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Comparison of the Fecal Bacterial Microbiota Composition Between Wild and Captive Bolson Tortoises (*Gopherus flavomarginatus*)

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Abstract.—**Research analyzing fecal microbiota provides important insights into the biological mechanisms affecting the health of animals. The Bolson Tortoise (*Gopherus flavomarginatus*) is endemic to northern Mexico and is threatened mainly by habitat loss and collecting by humans. Here, we characterized the fecal bacterial communities of wild (10 samples) and captive (10 samples) *G. flavomarginatus*, and compared their microbiota analyzing the V3–V4 region of 16S rRNA gene using high-throughput sequencing technology on Illumina Miseq platform. We identified 21 phyla, 44 classes, 66 orders, 139 families, and 463 genera for all *G. flavomarginatus* fecal samples. Bacterial communities showed significant differences between wild and captive tortoises at each taxonomical level. Firmicutes and Cyanobacteria were the most predominant phyla in wild tortoises. In captive tortoises, Firmicutes was the dominant phylum, followed by Fibrobacteres. At the genus level, *Hydrogenispora*, GQ422712_g, and *Acetivibrio* were significantly more abundant in the wild population, whereas LDWW_g, HQ716403_g, AB239481_g, and *Sporobacter* predominated in the captive population. Diet could be the main factor responsible for differences between fecal microbiotas. All the individuals we analyzed were clinically healthy, which would mean that their bacterial communities are in apparent equilibrium. Nevertheless, the differences between the fecal bacteria of wild and captive tortoise populations indicate that, prior to releasing captive animals into the wild, inoculating them with wild-tortoise bacteria may improve their success at adjusting to a wild diet**.

Key Words.—Fibrobacteres; Firmicutes; high-throughput sequencing; 16S rRNA gene

Introduction

The gut microbial community of animals is composed of archaea, bacteria, fungi, yeasts, protozoa, viruses, and bacteriophages (Turner 2018). They have co-evolved with each host species affecting digestion, nutrition absorption, maintenance of intestinal mucosal integrity and gut peristalsis, development of immunity and immunomodulation, metabolism of xenobiotics, and disease resistance (Berg 2014; Clarke et al. 2014; Shang et al. 2018; Turner 2018). Also, the gut microbiota affects other organ systems (liver, lungs, brain, and skin) influencing their function (Kamada et al. 2013).

Herbivorous reptiles have a hindgut containing high concentrations of bacteria working in an endosymbiotic relationship (Hong et al. 2015). This microbiota produces enzymes needed to ferment carbohydrates such as structural cellulose and hemicellulose that comprises much of their diet (Stevens and Hume 2004). This fermentation produces short chain fatty acids like acetate, propionate, and butyrate (they provide more than 30% of the daily energy that is required for this type of animal), as well as vitamins and amino acids (Mackie et al. 2004; Stevens and Hume 2004).

Knowledge of the gut microbial communities of *Gopherus* tortoises is limited (Yuan et al., 2015). Tortoises are considered keystone species, with ecosystem functions that include burrow construction, which provides habitat for many other vertebrates (Dziadzio and Smith 2016), and seed dispersal due to their herbivore/frugivore diets (Carlson et al. 2003). The Bolson Tortoise (*Gopherus flavomarginatus*; Fig. 1) is endemic to north-central Mexico (Ureña-Aranda et al. 2015). Loss of habitat and gathering

tortoises from the wild for private collectors and food are the main factors that cause this tortoise to be considered endangered according to the Norma Oficial Mexicana 059 (Secretaría de Medio Ambiente y Recursos Naturales, 2010), and is listed as Critically Endangered on the Red List of the International Union for Conservation of Nature (Kiester et al. 2018). Its geographical distribution is restricted to the Bolson de Mapimi in the Chihuahuan Desert of Mexico where it is currently protected in the Mapimi Biosphere Reserve (Comisión Nacional de Áreas Naturales Protegidas 2006). Additionally, there are several populations of *G. flavomarginatus* in captivity; one of them is located in the Unidad Regional Universitaria de Zonas Áridas (URUZA), Universidad Autónoma de Chapingo, in Tlahualilo, Durango, (Castro-Franco et al. 2007). This population was founded in 1997, when approximately 25 *G. flavomarginatus* adult individuals were seized from dealers on the outskirts of the Reserve; this population has been maintained for the purpose of conservation and education (Rafael Castro-Franco, pers. comm.).

Maintaining the health of captive individuals, providing all the necessary requirements to sustain them outside their natural habitat remains a challenge. Analyses of the intestinal bacterial microbiota can provide useful information to establish the health status of the hosts and improve strategies for conservation and management of threatened species (Amato 2013; Xie et al. 2016). Intestinal microbiota improve nutrition and energy acquisition, synthesis of essential vitamins, immune system stimulation, and defense from invasive pathogens in the host (O'Hara et al. 2006; Nizza et al. 2014; Pagliari et al. 2015; Shapira 2016).

Intestinal bacterial microbiota in vertebrates vary according to differences in their environment and this also might be true for wild versus captive individuals (Xenoulis et al. 2010; Wienemann et al. 2011; Guan et al. 2017). Keeping individuals in captivity may alter the microbial communities compared to wild populations. Therefore, bacterial microbiota may be an important consideration for conservation programs seeking reintroduction of specimens to their natural habitat (Xie et al. 2016). Information on the intestinal microbial diversity is not available for *G. flavomarginatus* and for most reptiles, however, so our objective for this study was to characterize and compare the fecal bacterial composition between two populations of the *G. flavomarginatus* (wild and captive), providing information that supports decision making for the conservation of this species.

Materials and Methods

Study sites.—We sampled wild tortoises at the 3,423.9 km^2 Mapimi Biosphere Reserve, which includes part of the states of Chihuahua, Coahuila, and Durango (26°00' and 26°10'N, 104°10' and 103°20'W), Mexico. We sampled captive tortoises at URUZA (25°33'51"N, 103°36'43"W). Both areas have warm, very arid climate (García 2004), with an average annual temperature of 25.5° C, and an average annual precipitation of 264 mm (Cornet 1988). The predominant vegetation in the reserve is rosette and microphile scrub, as well as halophyte, and gypsophila plants (Rzedowski 2006).

