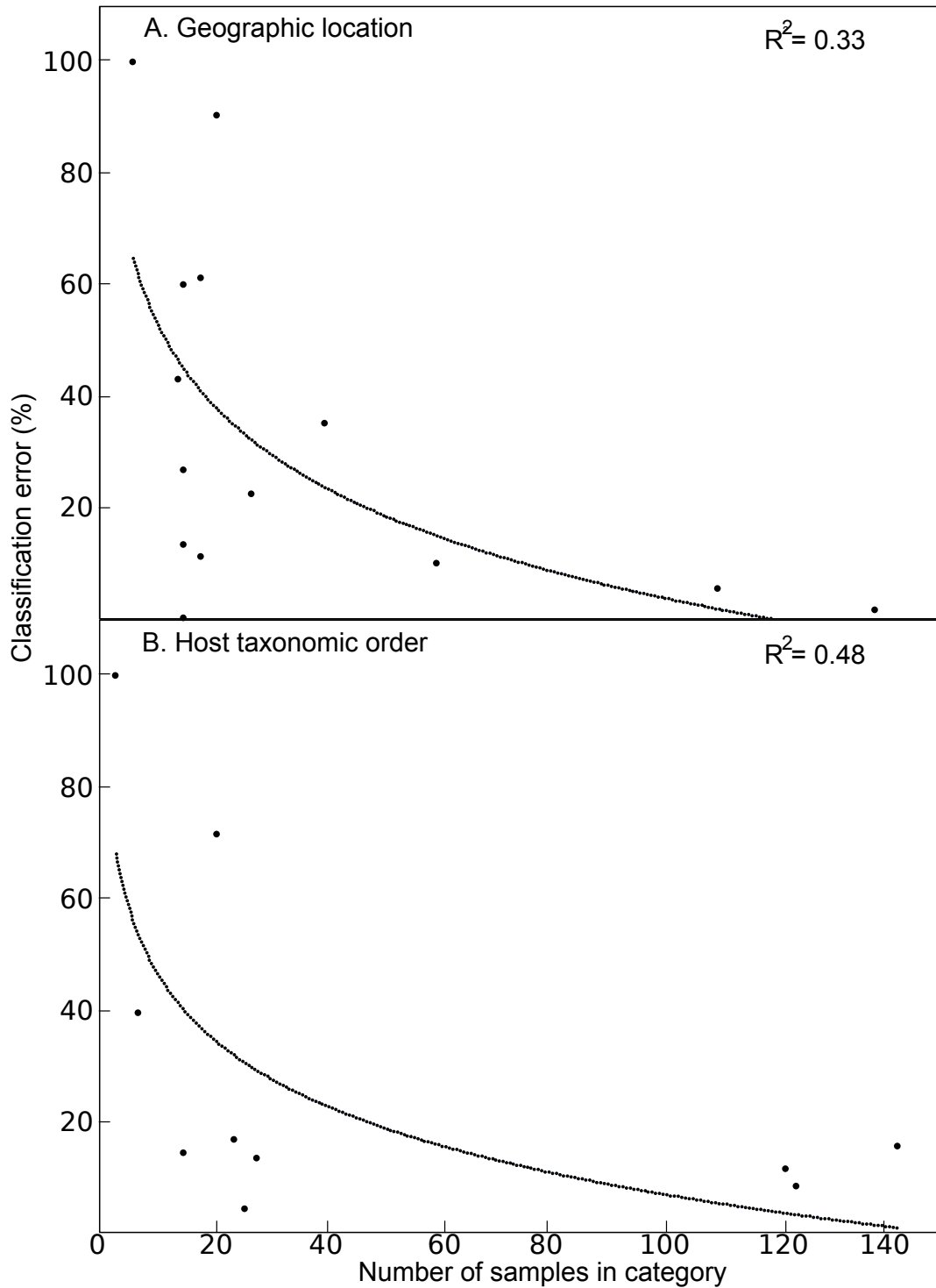
Figure 21: Plot of classification error rate compared to the number of samples included in the random forest modelling analysis for A: geographic location, B: host taxonomic order. An exponential regression provided the most accurate line of best fit.

The ability to classify accurately from specific locations may be in part due to the soil that is present in the habitat. Indeed, a study that analyzed the similarity of skin bacterial communities between salamanders and their environment noted that certain taxa are shared between the skin microbiota and the abiotic environment (84), in part due to contact with forest litter. A previous study has noted that the host was the most important factor influencing the skin microbiome of amphibians, whereas geographic location was the second most important significant factor (79), which aligns closely with both the PERMANOVA and random forest model findings from this study.

Other factors that have been demonstrated to influence the human skin microbiome, such as individuality, biological sex, and body region, exhibited less of an effect on animals. Both taxonomic order and geographic location were significantly classified more accurately than biological sex (65.2±4.5%), body region (39.9±6.0%), or individual animal (36.7±36.7%). The inability to classify the individual is in contrast to human studies that have shown that individuality is one of the most important factors influencing the human skin microbiome. However, many of these studies used >15 samples per individual (17, 42, 46). It is therefore still possible that animals' skin microbiota are relatively unique among individuals, but this cannot be observed with only three samples per animal.

To address whether biological sex influenced the skin microbiome within a species, cats, dogs, and horses were analyzed because they contained a relatively large number of sampled individuals and a balanced biological sex split. Biological sex was not a significant factor for any of these species (PERMANOVA; Cat: $F_{1,15} = 1.15$, $p = 0.20$; Dog: $F_{1,11} = 0.79$, $p = 0.77$; Horse: $F_{1,20} = 0.94$, $p = 0.44$), even when analyzing within body locations, such as the thigh regions that exhibit strong sex variation in humans (203). The only animal where biological sex explained the

most variation was the red kangaroo (PERMANOVA; $F_{1,16} = 2.21$, $p = 0.002$), which also

exhibited a visual split between males and females in an ordination (PCoA; Figure 22). Larger

variations in the microbiota among different body regions were observed within Perissodactyla

(PERMANOVA; $F_{2,121} = 4.26$, $p < 0.001$; Figure 20) and Proboscidea (PERMANOVA; $F_{2,12} =$

2.38, $p = 0.02$; Figure 20) than within other orders. The overall low effect from body region is

likely due to the body regions sampled. The back, inner thigh, and torso are all covered with hair.

A previous study on dogs demonstrated that fur-covered regions had higher species richness and

diversity (51) compared to mucosal surfaces. Therefore, sampling mucosal surfaces would be