Field work.—From May to September 2015, we collected fecal samples from 20 physically healthy adult individuals (10 wild and 10 captive). For each individual, we recorded their straight-line carapace length (CL) and width (CW; each to 0.1 cm) using a tape measure, and weight (W) using a digital scale (0.01 kg). We evaluated each tortoise for clinical health using standard protocols (Homer et al. 1998; Wendland et al. 2009; Jacobson 2014; USFWS 2016). Individuals often defecated during data collection and we collected their full excrement immediately using sterile vials. From each fecal sample, we collected 0.25 g from the center of the sample and deposited in BashingBead™ cell lysis tubes (Zymo Research Corp., Irvine, California, USA) adding 750 µl of lysing/stabilizing solution. Each tube was processed in a TerraLyzer™ cellular disruptor (Zymo Research Corp.) for 20 sec according to the specifications of the manufacturer. We then released tortoises at the site of capture.

Laboratory work.—We extracted DNA from the samples using the Xpedition™ Soil/Fecal DNA MiniPrep kit (Zymo Research Corp.) in a laminar UV flow hood in sterile conditions. We ran the extracted DNA on a 1.2% agarose gel at 80V for 45 min in a BIO-RAD electrophoresis chamber (Bio-Rad Laboratories, Inc., Hercules, California, USA) to visualize the presence of high molecular weight DNA. The visualization was carried out in a GelMax™ photo documenter (UVP LLC; Upland, California, USA). The amount of DNA obtained was measured in a Qubit™ fluorometer (Invitrogen, Carlsbad, California, USA). We amplified the V3-V4 region of the 16S rRNA gene using the following primers (Klindworth et al. 2013): S-D-Bact-0341-b-S-17, 5′-CCTACGGGNGGCWGCAG-3′ and S-D-Bact-0785-a-A-21, 5′-GACTACHVGGGTATCTAATCC-3′, which produces an amplicon of about 460 bp. These primers were synthetized along with overhang adapters: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′ (amplicon of about 550 bp) according to the Illumina protocol (Illumina. 2013. 16S Metagenomic Sequencing Library Preparation (15044223 B). Available from https://support.illumina.

Figure 1. A juvenile Bolson Tortoise (*Gopherus flavomarginatus*) from the Mapimi Biosphere Reserve, Mexico. (Photographed by Cristina García-De la Peña).

com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf [Accessed 15 March 2018]).

We performed the Illumina PCR protocol (Illumina 2013, *op. cited*) by using 12.5 µl of MyTaq™ Ready Mix 1X (Bioline, London, UK), 1 µl of each primer (10 nM), 5 µl of DNA (50 ng total) and 5.5 µl of molecular grade H_2O. We used the following cycle: 95° C for 3 min; 25 cycles of 95° C for 30 sec, 55° C for 30 sec, 72° C for 30 sec; and 72° C for 5 min in a Labnet Multigene™ Gradient PCR thermal cycler (Labnet International, Inc. Global, Edison, New Jersey, USA). We placed 1 µl of each PCR product in an Agilent Bioanalyzer DNA 1000 chip (Agilent Technologies, Inc., Santa Clara, California, USA) to verify amplicon size (about 550 bp). We then purified the amplicons with Agentcourt® AMPure® XP 0.8% beads (Beckman Coulter Inc., Brea, California, USA). Next, we used Nextera XT Index Kit™ (Illumina, Inc., San Diego, California, USA) to create the library, following the Illumina protocol (Illumina. 2016. Nextera DNA Library Prep Reference Guide (15027987 v01). Available from https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nexteradna/nextera-dna-library-prep-reference-guide-15027987-01.pdf [Accessed 15 March 2018]) using 25 µl of MyTaq™ Ready Mix 1X (Bioline®), 5 µl of each primer (N7xx and S5xx), 5 µl of DNA, and 10 µl of molecular grade H_2O. We put each sample through the following cycle: 95° C for 3 min; 10 cycles of 95° C for 30 sec, 55° C for 30 sec, 72° C for 30 sec; 72° C for 5 min. We purified the libraries with Agencourt® (AgenCourt Bioscience Corporation, Beverly, Massachusetts, USA) AMPure® XP 1.2% beads. We randomly selected 1 µl of the final PCR products library and placed it on a Bioanalyzer DNA 1000 chip to verify the size of the amplicon waiting for a size of about 630 bp. Finally, we performed quantification, normalization (equimolarity), and next generation massive sequencing (MiSeq; Illumina, San Diego, California, USA) of 2 × 250 paired final readings following the 16S metagenomic protocol (Illumina 2013, *op. cited*).

Bioinformatic analysis.—We analyzed DNA sequences on MGLinux, in a VM Oracle VirtualBox 5.1.14 using Quantitative Insights into Microbial Ecology bioinformatics software (QIIME) v.1.9.0 (Caporaso et al. 2010). We assembled forward and reverse sequences using PEAR program (Zhang et al. 2014) with an overlap of 50 bp, a minimum reading length of 430 bp and a maximum of 470 bp, a quality criterion Q30 (one false base for every 1000 bases) with $P < 0.0001$. Then we converted the files to FASTA format and eliminated chimeric sequences with USEARCH (Edgar 2010). We selected operational taxonomic units (OTUs) with the UCLUST method (Edgar 2010) at 97% similarity; we obtained a representative sequence for each OTU and the taxonomy taking as reference the EzBioCloud database (Yoon et al. 2017). We built OTUs tables in Biom format (Biological observation matrix; McDonald et al. 2012) separating domains and eliminating singletons (Navas-Molina et al. 2013). We obtained absolute abundance of OTUs tables and graphed the number of sequences by the number of taxa to genus level to see if we achieved an adequate coverage (asymptote curves); this graph was made in PAST ver 3.15 (Hammer et al. 2001). We made a simple random rarefaction process of 55,000 sequences (Weiss et al. 2017) to obtain a standardized Biom file for all samples.

We represented relative abundance of phylum, class, and order in Excel, using heatmaps to identify family and genus levels (we represented only taxa whose relative abundance was greater than 0.01% for family and 0.1% for genus); for heatmaps, the method of hierarchical conglomerates with Euclidean measurement was used to visualize samples dendrogram. We made heatmaps in Morpheus software (https://software.broadinstitute.org/GENE-E/). We used Bray-Curtis similarity percentage analyses (SIMPER; Clarke 1993) with Mann-Whitney U test ($P < 0.05$) to find the bacterial taxa that largely contributed to the differentiation of fecal microbiota between populations; we considered only taxa with relative abundance > 1%. We made these analyses in PAST, ver 3.15.