expected to result in more distinct differences between body regions within a species.
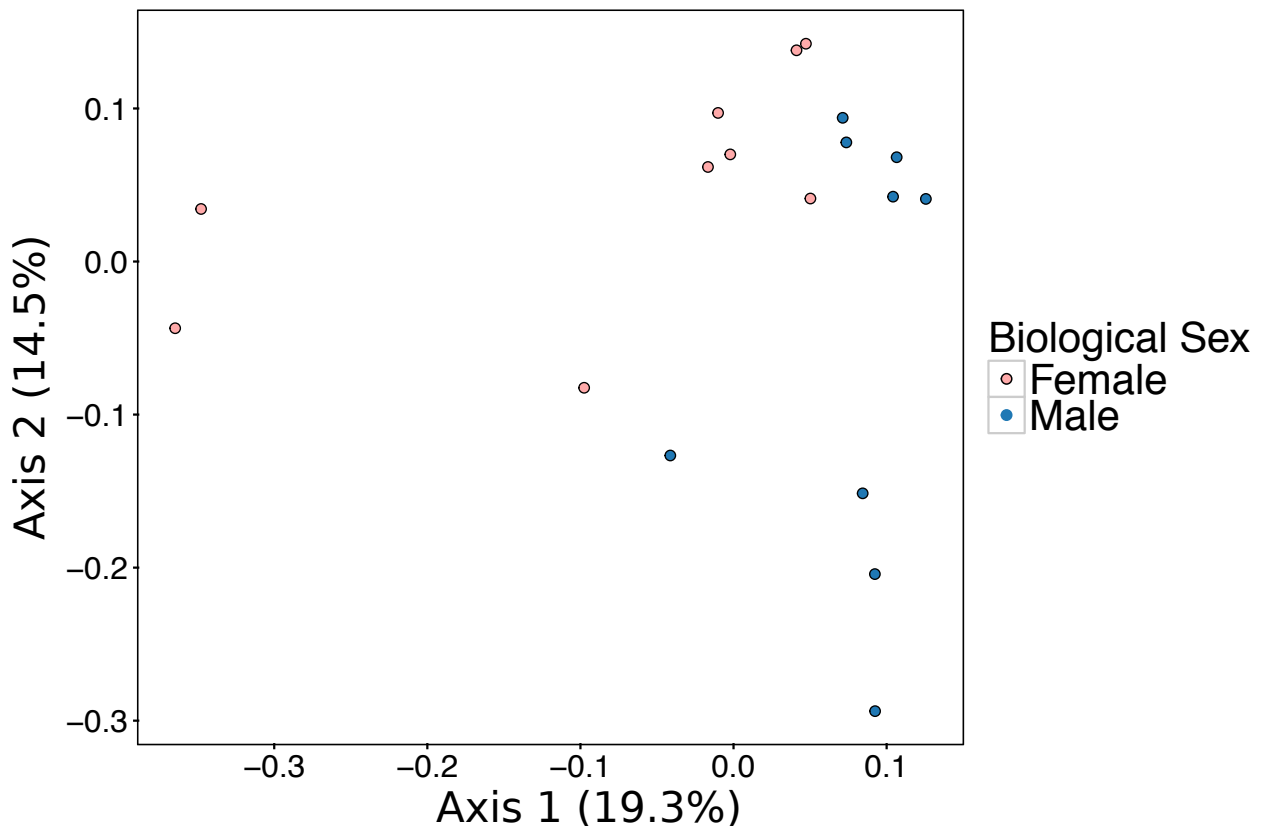
Figure 22: Ordination (PCoA) generated by using the Bray-Curtis dissimilarity metric for each of the three body locations of red kangaroos. Samples are colored according to biological sex.

To ensure that the importance of the host's taxonomic order on skin microbiota was not overly influenced by orders with fewer samples and locations, the three orders with a large number of samples, Artiodactyla, Carnivora, and Perissodactyla, were analyzed with all other orders removed. Each of these orders had samples sourced from six to eight different locations. Removing orders with fewer samples increased the influence of order (PERMANOVA; $F_{2,385}$ = 15.1, $p < 0.001$) and decreased the impact of geographic location (PERMANOVA; $F_{3,384}$ = 8.7, $p < 0.001$). Therefore, the effect on microbial communities exhibited by the mammalian host exists despite varying geographic locations, and cannot be fully attributed to certain species only being sampled in a single location.

3.3.3 Living with a dog may shift the feline skin microbiome

Although the majority of animals possessed a microbial skin community that was distinct from humans, a subset of pets had similar microbiota to humans. In particular, of the 17 pet samples that grouped with humans, 15 of them were obtained from indoor housecats, whereas the remaining two samples belonged to the backs of dogs that were frequently bathed and groomed (Table 5). None of these 17 pet samples belonged to animals that had been exposed to antibiotics. In total, 75% of these samples belonged to animals that were owned by humans who themselves participated in the study. All cats with similar microbial communities to humans had at least two of the three sampled body locations possess this "human" community composition (Table 5), whereas the two dogs only possessed the human microbial community on their backs. The remaining 11 dogs had similar communities to the other animals, as did all cats that lived outdoors on farms and a single cat that lived in the city, without a dog (Figure 18 inset). Interestingly, 11 of 12 cats that lived with a dog possessed similar microbial communities to the other animals, which was true for all samples. It may be that owning a dog that is walked daily

may result in an influx of soil microbiota into the home, which in turn is transferred to the

exclusively indoor cats through either contact with the built environment or personal contact.

Indeed, several studies have shown that owning a dog shifts the human microbiome as well as

built environment surfaces (150, 187). This study only sampled from 13 dogs and 19 cats (87

samples). Future studies might include a larger sample size of animals that would help further

elucidate how owning a dog impacts other inhabitants within a household.

Table 5: Table containing the animal samples that had similar microbial communities to humans.

Animal ID #	Species	Owner is also in study?	Number of samples grouped with humans	Body regions that grouped with humans
52	Cat	Yes	3	Back, inner thigh, torso
54	Cat	Yes	2	Back, torso
55	Cat	Yes	2	Back, inner thigh
56	Cat	Yes	2	Inner thigh, torso
57	Dog	Yes	1	Back
58	Cat	Yes	3	Back, inner thigh, torso
69	Dog	No	1	Back
70	Cat	No	3	Back, inner thigh, torso

3.3.4 Predicted functions of skin microbiota vary between human and animal samples

The predicted functions based on the prokaryotic clades were determined using

FAPROTAX (208) and demonstrated that there were several conserved functions on mammalian

skin (Figure 23). Many of these match functions predicted from human samples from the Human

Microbiome consortium that underwent metagenomic sequencing (47). Animal symbionts and

human pathogens were expected because the samples were derived from mammalian hosts. Urea

is a component of sweat and provides a nitrogen source, which could explain ureolysis as a

predicted skin function (219). However, there were several functions that were significantly

different between humans and animals. Humans had elevated levels of predicted manganese

oxidation. Human sweat contains on average 100 ppb manganese, which would result in approximately 200-300 mg of manganese secreted each day (220). This concentration is low compared to other trace metal elements, for instance zinc and copper (221). Manganese oxidation was predicted to occur from the core human OTU *P. acnes* (222). In contrast, animals had higher levels of predicted functions involved in the nitrogen cycle and single-carbon compound degradation. Methanol oxidation was attributed to the core OTUs affiliated with *Arthrobacter,* and methylotrophy with *Methylobacterium* OTUs, according to the FAPROTAX database. Nitrogen respiration was associated with numerous organisms, such as *Paracoccus* and *Pseudomonas*. In accordance with lower diversity (Figure 19), humans had a significantly lower number of predicted functions (34.2±8.4 compared to 51.8±9.4 in animals; *p* < 0.001). Predicting functions based on taxonomy is the first step to elucidating how biochemical processes from skin microbiota are influencing host skin health. Future studies using metagenomic sequencing may help confirm which of these predicted conserved microbial functions are core to mammalian skin, versus functions that are variable amongst different host species.
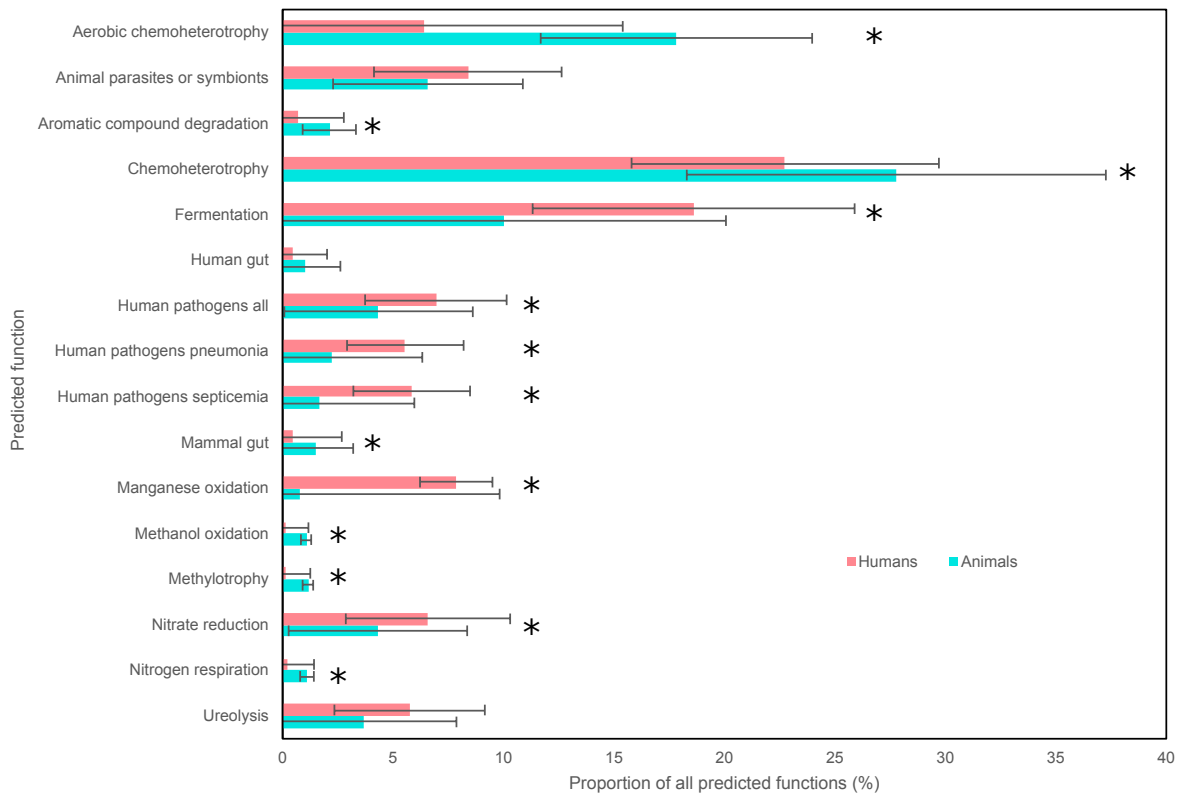
Figure 23: Barplot of predicted functions based on FAPROTAX database. Stars indicate *p* < 0.05 among mammalian and human samples after Bonferroni correction was applied. Error bars denote the standard deviation of animal (n=512) and human (n=77) samples.