We calculated Shannon and Simpson alpha diversity indexes using the standardized Biom file and used Monte Carlo permutations (999) to calculate the *P*-value (compare_alpha_diversity.py script from QIIME) to test significant difference between populations. We presented alpha diversity indexes as the means ± one standard deviation (SD). We calculated beta diversity matrix using the Bray-Curtis index (Beals 1984); we used this matrix to make a PERMANOVA test ($P < 0.05$) to show significant difference of the fecal

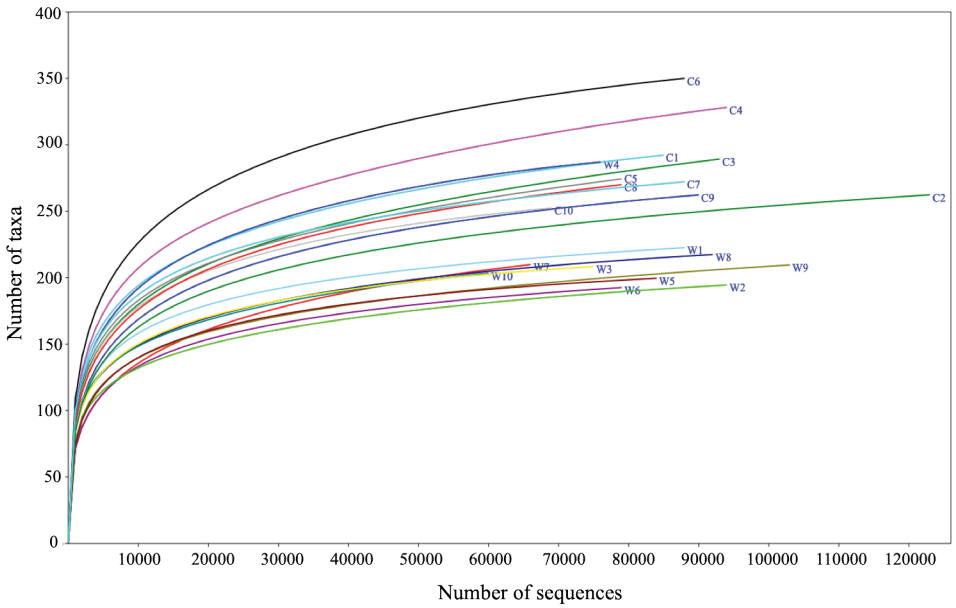

Figure 2. Rarefaction curves for fecal bacterial taxa identified from wild (W) Bolson Tortoises (*Gopherus flavomarginatus*) in Mapimi Biosphere Reserve, Mexico, and from captive (C) tortoises in the Unidad Regional Universitaria de Zonas Áridas (URUZA).

microbiota between tortoise populations. Finally, we made Principal Coordinate Analyses (PCoA) for both populations, and visualized it using Emperor software (Vázquez-Baeza et al. 2013).

Results

Wild tortoises had an average carapace length of 31.8 cm ± 5.8 (SD), carapace width of 26.3 cm ± 4.3, and weight of 9.7 kg ± 2.9; captive tortoises were 29.2 cm ± 4.2 CL, 25.1 cm ± 3.7 CW, and 9.2 kg ± 2.4 W. The average number of sequences assembled in fecal samples was 118,002 for wild tortoise and 126,400 for captive tortoises. After taxonomic designation and singletons removal, we obtained an average of 84,430 bacterial sequences for wild and 169,271 for captive tortoises. The average number of OTUs was 6,002, and 6,674, respectively (Table 1). Rarefaction curves for the OTUs detected in this study showed that the quantity of observed species increased as the sequencing depth increased; curves reached asymptotes near 60,000 sequences (Fig. 2).

We identified organisms belonging to 21 phyla, 44 classes, 66 orders, 139 families, and 463 genera for all *G. flavomarginatus* fecal samples. We recorded 19 bacterial phyla in the wild tortoise population, and 21 in the captive population. Firmicutes was the most abundant phylum in both populations averaging 93% and 80%, respectively. Fibrobacteres was the second most abundant phylum in the captive population (11%), while Cyanobacteria dominated in the wild (2.6%, Fig. 3). According to SIMPER analysis, both populations differed in relative abundance of eight phyla: Firmicutes, Cyanobacteria, and Lentisphaerae were significantly more abundant in the wild population, whereas Fusobacteria, Fibrobacteres, Parcubacteria_OD1, Proteobacteria, and Spirochaeta predominated in the captive population (Table 2).

We recorded 33 bacterial classes in the wild tortoise population and 42 in captivity. Clostridia was the most abundant in both populations averaging 73% and 79%, respectively; Negativicutes was the second most abundant in wild population (19%), whereas Chitinivibrionia (11%) was in the captivity population (Fig. 4). According to SIMPER analysis both populations differed in relative abundance of six classes; Negativicutes and Vampirovibrio_c were significantly more abundant in the wild population, whereas Chitinivibrionia, Spirochaetes_c, Paceibacter_c and Epsilonproteobacteria predominated in the captivity population (Table 2).

We recorded 53 bacterial orders in the wild tortoise population, and 60 in the captive population. Clostridiales was the most abundant in both populations averaging 73% and 79%, respectively; Hydrogenispora_o was the second most abundant in wild population (19%), whereas Chitinivibrionales (11%) was in the captive population (Fig. 5). According to SIMPER analysis both populations differed in relative abundance of six orders; Hydrogenispora_o and FR888536_o were significantly more abundant in wild population, whereas Chitinivibrionales, Spirochaetales, CP011215_o, and Campylobacterales predominated in the captive population (Table 2).

Table 1. Fecal sequences of Bolson Tortoises (*Gopherus flavomarginatus*) from wild (W) and captive (C) populations. Abbreviations are ChS = chimeric sequences eliminated, QS = quality sequences after chimeras elimination, BS = bacteria sequences after taxonomy designation, BSS = bacteria sequences after singletons elimination, and OTUs = operational taxonomic units.

Sample	Total	Assembled	Discarded	ChS	QS	BS	BSS	OTUs
W1	249,434	113,497	135,937	479	113,018	102,500	79,103	6,506
W2	230,612	127,352	103,260	1043	126,309	118,122	90,824	6,423
W3	218,503	127,935	90,568	474	127,461	116,933	86,752	6,675
W4	259,329	112,107	147,222	843	111,264	103,799	81,675	5,781
W5	273,855	95,763	178,092	396	95,367	83,183	68,573	4,607
W6	266,287	132,391	133,896	289	132,102	119,844	95,256	6,722
W7	277,616	143,212	134,404	348	142,864	121,576	105,606	6,699
W8	256,784	132,718	124,066	141	132,577	132,231	96,804	6,355
W9	271,927	105,796	166,131	155	105,641	96,257	77,363	5,194
W10	204,021	89,246	114,775	118	89,128	78,704	62,344	5,060
Mean	250,837	118,002	132,835	429	117,573	107,315	84,430	6,002
C1	268,330	121,233	147,097	521	120,712	109,746	870,000	6,811
C2	333,395	176,399	156,996	1250	175,149	160,187	125,347	7,557
C3	325,546	132,334	193,212	325	132,009	119,590	94,741	6,990
C4	331,566	134,319	197,247	546	133,773	122,822	96,344	7,010
C5	297,758	115,372	182,386	238	115,134	104,037	80,542	6,454
C6	267,587	123,818	143,769	540	123,278	115,223	90,337	7,162
C7	312,239	120,170	192,069	513	119,657	111,358	90,777	6,337
C8	294,412	113,283	181,129	549	112,734	104,514	81,360	6,351
C9	293,750	126,376	167,374	1013	125,363	116,624	92,611	6,228
C10	277,480	100,699	176,781	284	100,415	92,539	70,651	5,843
Mean	300,206	126,400	173,806	578	125,822	115,664	169,271	6,674

We recorded 95 bacterial families in the wild tortoise population and 126 in captivity. Ruminococcaceae was the most abundant in both populations averaging 26% and 27%, respectively; followed by Christensenellaceae averaging 24% and 25%, respectively. Hydrogenispora_f was the third most abundant in the wild population (19%), whereas Lachnospiraceae (18%) was in the captive population (Fig. 6). According to SIMPER analysis both populations differed in relative abundance of five families; Hydrogenispora_f and FR888536_f were significantly more abundant in the wild population, whereas LDWW_f, Leptospiraceae, CP011215_f, and FR888536_f predominated in the captive population (Table 2).

We recorded 344 bacterial genera in the wild tortoise population, and 425 in captivity. *Hydrogenispora* was the most abundant in the wild population (19%), and in captivity predominate an unknown genus of the LDWW family (11%), *Clostridium* (4.3%), and *Sporobacter* (3.8%; Fig. 7). According to SIMPER analysis both populations differed in relative abundance of 17 genera; *Hydrogenispora*, GQ422712_g, and *Acetivibrio* were significantly more abundant in the wild population, whereas LDWW_g, HQ716403_g, AB239481_g, and *Sporobacter* predominated in the captive population (Table 2). Most genera determined for both species possess only an identification key since only 191 have taxonomic name.

The Shannon diversity index for wild tortoises was 8.20 ± 0.60 (SD), compared to 8.59 ± 0.52 in captive tortoises. The Simpson diversity index for wild tortoises was 0.96 ± 0.02, compared to 0.97 ± 0.01 in captive tortoises. There was no significant difference between populations in alpha diversity indices (Shannon: $P = 0.109$; Simpson: $P = 0.220$). There was significant difference between both populations, however, using Bray-Curtis beta diversity matrix (PERMANOVA: pseudo-$F = 8.280$, $P < 0.001$); the separation between groups was evident in the PCoA (Fig. 8).

Discussion

The dominant phyla of fecal bacteria in both wild and captive populations of *G. flavomarginatus* was Firmicutes, which plays an important role in the metabolism and digestion in the host, comprising part

Table 2. Percentage similarity analysis (SIMPER) of fecal bacteria from Bolson Tortoises (*Gopherus flavomarginatus*) at phyla, class, order, family, and genus levels. Abbreviations are Av. dissim = average dissimilarity, W = wild, C = captive; and an asterisk (*) = significant difference based on Mann-Whitney U test.

	Taxon	Av. dissim	Contribution %	Cumulative %	Mean W	Mean C	*U*	*P*
Phylum	Firmicutes	6.410	40.78	40.78	0.931	0.805	6	0.001*
	Fibrobacteres	5.120	32.61	73.39	0.016	0.117	12	0.004*
	Spirochaetes	0.929	5.91	79.30	< 0.001	0.018	0	0.000*
	Parcubacteria_OD1	0.908	5.78	85.09	< 0.001	0.018	0	0.000*
	Cyanobacteria	0.830	5.29	90.38	0.026	0.010	9	0.002*
	Proteobacteria	0.387	2.46	92.85	0.002	0.009	5	<0.001*
	Synergistetes	0.381	2.42	95.28	0.010	0.010	44	0.677
	Elusimicrobia	0.285	1.81	97.09	0.005	0.002	42	0.570
	Lentisphaerae	0.158	1.00	98.10	0.004	0.001	18	0.017*
	Actinobacteria	0.155	0.99	99.09	0.001	0.003	28	0.104
	Tenericutes	0.092	0.58	99.68	0.001	0.002	28	0.104
	Fusobacteria	0.024	0.15	99.84	< 0.001	< 0.001	11.5	0.002*
Class	Negativicutes	9.165	37.93	37.93	0.192	0.008	0	<0.001*
	Clostridia	5.413	22.40	60.33	0.737	0.794	35	0.273
	Chitinivibrionia	5.123	21.20	81.53	0.016	0.117	12	0.004*
	Spirochaetes_c	0.929	3.84	85.38	< 0.001	0.018	0	<0.001*
	Vampirovibrio_c	0.784	3.24	88.62	0.024	0.009	14	0.007*
	Paceibacter_c	0.698	2.89	91.52	< 0.001	0.014	0	<0.001*
	Synergistia	0.381	1.57	93.09	0.010	0.010	44	0.677
	Epsilonproteobacteria	0.322	1.33	94.43	< 0.001	0.006	0	<0.001*
	Elusimicrobia_c	0.285	1.18	95.61	0.005	0.002	42	0.570
Order	Hydrogenispora_o	9.165	37.66	37.66	0.192	0.008	0	<0.001*
	Clostridiales	5.372	22.07	59.73	0.732	0.790	35	0.273
	Chitinivibrionales	5.123	21.05	80.78	0.016	0.117	12	0.004*
	Spirochaetales	0.929	3.81	84.60	< 0.001	0.018	0	<0.001*
	FR888536_o	0.784	3.22	87.82	0.024	0.009	14	0.007*
	CP011215_o	0.698	2.87	90.70	< 0.001	0.014	0	<0.001*
	Synergistales	0.381	1.56	92.26	0.010	0.010	44	0.677
	Campylobacterales	0.322	1.32	93.59	< 0.001	0.006	0	<0.001*
	Endomicrobium_o	0.285	1.17	94.76	0.005	0.002	42	0.570
Family	Hydrogenispora_f	9.165	26.83	26.83	0.192	0.008	0	<0.001*
	Christensenellaceae	5.576	16.32	43.15	0.250	0.252	50	0.969
	LDWW_f	5.123	15.00	58.14	0.016	0.117	12	0.004*
	Lachnospiraceae	3.874	11.34	69.48	0.135	0.190	27	0.088
	Clostridiaceae	2.581	7.55	77.04	0.067	0.051	49	0.969
	Ruminococcaceae	2.424	7.09	84.13	0.264	0.277	45	0.733
	Leptospiraceae	0.929	2.72	86.85	< 0.001	0.018	0	< 0.001*
	CP011215_f	0.698	2.04	88.90	< 0.001	0.014	0	< 0.001*
	FR888536_f	0.687	2.01	90.91	0.021	0.009	18	0.017*
	Synergistaceae	0.350	1.02	91.94	0.010	0.008	39	0.427
Genus	*Hydrogenispora*	9.167	17.94	17.94	0.192	0.008	0	< 0.001*
	LDWW_g	5.118	10.02	27.96	0.016	0.117	11	0.004*
	GQ422712_g	2.726	5.33	33.29	0.068	0.017	17	0.014*