3.3.5 Phylosymbiosis is evident in the orders Perissodactyla and Artiodactyla, but not across all mammals

Comparing the known host mammalian phylogeny to dendrograms of the microbial communities for each host species provides evidence that skin communities on animals from the orders Perissodactyla and Artiodactyla experience shifts that match mammalian evolution. Phylosymbiosis postulates that closely related clades of animals will have more closely related microbial communities (209). This can be measured using normalized Robinson-Foulds values, which compares the congruence between two phylogenetic trees. A score of zero indicates the trees are identical, whereas a score of one indicates there is no congruence between the two trees. Comparisons were made at both the 97% and 99% prokaryotic OTU threshold, using Bray-

Curtis, unweighted, and weighted UniFrac distances. Previous studies have shown that shifts in microbial communities have matched host evolution within insects (206, 209), which were more apparent at the 99% threshold.

Perissodactyla exhibited phylosymbiosis with all thresholds and distance measures, because the only discrepancy in each test case was the microbial community of horses and Przewalski's horses (Figure 24C). The split between the equestrian and rhinoceros' clades cannot be attributed to differences in location, such as farm or zoo habitats, because the Przewalski's horses were sourced from the Toronto Zoo. Although Artiodactyla (Figure 24A) possessed a relatively poor normalized Robinson-Foulds score of 0.71 (Table 6) it still demonstrated significant congruence between the host phylogeny and microbial dendrogram with both the Bray-Curtis and weighted UniFrac metrics. The largest discrepancy was with the goats because they grouped with the giraffe and reindeer instead of with sheep. The host species did not group according to the geographic locations they were sourced from. In contrast, the order Carnivora (Figure 24B) did not exhibit significant phylosymbiosis. Within the carnivores, one would expect the cat and dog clades to have distinct microbial communities from one another if phylosymbiosis was occurring; however, this was not observed. This observation did not change when all cat and dog samples were removed from the dataset. Therefore, the microbial dendrograms were not being unduly influenced by household animals that undergo frequent grooming and spend the majority of time indoors. It is possible that phylosymbiosis may be more strongly observed within clades of animals that share similar diets. All of the sampled animals within Perissodactyla and Artiodactyla were herbivores that graze on local grasses. In contrast, the animals within order *Carnivora* had a more diverse diet, such as the herbivorous giant pandas, the carnivorous lions, and the pets that were fed an omnivorous diet. Similar to how diet

influences the gut microbiome (7), it may be that the skin microbiome is impacted by diet, as has

been shown for skin microbial communities of amphibians (95).

Table 6: Phylosymbiosis analysis of main mammalian clades. The normalized Robinson-Foulds scores were calculated at the 97% and 99% threshold. (BC: Bray-Curtis distance metric; nRF: normalized Robinson-Foulds score; UU: unweighted UniFrac distance metric; WU: weighted UniFrac distance metric). Significant normalized Robison-Foulds scores are starred.

Clade	Distance metric	97% threshold	99% threshold
All samples	BC	0.97	0.97
	UU	1.00	0.93*
	WU	0.97	0.97
All mammals:	BC	0.93*	0.94*
humans removed	UU	0.96*	0.94*
	WU	0.93*	0.93*
Artiodactyla	BC	0.71*	0.71*
	UU	0.86	0.86
	WU	0.71*	0.71*
Carnivora	BC	0.83	0.83
	UU	1.00	1.00
	WU	0.83	0.83
Carnivora:	BC	1.00	0.75
pets removed	UU	1.00	1.00
	WU	0.75	0.75
Perissodactyla	BC	0.33*	0.33*
	UU	0.33*	0.33*
	WU	0.33*	0.33*
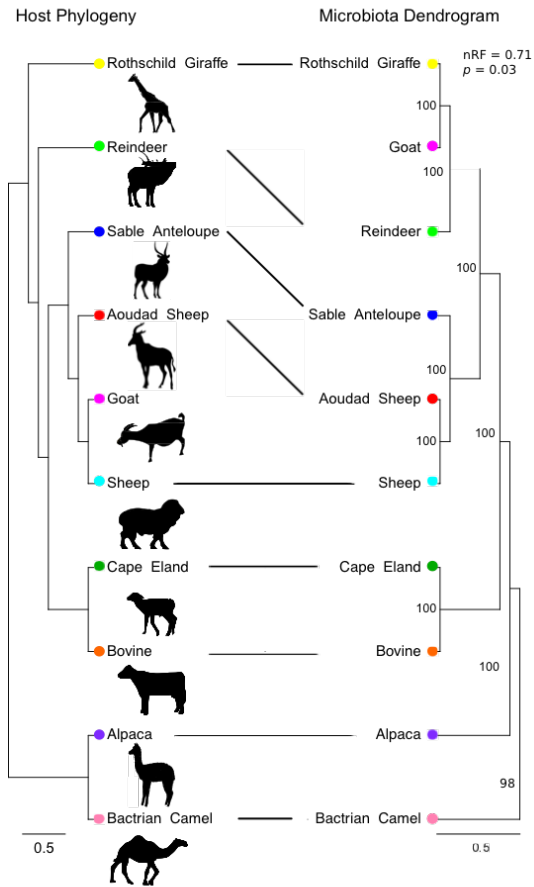
Figure 24: Microbiota dendrograms created using the Bray-Curtis distance metric and a 99% OTU threshold compared to the known host phylogenies of A: Artiodactyla, B: Carnivora, C: Perissodactyla. Congruences were measured using normalized Robinson-Foulds scores (nRF). Horizontal lines denote species that have concordance between the host phylogeny and microbial dendrogram. All images of are courtesy of Tracey Saxby, Integration and Application Network, University of Maryland Centre for Environmental Science, except for the alpaca (Meaghan Mechler), bovine (John C. Fisher), goat (Jane Hawkey), and sheep (Tim Carruthers)

There was no significant phylosymbiosis observed when all mammalian orders and humans were analyzed, except for the unweighted UniFrac measure at the 99% threshold. Although in this single case animals could be matched significantly better than 100,000 randomized trees of the 38 species, there was very little congruence observed (Table 6), as indicated by the normalized Robinson-Foulds score of 0.93. When humans were removed from this dataset, congruence was increased modestly, although the host tree and bacterial dendrograms still exhibited little congruence. This can be explained by the significantly different microbial community that humans have, because human skin microbial community node was positioned near the root of the tree, instead of within the primate clade.

The unweighted distance metric was overall the worst at demonstrating host-microbiota congruence, whereas using a 99% threshold only lead to a modestly improved result in three of the 18 tests. This indicates that using higher taxonomic resolution to assign prokaryotic OTUs did not have an effect on the analysis. Furthermore, the poor performance by the unweighted distance measure in most cases implies that both the types of prokaryotic OTUs and their abundance should generally be taken into consideration when comparing microbial communities to their host phylogenies. Although previous studies have been able to demonstrate phylosymbiosis, they did so under highly controlled laboratory conditions and with fecal samples (209). Skin represents a more transient environment that is influenced by shedding and contact with other surfaces. The animals in this study had several confounding factors, such as different locations and age. It is possible that if mammals were sampled at similar timepoints in their life history, and inhabited the same geographic location that a more distinctive congruence would be observed. Additionally, the potentially transient soil microorganisms that were abundant on mammalian skin may mask phylosymbiosis when all sequenced OTUs are being considered as a

community in the phylosymbiosis analysis (Figure 17). Future studies should potentially sample before and after washing the skin to observe how this treatment would influence the analysis. I postulate that reducing the number of transient, auxiliary organisms from the environment would strengthen the finding of phylosymbiosis because the transient organisms that would not co-evolve with a host would be removed from the analysis. Phylosymbiosis between the skin microbial community and host would not be unexpected. Multiple studies have observed this phenomenon in the gut of mammals (7, 223). These findings illustrate that despite multiple confounding factors that would potentially mask phylosymbiosis, that it is still significantly observed in multiple mammalian clades. Further studies should determine if this finding is strengthened when the hosts within a clade experience equivalent extrinsic factors.

3.3.6 Archaea are present on mammalian skin at low abundance levels

Archaea comprised only 6,509 of the total 6,550,625 non-rarefied sequences (0.001%). Several archaeal clades were present, such as the salt tolerant *Halobacteria*, the methanogen *Methanobrevibacter*, and the ammonia-oxidizing *Thaumarchaeota* (Figure 25). Methanogens potentially represent fecal contamination because *Methanobrevibacter* is the dominant archaea present in the gut (224). However, *Halobacteria* and thaumarchaeotes, such as *Nitrososphaera*, have the potential to be resident skin microbiota. The *Halobacteria* are able to tolerate the salt concentrations from sweat (225), whereas ammonia-oxidizing organisms have been observed on human skin (43, 63).
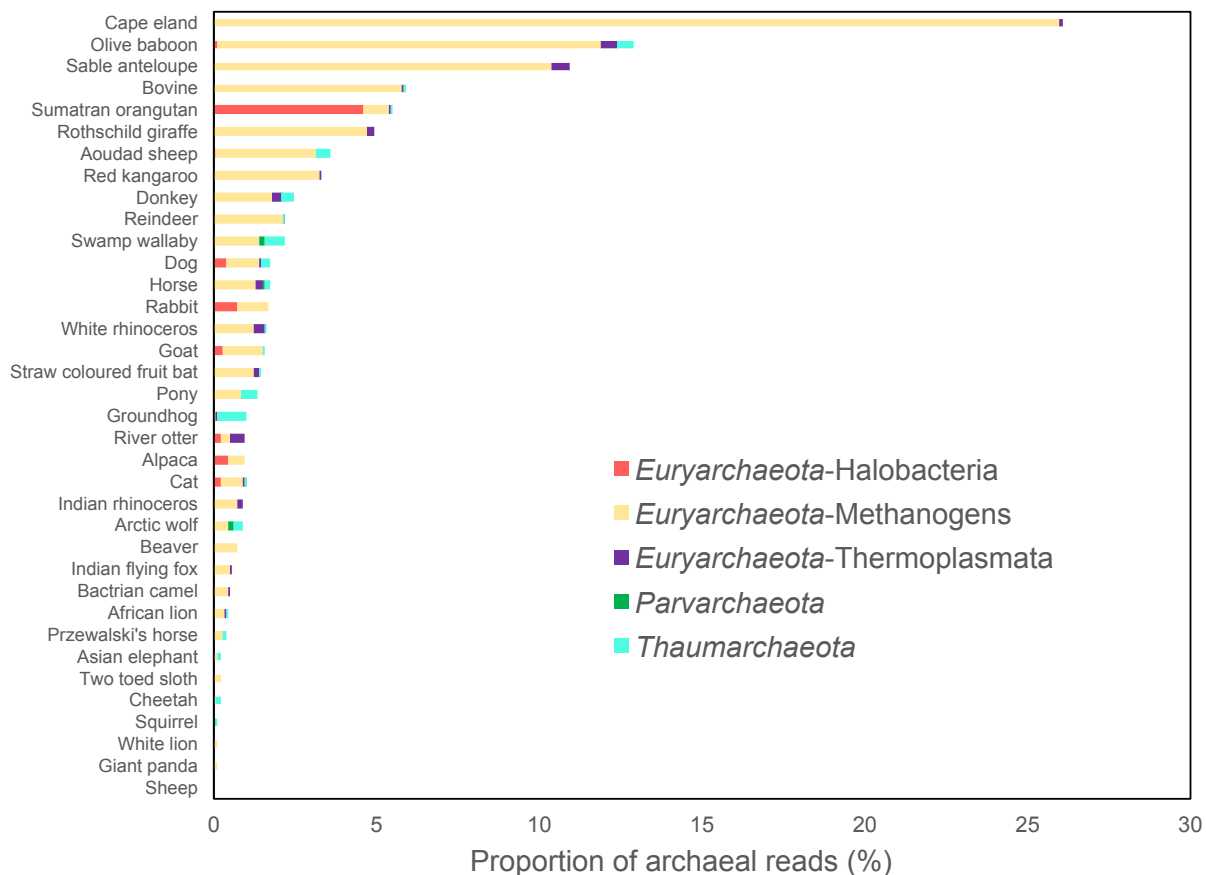
Figure 25: Barchart of archaeal sequence reads. The proportion of reads represents the total proportion of the 6509 archaeal reads. Each mammalian species was corrected by the number of samples collected to account for an unequal sampling depth.

Archaeal reads were disproportionately present on cape elands (26.1% of all archaeal sequences), olive baboons (12.9%), the sable antelope (10.9%), and bovine (5.8%). The methanogens from the phylum Euryarchaeota, were the dominant archaeal clade. However, the ammonia-oxidizing Thaumarchaeotes were at elevated levels in groundhogs (0.9%), the swamp wallaby (0.6%), olive baboons (0.5%), and the pony (0.5%), providing further evidence to previous research that these organisms are a part of the skin microbiome at low abundances (43, 63).

The low relative archaeal abundance compared to bacteria in this study is likely an underrepresentation of the actual archaeal abundance because of primer mismatches to archaeal 16S rRNA genes (175). Indeed, the performance of the Pro341F/Pro805R primer was analyzed using TestPrime v. 1.0 on the SILVA database (Table 7). Only 64.8% of archaeal 16S rRNA genes had zero mismatches to the primer used in this study. Disconcertingly, the ammonia-oxidizing thaumarcheotes only had 11.9% of taxa with zero mismatches, in contrast to 85.7% of all bacteria. When the number of mismatches was increased to two, 94.9% of all archaea, and 95.5% of thaumarcheotes were matched. A recent study on the gut microbiome of great apes that used both universal prokaryotic and archaeal specific primers determined that the distribution, diversity, and prevalence of archaea in mammalian gut samples is underestimated by up to 90% (175). Currently archaeal specific primers, namely Arch516F/Arch915R, offer more accurate representation of this domain. Therefore, this study provides evidence that archaea are present in relatively high abundance on cape elands, olive baboons, sable antelope, and bovine compared to other species, which requires further examination with archaea-specific primers.

Table 7: TestPrime comparison of Pro341F/Pro805R primer mismatches to archaea, thaumarchaeotes, and bacteria.

# mismatches	% archaea covered	% thaumarchaeotes covered	% bacteria covered
0	64.8	11.9	85.7
1	89.0	93.2	94.6
2	94.9	95.5	96.1

3.3.7 Comparison to Yanomami uncontacted Amerindians

In 2015 an intriguing microbiome study was published describing a tribe of Amerindians in Venezuela (103). This previously uncontacted tribe did not use antibiotics, spent the majority of their time outdoors, and did not use modern hygiene products. Therefore, these individuals

experienced vastly different environmental influences than the majority of westernized individuals that had been included in microbiome studies to date. The skin microbiome of the Amerindian forearm had a higher diversity than any known human study to date.