Table 2 (continued). Percentage similarity analysis (SIMPER) of fecal bacteria from Bolson Tortoises (*Gopherus flavomarginatus*) at phyla, class, order, family, and genus levels. Abbreviations are Av. dissim = average dissimilarity, W = wild, C = captive; and an asterisk (*) = significant difference based on Mann-Whitney U test.

Taxon	Av. dissim	Contribution %	Cumulative %	Mean W	Mean C	*U*	*P*
Clostridium	2.581	5.05	38.34	0.067	0.051	49	0.970
HQ716403_g	2.375	4.64	42.99	0.052	0.092	18	0.017*
AB239481_g	2.124	4.15	47.15	0.029	0.070	7	0.001*
Other	1.997	3.90	51.06	0.062	0.091	8	0.002*
Acetivibrio	1.483	2.90	53.96	0.031	0.001	1	< 0.001*
AB009176_g	1.345	2.63	56.59	0.059	0.045	38	0.385
HM124244_g	1.128	2.20	58.80	0.031	0.027	42	0.571
Sporobacter	0.993	1.94	60.74	0.029	0.047	18	0.017*
Ruminococcus	0.993	1.94	62.69	0.016	0.030	32	0.186
GQ468580_g	0.929	1.81	64.51	< 0.001	0.018	0	< 0.000*
AB240379_g	0.768	1.50	66.01	0.017	0.002	19	0.021*
Cellulosilyticum	0.726	1.42	67.43	0.012	0.018	35	0.273
LBRP_g	0.698	1.36	68.80	< 0.001	0.014	0	< 0.001*
FR888536_g	0.687	1.34	70.15	0.021	0.009	18	0.017*
HQ697740_g	0.685	1.34	71.49	0.016	0.003	19	0.021*
Other	0.646	1.26	72.75	0.015	0.026	21	0.030*
Hungatella	0.627	1.22	73.98	0.016	0.016	44	0.678
EU843993_g	0.625	1.22	75.20	0.006	0.017	18	0.017*
GU302849_g	0.605	1.18	76.39	0.009	0.021	5	0.001*
GQ448104_g	0.549	1.07	77.47	0.012	0.001	8	0.002*

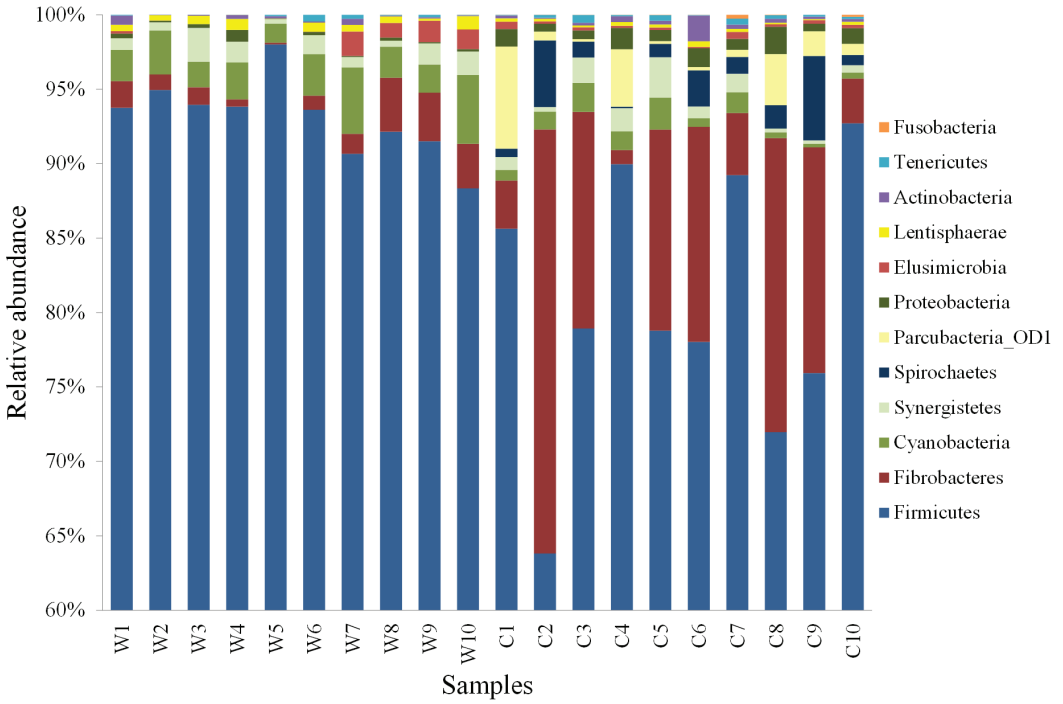

Figure 3. Relative abundance (%) of fecal bacterial taxa at phylum level from wild (W) Bolson Tortoises (*Gopherus flavomarginatus*) in Mapimi Biosphere Reserve, Mexico, and from captive (C) tortoises in the Unidad Regional Universitaria de Zonas Áridas (URUZA).