The raw sequences of this study were obtained and analyzed using the AXIOME pipeline to compare to both the human and mammalian skin samples included in this study. The most abundant OTUs from the Amerindians were different from both the human and animals included in the current research (Table 8). The Canadian human skin was dominated by *Staphylococcus epidermidis*, *Corynebacterium*, and *Propionibacterium acnes* (Chapter 2), whereas the Amerindians were dominated by *Weissella cibaria (*5.5% relative abundance), *Janibacter* (4.2%), and *Streptococcaceae* (3.3%) (Table 8). *Weissella* was only present in the Canadian mammals (0.5%), whereas *Janibacter* was not present in either the Canadian animal or human dataset. Only *Streptococcaceae* was present at a comparable relative abundance of 3.1% in humans and a reduced 1.0% in animals.

Table 8: Core OTUs present >90% of samples from the Amerindian dataset. Relative abundances (%) of each OTU are listed for each dataset. Amerindian OTU that are also core to other datasets are highlighted. Repeated OTUs represent a different strain within the same genus.

OTU	Abundance in Amerindians	Abundance in Canadian humans	Abundance in Canadian mammals
Weissella cibaria	5.5	0.0	0.5
Janibacter	4.2	0.0	0.0
Streptococcaceae	3.3	3.1	1.0
Nesterenkonia	3.2	0.01	0.03
Kocuria	3	0.3	0.4
Streptococcus	2.2	3.5	0.7
Rothia mucilaginosa	2.1	0.4	0.04
Kocuria	2	0.3	0.4
Granulicatella	2	0.2	0.01
Actinomycetales	1.6	0.2	0.5
Neisseria subflava	1.4	0.2	0.0
Streptococcaceae	1.1	3.1	1.0
Propionibacterium acnes	0.9	12.4	0.5
Porphyromonas	0.8	0.1	0.07
Gemellaceae	0.6	0.4	0.06
Brachybacterium	0.6	0.1	0.0
Neisseria	0.6	2.1	0.05
Solirubrobacterales	0.6	0.0	0.03
Aerococcus	0.5	0.0	0.6
Janibacter	0.5	0.0	0.0
Prevotella	0.5	0.0	0.03
Nesterenkonia	0.5	0.01	0.03
Brevibacterium aureum	0.4	0.0	0.1
Streptococcus	0.4	3.5	0.7
Nesterenkonia	0.4	0.01	0.03
Actinomycetales	0.4	0.2	0.5
Nocardioidaceae	0.3	0.0	0.1
Brachybacterium	0.3	0.1	0.0
Alkalibacterium	0.1	0.01	0.01
Dietziaceae	0.1	0.0	0.4

The Amerindians had a higher number of core OTUs than the Canadian humans (Table 8), however this may in part be attributed to cohabitation within the tribe, compared to the Canadians couples who were geographically separated. The Venezuelans had eight core OTUs that were present in all samples, and an additional 22 OTUs present above the 90% threshold used in the mammalian study (Table 8). These core OTUs included the traditional human commensal *P. acnes*, and ten OTUs that were core to mammals such as *Kocuria* and *Aerococcus* (Figure 16). However, 11 of the core Amerindian skin OTUs were entirely absent from either the Canadian humans or animal datasets, including *Janibacter*, which was the second most abundant OTU on Amerindian skin. Therefore, the Amerindians have a distinct microbial profile from the Canadian humans and animals in the current study.

The Venezuelans had a higher microbial skin diversity than the Canadian participants according to the Shannon index (4.0 ± 0.4 vs $2.7 \pm 0.9\%$, $p < 0.001$), but was significantly less diverse than the sampled animals (4.0 ± 0.4 vs. $4.5 \pm 1.2\%$, $p < 0.001$) (Figure 26). This finding supports the main conclusion from the Amerindian paper that these natives possessed more diverse skin than those analyzed from other human skin microbiome studies (103). Their outdoor lifestyle, coupled with lack of antibiotic and skin products usage likely increased their skin diversity level closer to the average mammalian level diversity, albeit remaining significantly less diverse than the average mammal sampled. Thus, this comparison provides further evidence that Westernized humans have distinct skin microbial profiles, which may potentially be linked to recent changes in lifestyle, instead of solely due to evolutionary history of all humans as a species possessing lower skin diversity.
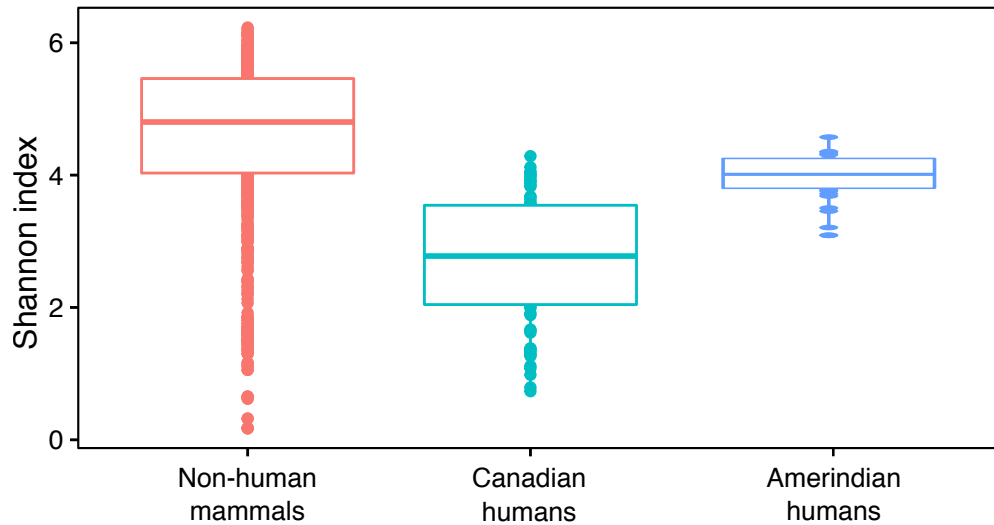
Figure 26: Boxplot of Shannon indices for all animals, and Canadian and Venezuelan humans. All three categories possess significantly different diversity levels from each other.

It must be noted that a different body region was sampled from the Amerindians (forearm vs. back, torso, and inner thigh), representing a dry region compared to the moist and oily regions sampled in this study, which may contribute to the discrepancies in the observed diversity levels and microbial profile. Dry body regions generally have a higher diversity level than oily or moist body regions (Chapter 2). Additionally, the Amerindian study sequenced the V4 region of the 16S rRNA gene, in contrast to the larger V3-V4 region sequenced in this thesis. Indeed, it has been shown that sequencing different regions may result in biases in relative abundances (155, 158), but cannot explain the observed variations in the types of OTUs present. Lastly, one additional contributing factor is likely the geographic distance between the studies. The Amerindians were all sourced from Venezuela, whereas all humans and animals in the current study were from South-Western Ontario, Canada. The large geographic distance between North and South America would likely result in variations in soil, temperature, and humidity that could feasibly influence the external skin environment.

3.3.8 Limitations

This study possesses several inherent limitations. The majority of the animals were collected based on opportunistic availability. For example, animals from the Toronto Zoo were sampled during routine veterinary checkups. The nature of sample collections resulted in an inability to collect an equal number of represents from each host taxonomic order and species. For example, the following species only had a single representative sampled: alpaca, beaver, pony, sheep, sable antelope, spotted hyena, swamp wallaby, and two-toed sloth. Although it is recognized that no significant conclusions can be made about a single host animal within a species, these animals were included in the analysis to have the most in depth coverage of each mammalian order. This study represents an initial survey of the mammalian skin microbiome. Much work remains to be conducted within each species to determine intra-specific effects of individuality, body region, and biological sex.

Animals were sampled across an entire year and frozen until DNA extraction. It is possible that the skin microbiota of outdoor animals may undergo seasonal shifts, especially between the relatively cold winter and warm humid summer in Canada; however, this cannot be tested using a single sampling time for each animal. Future investigations should sample the same individuals across a year to determine if changes in temperature and resulting skin secretion levels might exhibit an effect on the microbiota. Moreover, the significant difference in geographic location that was observed may be more pronounced if animals with greater geographic distance were sampled. All of the animals were sampled in South-Western Ontario. Sampling the same species from multiple continents is postulated to result in more pronounced variations in communities according to location due to significant changes in extrinsic factors, for instance soil microorganisms.

Lastly, the rodents collected in this study were sourced from the wild and deceased. Although these samples still grouped with the remaining live animals (Figure 18), the high level of diversity (Figure 19) may be in part from the initial changes in skin community from decomposition, which have been shown to progress in a clock-like manner (226). Deceased rodents were collected during the first day of death and did not have any visible injuries that would result in internal microorganisms from the GI tract contaminating the skin.

3.4 Conclusion

To the best of our knowledge, this study represents the largest mammalian skin microbiome project to date that did not sample humans exclusively, and is the first known study to elucidate the skin microbiota for 32 distinct species. Human samples were dominated by *S. epidermidis*, *Corynebacterium*, and *P. acnes*. In contrast, other animals were significantly more diverse and have higher levels of OTUs that typically inhabit soil that likely represent transient organisms from their enclosure or natural habitat. These findings are the first to demonstrate that human skin is distinct, not only distinct from other primates, but from all 10 mammalian orders sampled. Given the recent evolutionary divergence of humans as distinct species from other non-human primates, these results suggest that modern human practices, such as living within a built environment, wearing clothing, and washing with soap, have strongly impacted the diversity and composition of the skin microbiota that can be sampled with sterile swabs.

3.5 Data Availability

The sequence data associated with all mammal samples are available in the Sequence Read Archive (SRA) under BioProject ID PRJNA385010. The sequence data for all human participants (published previously) are available in the Sequence Read Archive (SRA) under BioProject ID PRJNA345497.

Chapter 4
Conclusions & Future Directions

4.1 Contributions

      This research adds to a rapidly growing body of literature on the skin microbiome. Skin is the largest mammalian organ, and represents the external barrier between an animal and its environment. The advent of high-throughput sequencing in the past two decades has enabled researchers to characterize the vast diversity of skin microorganisms that previously could not be detected using culture-based techniques. This exciting field has generated new understanding about the interactions between a host and its external microbiota, the impact on host immunity, skin disorders, as well as the distribution of these microbial communities in the context of mammalian skin functioning as a microscopic ecosystem. This research has contributed to two main aspects of these fields. First, the cohabiting couple study contributes to knowledge of the human skin microbiome and the factors that influence prokaryotic distribution across body locations and individuals. Second, the research on the mammalian skin microbiome constitutes one of the first non-human skin microbiome studies and represents, to the best of my knowledge, the world's largest non-human mammalian skin dataset. This data provides a valuable baseline survey of the microorganisms present on mammalian skin that can be applied to veterinarian and dermatology research.

4.1.1 Cohabiting couple study contributions

      The research on cohabiting couples represents the first study to test the accuracy with which couples can be linked based on their skin microbiome. The results showed that living with a partner has a significant impact on the skin microbiome, albeit less than the individual or body region sampled. The strong influence exhibited by the individual and body locations provides

additional confirmation to previous studies that observed these influences (18, 20, 42). Notably, couples could be correctly matched 86% of the time, and could always be matched better than a randomized dataset of incorrect couples. By sampling 17 body locations, the optimal body locations for matching couples could be determined. The feet provided the most classification accuracy, likely due to the presence of transient microorganisms from the floor of their shared environment. Indeed, a longitudinal study on the families and their houses concluded that after moving, the surfaces of a new home rapidly begin to resemble the skin communities of its inhabitants (71).

This study also observed the influences on human skin microbiota from biological sex and lifestyle choices. A notable discovery was that thigh microbial communities are strongly shaped by human biological sex. Although a previous study on hands discovered differences between biological sex (45), the inner thighs included in this study exhibited the most stark difference between the biological sexes of any known skin study to date, due to the influence from the proximal vaginal microbial community of female participants. Furthermore, lifestyle factors also significantly influenced the distribution of prokaryotic organisms. Higher alcohol consumption was correlated with lower skin diversity. Higher skin diversity was associated with pet ownership, which confirms the findings from previous research (150, 187). Moreover, using skin products, such as cosmetics and moisturizers was also associated with higher skin diversity, which may be attributed to the resulting higher moisture levels on the skin environment (188).

4.1.2 Mammalian microbiome study contributions

My research adds to current literature on the mammalian skin microbiome. The primary finding was that humans possess a distinct skin microbial community, not only from other primates, but from almost all other mammals. Human skin had lower community diversity than

animals in the wild, farms, and in zoos, according the Shannon index. However, human skin had similar diversity levels to cats that lived exclusively indoors. This finding suggests that humans have potentially fundamentally shifted their skin microbiome by living indoors, undergoing frequent baths, applying modern skin products, and cosmetics. Pets that live exclusively indoors have a similar microbiome to humans. However, having a dog that frequently goes outdoors results in inclusively indoor cats having similar microbiota to animals that live outdoors.

The mammalian host order was the most significant influence on the skin community, followed by the geographic location according to PERMANOVA F statistics. My research demonstrates that although the skin is external to the host and in contact with the surrounding environment such as soil, that intrinsic factors including the host order and species also significantly influence the microbial community. Previous studies have demonstrated that coevolution has likely occurred between the mammalian gut microbiome and it hosts (7, 143). This study provides the first evidence of phylosymbiosis of skin microbiota within the orders Artiodactyla and Perissodactyla. The microbial dendrograms exhibit significant congruence with known host phylogenies that do not cluster according to geographic location, indicating that skin communities may have co-evolved with their host.

4.2 Future Directions

4.2.1 Cohabiting couple future directions

A natural extension of the cohabiting couples' study (Chapter 2) is to sample a larger number of couples to determine how accurately they can be classified. Although 17 body locations were sampled, 10 couples constitute a very small sample size. The experimental design included a small number of individuals because the goal of the study was to determine which body locations would be best for identifying couples, which required many samples per

individual. Certain body locations, namely the feet, were determined to have high classification abilities, whereas other locations, including the thigh, could not be used to classify couples. Future studies can now sample a larger number of couples using fewer body locations per couple, without the risk of sampling the incorrect body regions for the analysis. Another extension from the research is to include more ethnically diverse participants. Of the 20 participants, 18 were of Caucasian descent. Volunteers were recruited on a first-come basis, without setting ethnicity thresholds. Future studies with larger participant sizes should include a wide range of backgrounds because other studies have shown that differences in skin communities exist between ethnic groups (195, 227).

Future studies should include homosexual couples. Studies with small participant sample sizes are currently unable to include these groups because of the potential ability to identify couples within a specific geographic location. However, large scale studies would be able to maintain patient confidentiality, while answering intriguing questions about how cohabiting with a member of the same biological sex affects the microbiome. For example, skin regions that are strongly influenced by biological sex, such as the thigh (Chapter 2), or hands (45), may be highly successful at classifying homosexual couples because biological sex would no longer be a confounding factor.

Couples with children of varying ages should be sampled in future studies. Intriguingly, a single couple in this study had elevated levels of bacteria associated with the GI tract. This couple had an infant in diapers, and had also recently overcome a gastrointestinal illness. Further work is therefore needed to be completed on families with children of varying ages to determine how living with a child shifts the adult microbiome. Longitudinal studies could be completed over several years that monitor the changing microbiome on both the infant and the parents.

Although previous studies have sampled the skin microbiome of families (150) and identified effects of maternal transfer (114, 119), they did not analyze how the offspring change the ability to match cohabiting couples over time. Additionally, a future study could analyze the effect a gastrointestinal illness has in shifting the skin microbiome. It would be intriguing to determine how long after an illness the causative agent persists on the skin. These findings would have implications for epidemiological tracking of foodborne illnesses.

Furthermore, other sequencing initiatives can be conducted to broaden our understanding of the cohabiting couple skin microbiome, such as sequencing the mycobiome or virome. Fungi and viruses require different primer sets from the 16S rRNA gene to determine their taxonomy. Although human fungal (68) and viral (66, 67) microbiomes have been sequenced, no study has yet determined how similar these are between cohabiting couples. These studies would be able to determine if couples can be matched better using their fungal or viral communities in place of prokaryotic microorganisms.