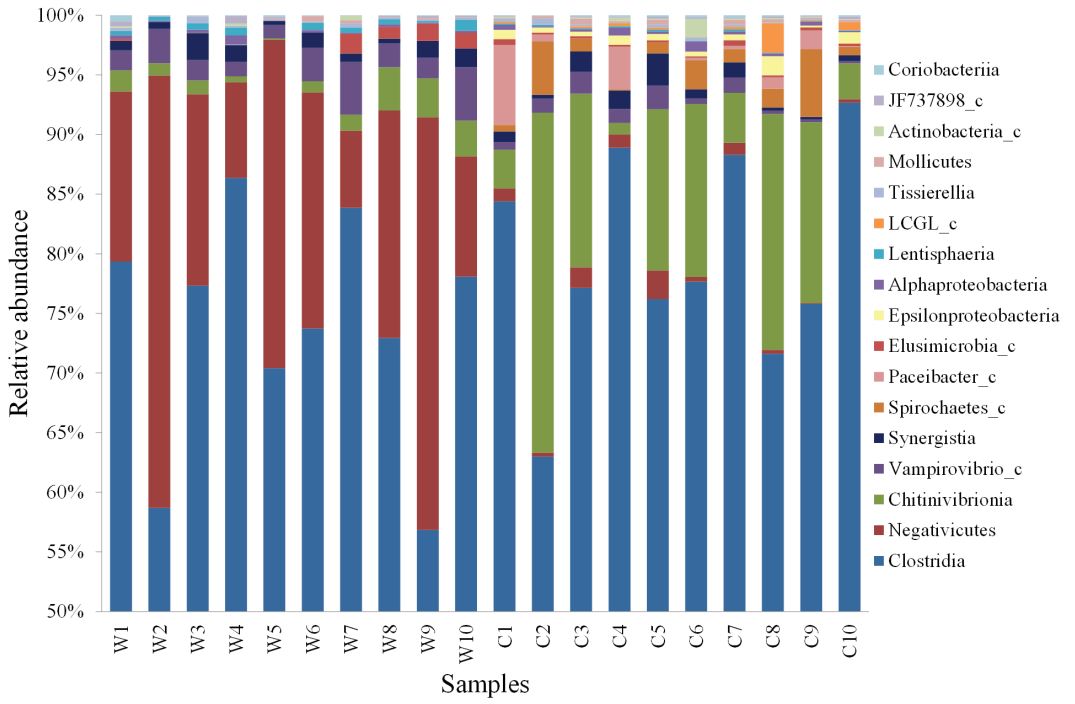

Figure 4. Relative abundance (%) of fecal bacterial taxa at class level from wild (W) Bolson Tortoises (*Gopherus flavomarginatus*) in Mapimi Biosphere Reserve, Mexico, and from captive (C) tortoises in the Unidad Regional Universitaria de Zonas Áridas (URUZA).

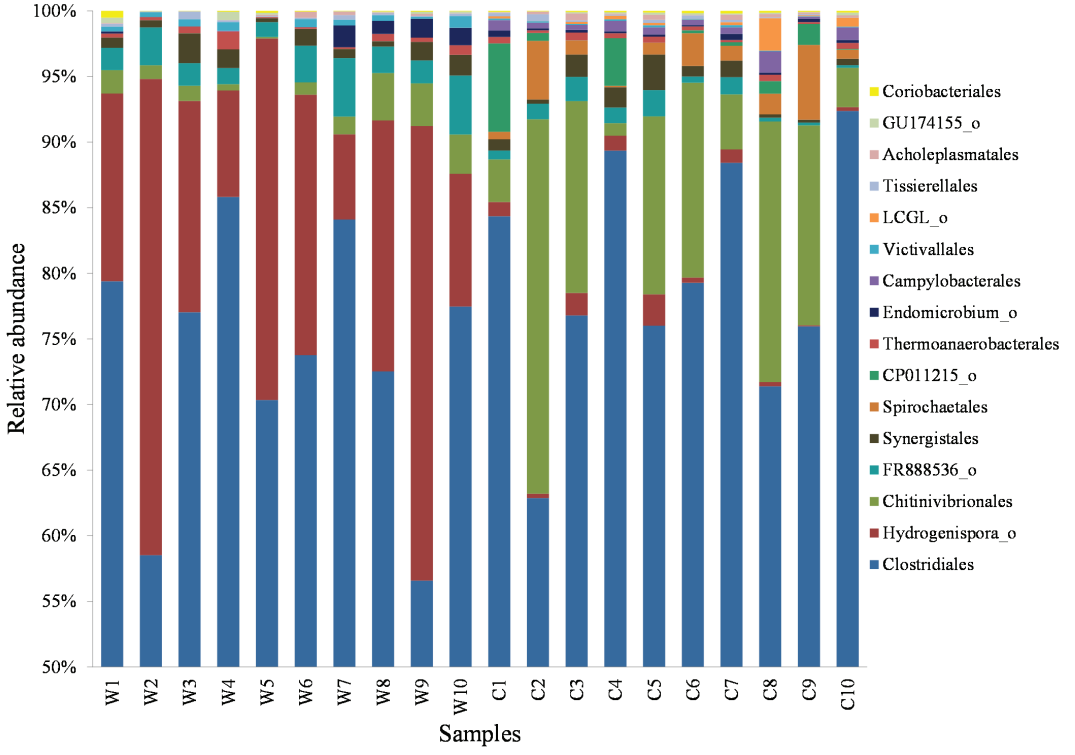

Figure 5. Relative abundance (%) of fecal bacterial taxa at order level from wild (W) Bolson Tortoises (*Gopherus flavomarginatus*) in Mapimi Biosphere Reserve, Mexico, and from captive (C) tortoises in the Unidad Regional Universitaria de Zonas Áridas (URUZA).