Studying the human skin microbiome has the potential for numerous applications such as product development that will improve skin health. Indeed, it has been shown that microbial skin diversity can be used as a measure of skin health (228). Companies are currently trying to create cosmetic products that account for the skin microbiota. One method is to use natural products that have been shown to not shift the microbial community, whereas a second strategy is to apply a live culture of skin microbiota as a probiotic similar to how yogurt has been formulated to improve gut health. The aim of these products is to reduce allergies, decrease body odour and the need for daily showers, as well as to improve aesthetics and prevent premature skin aging.

4.2.2 Mammalian skin microbiome future directions

The mammalian portion of this research (Chapter 3) presented a baseline dataset of the bacteria and archaea located on mammalian skin. An extension of this project would be to use the already acquired extracted DNA from the skin swabs included in this study for additional sequencing studies. Each sample was aliquoted after DNA extraction, resulting in DNA samples that have not undergone freeze-thaw cycles. These samples have the potential to provide more information about the mammalian skin microbiome. Specific samples could be selected for metagenomics sequencing. Metagenomic sequencing is a method that uses shotgun sequencing to create draft genomes and provides the genetic information for all genes present in an environmental sample, instead of only the 16S rRNA gene that allows for the prokaryotic taxonomy to be elucidated (229). Indeed, metagenomics represents a rapidly growing field in microbiome research, and has led to numerous advances including the discovery of novel microorganisms (230), information on biogeochemical cycles (231), viral population dynamics (232), microbial evolution (233) and resulted in a recent major update on the tree of life (234). Metagenomic sequencing would be a natural extension to the FAPROTAX analysis presented in Chapter 3, and would enable comparison between putative functions and the actual functions from within a single sample. Although it would not be feasible to complete metagenomic sequencing on all samples, due to current cost and low biomass limitations, several samples with sufficient DNA concentrations could be selected from a range of animals in the wild, zoos, farms, and households to elucidate the biochemical functions of the organisms that are present on mammalian skin.

Additional uses for the library of mammalian skin samples include conducting further tests to more accurately analyze the distribution of archaea, to find stronger evidence for

coevolution, and to sequence mammalian genomes. Known mismatches exist between the prokaryotic primer set and archaea specific primers, resulting in an underestimation in the number of archaea present (175). Samples could be sequenced using an archaeal specific primer set, such as Arch516F/Arch915R, to better capture archaea distribution. Specifically, cape elands, olive baboons, sable antelope, and bovine samples are ideal animals to sequence because they possessed higher number of archaeal sequences than any other mammalian species included in this study. Another exciting usage for the current library would be to further explore the possibility of evolution. Although the 16S rRNA gene provides initial microbial data to study phylosymbiosis, the resulting datasets do not possess the strain-level resolution required to observe if the evolution of specific bacterial strains match that of their mammalian hosts. DNA gyrase subunit B (*gyrB*) has been used previously to determine that coevolution has occurred within the guts of hominids (143). Several bacterial families of interest, such as *Staphylococcaceae* (e.g., *Macrococcus)* present on the skin of animals within the mammalian order Perissodactyla, would be sequenced using *gyrB* primers. Both the order Perissodactyla and Artiodactyla should be examined based on the phylosymbiosis analysis presented in Chapter 3. A final use for the skin library would not involve the study of prokaryotic organisms. Each extracted DNA sample contains a mammalian host DNA obtained from the skin swabs. Approximately half of the mammalian species included in this study do not have complete genomes sequenced. Closing several host genomes using samples with higher DNA concentrations would further contribute the field of mammalian genetics and provide valuable information about the evolution of specific genes. Animals with no known completed genome according to the NCBI genome database include reindeer, arctic wolves, red kangaroos, Asian elephants, and squirrels.

An additional consideration is to conduct studies using an expanded number of hosts and locations. Many of the species included in this study had a low number of animal participants due to the opportunistic nature of access to animals. More animals from each species need to be sampled to elucidate an accurate skin microbiome within each species. New sampling should strive to include an equal balance of both biological sexes to have the statistical resolution required to determine which species are influenced by factors attributed to biological sex, for example hormones and secretion levels. Moreover, more body regions should be tested on each animal to elucidate if microbial communities shift by skin location, as is observed in human studies (20, 46, 47). Regions not covered by hair would be valuable to include in the future, as they have been observed to possess significantly lower levels of diversity than hair-covered locations on dogs (51).

Including an equal number of samples per metadata category of interest will improve the accuracy of random forest modelling because it will avoid overlearning on categories that have a larger number of samples (194). Additionally, including only small numbers of samples has the potential for many metadata categories to falsely appear to have an insignificant effect on the microbial community. It is possible that the null hypothesis is being incorrectly rejected (false negative) or accepted (false positive) (235), when instead more sampling is required to observe the true effect on the community. An additional limitation of small sample sizes includes biases caused by sampling error exhibiting a larger influence than in studies with many samples (236). The number of samples required for a microbiome study vary depending on the expected effect size (237), which can be estimated based on previous studies in the field where available. Currently, tools such as "Evident" exist to provide researchers with an approximate estimate of microbiome sample size that is required for their specific study design (237).

Microbiome studies that analyze the 16S rRNA gene involve sequencing DNA from both viable and non-viable microorganisms. The current study is unable to discern whether the OTUs are living and contributing to the skin microbiome, or if they represent DNA from dead cells. Protocol modifications exist to gain further information on the viable cells. Total RNA can be sequenced to characterize recently transcribed genes. Fluorescent in-situ hybridization with 16S rRNA gene probes have been used to enumerate human microbiome samples from the gut (238).

Future work could analyze in greater depth the effects of extrinsic factors, such as geographic location and abiotic factors within the environment. Animals would ideally be studied from multiple geographic locations, both locally and at a global scale to more accurately determine the effects from geographic location. Previous studies have shown that humans (73), amphibians (55, 79), komodo dragons (154), and bats (81) experience shifts in their skin microbiome over geographic distance, which implies that sampling animals over a greater geographic distribution results in larger shifts in skin microbial communities. Additionally, the current study did not include samples from the abiotic environment within cages. However, a previous study has shown significant similarity between forest debris and the skin microbiome of amphibians (84). Including soil and water samples from each enclosure would provide more evidence on the effects of surroundings on host skin. Indeed, random forest modelling could be used to determine if mammals can be correctly classified to their cage based on the soil and water present. Moreover, previous human studies have repeatedly sampled the same human participants over a long period of time to determine the levels of stability exhibited by the skin microbiome (18, 46, 71, 98). Temporal tests could be conducted on a wider range of mammalian hosts to determine how stable the environment is for microbial communities, as well as to determine if seasonal shifts occur. It may be that environmental factors influence animals that

live outdoors due to the significant annual variation in humidity and temperature in South-Western Ontario.

This research has resulted in practical and philosophical implications of sampling allocthonous and autochthonous microorganisms in microbiome studies. Although the majority of sequences were classified as non-environmental, humans had a much higher proportion of traditionally classified autochthonous skin organisms, compared to mammals with more OTUs that are considered to originate from soil. This finding leads to philosophical implications of what constitutes the true skin microbiome. Is the skin microbiome what remains after the skin has been washed? Or are microorganisms that originate from the soil an integral part of the mammalian skin microbiome, with the ability to survive on skin and interact with the host? This research has practical implications for how mammals should be sampled. Traditionally mammalian skin studies do not include a washing step, in contrast to amphibian skin studies. It is possible that by not washing the mammals before sampling that they cannot be accurately compared to humans that frequently shower and undergo modern hygiene regiments. Future studies are encouraged to explore the implications from this study and determine if a different sampling method yields a more accurate depiction of the true skin microbiome.