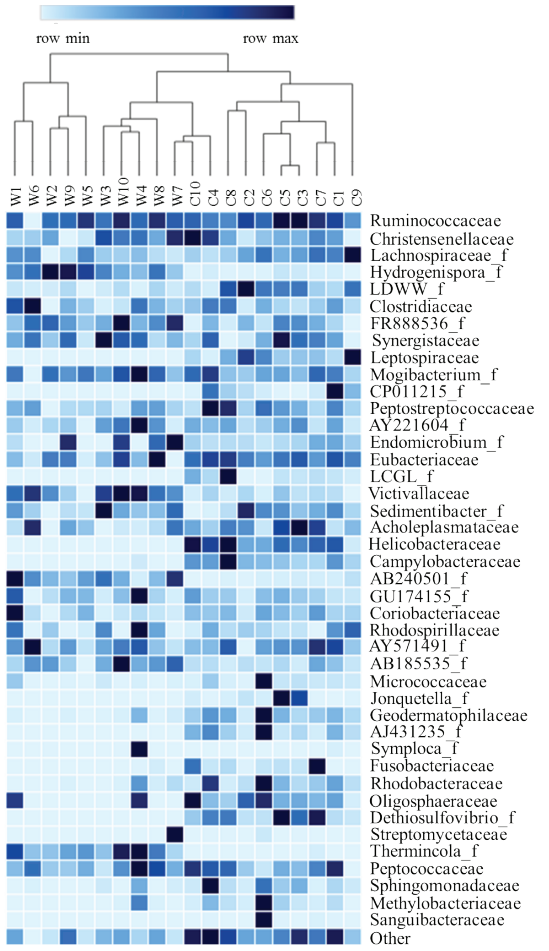

Figure 6. Heatmap of fecal bacterial taxa at family level from wild (W) Bolson Tortoises (*Gopherus flavomarginatus*) in Mapimi Biosphere Reserve, Mexico, and from captive (C) tortoises in the Unidad Regional Universitaria de Zonas Áridas (URUZA).

of the fecal bacterial microbiota described for other herbivorous reptiles (Hong et al. 2015), as well as for a wide variety of mammals (Ley et al. 2008). Clostridia, which is one of the most important bacterial taxa involved in the cellulose digestion in both herbivorous mammals and reptiles (Nelson et al. 2003; Flint et al. 2008; Ley et al. 2008), constituted more than 70% of the fecal bacterial microbiota in both populations of *G. flavomarginatus*. At these taxonomic levels there are important similarities between *G. flavomarginatus* and Gopher Tortoise (*G. polyphemus*) fecal bacterial composition; in the later tortoise species, Firmicutes is highly abundant (36%) and more than 97% of them were members of the Clostridia class belonging mainly to Ruminococcaceae and Lachnospiraceae families (Yuan et al. 2015). On the other hand, both populations

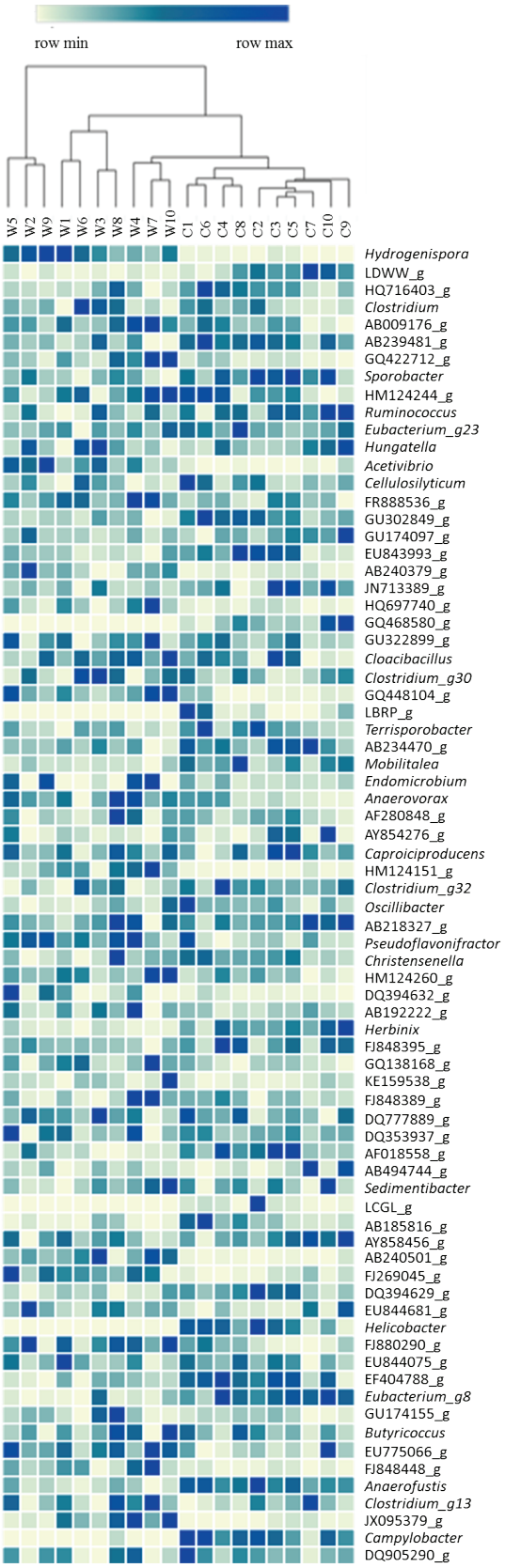

Figure 7. Heatmap of fecal bacterial taxa at genus level from wild (W) Bolson Tortoises (*Gopherus flavomarginatus*) in Mapimi Biosphere Reserve, Mexico, and from captive (C) tortoises in the Unidad Regional Universitaria de Zonas Áridas (URUZA).

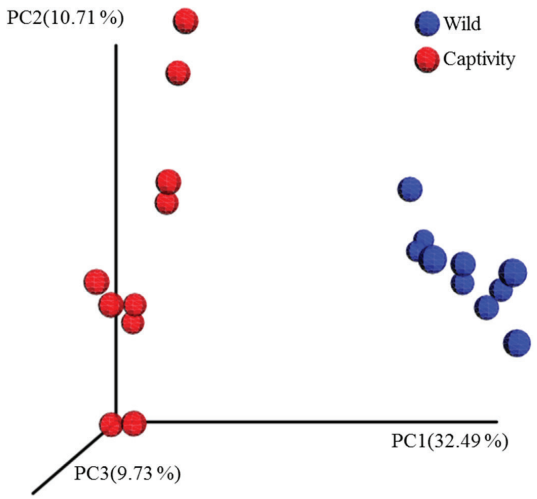

Figure 8. Principal Coordinate Analysis (PCoA) plot of fecal microbiota samples from wild (blue) and from captive (red) Bolson Tortoises (*Gopherus flavomarginatus*) based on Bray-Curtis index.

of *G. flavomarginatus* differed in relative abundance of fecal bacteria belonging to eight phyla, six classes, six orders, and five families.