Lastly, this research provides crucial baseline data of the healthy skin microbiome. Studies can now test the differences between healthy vs. diseased or allergic animals to determine which microorganisms experience shifts in relative abundance between disease states. This information would aid in determining if microorganisms contribute to skin disorders, such as canine atopic dermatitis, lick granulomas, autoimmune diseases, Alabama rot, or dermatophilosis, similar to what has been accomplished for bovine digital dermatitis (123). Understanding which microorganisms may contribute to disease states, and conversely which

can prevent disease, is valuable information that would contribute to both veterinary medicine and animal husbandry practices. It may be possible to create a probiotic of protective skin microorganisms to prevent disease in livestock, and therefore have significant economic advantages and improve the safety of the food supply.

To recapitulate, this thesis research explored the distribution of bacteria and archaea on mammalian skin. Cohabiting human couples can be successfully matched more often than randomly paired couples. Despite cohabitation being a significant influence on the skin microbiome, it was exceeded by the body region sampled and the effects from individuality. Other factors that modestly influenced the human skin microbiome included alcohol consumption, pet ownership, and the use of hygiene products. Furthermore, this work determined that human skin is unique not only from other primates, but from the majority of all mammalian orders. Humans were dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium*, and shared similar communities to pets that lived predominately indoors and were groomed frequently. In contrast, mammals sourced from farms, zoos, and the wild had higher levels of diversity and soil associated indicator organisms. The mammalian phylogeny within the orders Perissodactyla and Artiodactyla was significantly congruent with the microbial community dendrograms, providing the first known evidence that phylosymbiosis may be occurring between the skin microbiome and its hosts.

This body of work makes a significant contribution to the fields of microbial ecology and zoology by elucidating the distribution of microorganisms on the skin of 38 mammalian species. Ed Yong poeticized in his book I Contain Multitudes that "All zoology is really ecology. We cannot fully understand the lives of animal without understanding our microbes and symbioses with them. And we cannot fully appreciate our own microbiome without appreciating how those

of our fellow species enrich and influence their lives. We need to zoom out to the entire animal kingdom, while zooming in to see the hidden ecosystems that exist in every creature (239)." Indeed, this research has furthered our understanding of symbioses within the animal kingdom by delving "skin deep" into the multitudes we contain.

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Appendix

<u>Sample Collection Documents</u>

<u>Human Skin Microbe Survey</u>

Thank you for participating in the Human Skin Microbe study conducted by Dr. Josh D. Neufeld and Ashley Ross. The following questions will be used to understand which factors influence the organisms that live on human skin. This information is confidential and will be anonymized to a set of codes so that responses cannot be traced back to an individual. Please answer all questions, and as carefully as possible. If unwilling to answer any questions, this will result in withdrawal from the study, and you should do so if at all uncomfortable with the questions asked. In other words, please do not feel obliged to participate and feel free to withdraw from the study at any time.

ID #:

Date Sampled:

1. Gender:
   Female
   Male
   Other:_____ (optional)

2. Which of the following best represents your racial heritage?
   African
   Asian
   Caribbean
   Caucasian
   East Asian
   Hispanic/Latino
   Indigenous
   Middle Eastern
   Mixed race
   South Asian
   Other: _____

3. Indicate your age group:
   20-29
   30-39
   40-49
   50-59
   60+

4. Are you left or right handed?
   Left

Right

5. How long have you been cohabiting partners?

_____

6. Are you sexually active with your partner (defined as sexual intercourse > once per month)?
	Yes
	No

7. How many people are in your household?
_____
8. Do you live with any pets?
	Yes	List # and type:_____
	No

9. When is the last time you showered?
	This morning
	1-2 days ago
	Within the last week
	Within the last month
	Within the last year
	>1 year ago

10. How often do you shower on average?
	Every day
	Every other day
	Once per week
	Once per month
	Other:_____

11. How often do you exercise (>20 min, vigorous)?
	Once a week
	Twice a week
	Three times a week
	>4 times a week
	Never

12. Do you consider your skin type to be:
	Normal
	Dry
	Oily

Combination of the above. Describe:_____

13. Which of the following skin products do you use? Check all that apply.
       Soap
       Shampoo
       Conditioner
       Moisturizer
       Deodorant
       Antiperspirant
       Perfume
       Cosmetics
       Shaving cream
       Exfoliants/scrubs
       Antibacterial ointments
       Sunscreen
       Facial cleansers
       Anti-aging cream
       Hand sanitizer
       Other: _____

14. Have you experienced any of the following skin conditions in the last year (check all that apply)?
       Eczema
       Acne
       Excess sweat
       Sensitive skin
       Itchy skin
       Flaky skin
       Rashes
       Psoriasis
       Impetigo
       Warts
       Hair loss
       Rosacea
       Other:_____

15. Do you have any allergies?
       Yes:_____
       No

16. How do you characterize your usual diet?
       Omnivore
       Vegetarian
       Vegan

Other:_____

17. Do you smoke?
      Yes   How often do you smoke?_____
      No

18. How frequently do you consume alcohol?
      Multiple servings per day
      1 serving per day
      2-3 per week
      One serving per week
      One serving per month
      Rarely/Never
      Other:_____

19. Do you chew your nails?
      Yes
      No

20. Do you wear glasses?
      Yes
      No

21. Have you taken any antibiotics in the last month?
      Yes
      No

22. How much time on average do you spend outdoors daily?
      <1 hour
      1-3 hours
      4-6 hours
      7-10 hours
      >10 hours

Sampling Instructions

1. Wear provided gloves.

2. Remove pre-labeled sterile swab from packaging by twisting blue handle to break seal. Grasp swab by the blue handle and pull to release swab. Do not allow the swab to contact non-target surfaces. Discard if contaminated and use the spare swab provided instead (please label).

3. Rub one side of the applicator ~5 times on the skin (back and forwards, not side to side). Be sure to press down while swabbing adding pressure as you move it back and forth across the skin. Rotate the swab and repeat in adjacent regions for a total of ~20 strokes.

4. When area is complete, return swab to the initial plastic storage container and close tightly.

5. Repeat steps 1-3 for all skin sites (refer to attached diagram). Skin sites to be sampled include:

1. Left upper eyelid
2. Right upper eyelid
3. Left outer nose
4. Right outer nose
5. Left inner nostril
6. Right inner nostril
7. Left armpit
8. Right armpit
9. Torso
10. Back
11. Belly button
12. Left inner thigh
13. Right inner thigh
14. Bottom of left foot
15. Bottom of right foot
16. Left palm (entire surface including fingers; remove gloves before sampling)
17. Right palm (entire surface including fingers; remove gloves before sampling)

6. Fill out the associated survey.

7. Place swab and survey back in the zip-loc bag provided, then store in freezer until returned to the researcher.

Animal Skin Microbe Survey Sampling Sheet
Please sample healthy and sexually mature adult mammals that have not been exposed to antibiotics in the previous 6 months.

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Sampling Kit #: _____
Location and address (e.g., Pet owner name and address/Toronto zoo/Farm and address):

Animal type/species:

Internal Identification (e.g., Horse 1):

Gender:

Date sampled:

Age:

Health status (circle one): Healthy or Diseased   Details:

Co-habits with # animals and species:

Known exposure to antibiotics:

Diet (circle one): Carnivore or Omnivore or Herbivore

Neutered/Spayed (circle one): Yes or No

Hygiene history (e.g., monthly grooming, brushing, none):

Additional comments:

<h1 style="text-align:center">Animal Skin Sample Collection Protocol</h1>

1. Remove pre-labeled sterile swab from packaging by twisting blue handle to break seal. Grasp swab by the blue handle and pull to release swab. Do not allow the swab to contact non-target surfaces. Discard if contaminated. *Note that swabs are already labelled with the corresponding body sites.*

2. Gently move aside the fur to expose skin. While applying moderate pressure, rub one quarter of the applicator 10 times on the skin in a forwards and backwards motion. Rotate the swab and repeat in adjacent regions for a total of 40 strokes.

3. When area is complete, return swab to the initial plastic storage container and close tightly.

4. Repeat steps 1-3 for the two other skin sites. Skin sites to be sampled include: back, underchest, and inner thigh.

5. Fill out the included sampling sheet for each animal sampled.

6. Replace the sampled swabs and completed sheet in sampling bag and seal.

7. Place sampling bag in a freezer (avoid frost-free freezers if possible) until returned to the University of Waterloo.