Several studies have documented differences in microbiota abundance and diversity in feces between wild and captive populations in other animal species (Uenishi et al. 2007; Villers et al. 2008; Xenoulis et al. 2010; Wienemann et al. 2011). This difference has been attributed mainly to the type of diet (Brown et al. 2012; Vital et al. 2015; Li et al. 2017). Diet of *Gopherus* spp. is exclusively herbivorous (Morafka et al. 1989). Wild populations of these tortoises feed on a high diversity of plants, adjusting their diets according to plant availability (MacDonald and Mushinsky 1988; Jennings and Berry, 2015). Specifically, *G. flavomarginatus* feeding is based on grasses, shrubs, and annual herbaceous plants, which include more than 20 species (Morafka et al. 1989). In contrast, the captive population of *G. flavomarginatus* has been fed mainly with alfalfa hay (*Medicago sativa*) since its founding in 1997 (Castro-Franco et al. 2007). Kohl et al. (2017) indicated that captivity alters significantly the fecal bacterial community of lizards in a period of only eight weeks, where taxa are lost or added. Because captive *G. flavomarginatus* have been isolated almost 20 y with a diet low in variety, it is likely the abundance of several bacteria taxa of their intestinal microbiota has changed in comparison to wild *G. flavomarginatus*. As an example, Fibrobacteres was significantly more abundant in the captive population of *G. flavomarginatus* compared to the wild population. These are Gram-negative, strictly anaerobic, bacteria that degrade cellulose (Jami and Mizrahi 2012; Kameshwar and Qin 2016). Fibrobacteres have been recorded in the digestive tract of captive herbivorous mammals that were primarily fed hay (Tajima et al. 2001; Fernando et al. 2010). This could indicate that the diverse diet of wild tortoises in comparison to the diet of captive tortoises based mainly on hay caused the microbiota in captive *G. flavomarginatus* to gradually adapt to degraded food with higher fiber content, in the same way that has been reported for deer (Guan et al. 2017) and cattle (Fernando et al. 2010). Also, the genus *Hydrogenispora*, a strictly anaerobic bacteria that ferments sugars (Liu et al. 2014), was significantly low in abundance within the tortoise captive population, while it was the most abundant in the wild population. Several studies have stated possible factors that could explain the fecal microbiota differences between animal populations. Age, sex, type of soil, and vegetation in which each group is found, the degree of exposure to humans (high in the captive population) and to domestic animals (cats, rats or insects that can accidentally enter the captive population and leave scats), as well as the use of antibiotics, among others can all affect gut microbiota communities (Nelson et al. 2013; Tsukayama et al. 2018; Turner 2018). In our study, the only noticeable difference between both turtle populations was contact with people and with domestic animals, which together with the diet may be the most important causes of the difference found between fecal microbiotas. Other variables could be analyzed in detail in subsequent studies to complement the information that we have so far.

Although both populations showed significant differences in some taxonomic groups of fecal bacteria, all the individuals analyzed were clinically healthy, which would mean that their bacterial communities are in apparent equilibrium. It is necessary to take these differences into account, however, if a reintroduction program is planned for the original population because health problems may occur in the captive individuals (Redford et al. 2012). For example, Xie et al. (2016) reported a significant difference between the fecal bacterial microbiota of wild and captive populations of the threatened Japanese Crane (*Grus japonensis*) in East Asia. They argued that microbiota alteration of the captive group could increase the risk of acquiring an infection once they are in the wild, increasing the chances of reintroduction failure. Hence, detailed microbiological studies of all captive populations of *G. flavomarginatus* planned to be used in reintroduction programs is advisable, as well as a better understanding of gut dynamics along the year and in different locations for wild populations. Where significant differences are found, as we found in this study, there should be an effort to align the gut microbiota of the wild population and reintroduction candidates prior to reintroduction, perhaps by shifting the captive tortoise diet to be more similar to the wild tortoises, and/or introduce wild scat into the captive tortoise pens. A robust knowledge of tortoise microbial ecology is needed to carry out safe reintroduction programs.

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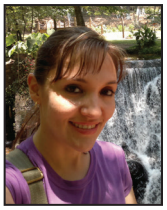

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Cameron W. Barrows research focuses on the impacts of environmental change in desert systems at scales that include populations, communities, habitat, and landscapes. Those changes may include land use, invasive species, habitat fragmentation, severing landscape connectivity, and climate. While focusing on conservation themes, his research provides insights into what drives the patterns of species richness and dynamics of natural systems. One theme of his ecological research is the importance of a foundation of natural history. In many areas of academia, natural history perspectives and skills are being lost; my goal is to continue to provide that foundational approach for new graduate students, undergraduates, and citizen scientists as an essential tool for addressing complex ecological questions. (Photographed by Michelle Murphy-Mariscal).

Bruno Gomez-Gil is a Lead Scientist at the Center for Research in Nutrition and Development (CIAD), Mazatlan Unit for Aquaculture and Environmental Management, Mexico. He received his Ph.D. from the Institute of Aquaculture, Stirling University, Stirling, Scotland. He established the bacteriology laboratory at CIAD and later the Microbial Genomics laboratory. For more than 20 y he has studied aquatic microbiology in aquaculture settings and in the environment. His entire career has been devoted to the study of vibrios, their taxonomy, pathogenicity, genomics, and prevalence in marine environments. (Photographed by Bruno Gómez Gil Rodríguez Sala).

Luis Manuel Valenzuela-Nuñez is a Researcher at the Juarez University of Durango State - Faculty of Biological Sciences in Gómez Palacio, Mexico. He received his Ph.D. from the Henri Poincaré University in Nancy, France. He established the Forest Biology and Ecology Laboratory at the Faculty of Biological Sciences. During 10 y he has studied the ecological interactions in temperate forest and vegetation in dry areas in northern Mexico. Furthermore, he has studied the effect of environmental factors on total non-structural carbohydrates and nitrogen compounds in natural vegetation and fruit trees. He has published more than 70 articles in peer-reviewed journals and more than 20 book chapters. (Photographed by Uriel Calderón Villegas).