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BACTERIAL MICROBIOTA FROM WILD FRESHWATER FISH UTILIZED FOR SUBSISTENCE IN WESTERN MEXICO

Microbiota bacteriana de peces silvestres de agua dulce utilizados para consumo en el oeste de México

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Key words: *Escherichia coli*, urban pollution, Manantlán, subsistence fishery

ABSTRACT

Microbiological analyses of freshwater fish utilized in subsistence fisheries are relatively scarce. These fisheries are widespread throughout Latin America, and support numerous communities in rural, impoverished areas. We present the results of genetically-based microbiological analyses of river water samples and fish tissue samples obtained from specimens (n = 128) captured in the wild from three sites in the Ayuquila river, west central Mexico. The system is affected by numerous human activities. We identify 11 bacterial taxa of which *Pseudomonas stutzeri*, *Shigella sonnei, Escherichia coli* and *Bacillus subtilis* were isolated from the most fish. *Citrobacter freundii* and *E. coli* were present in water samples from all sites. Average taxa richness was similar among rainy and dry seasons. Enterobacteriaceae dominate the bacterial microbiota of water and fishes of the Ayuquila river, suggesting there is significant influence from anthropic activities in the basin. Our findings provide a baseline upon which to improve our understanding of the microbiology of the Ayuquila river and similar systems in west central Mexico, especially related to risks for biota and human populations dependent on rivers for sustenance.

Palabras clave: *Escherichia coli*, contaminación, Manantlán, pesquería de subsistencia

RESUMEN

Los análisis microbiológicos en peces de agua dulce tomados directamente de hábitats naturales y que son utilizados en pesquerías de subsistencia son relativamente escasos. Estas pesquerías están muy extendidas en toda América Latina y son importantes para numerosas comunidades en áreas rurales empobrecidas. Presentamos los resultados de análisis microbiológicos y moleculares realizados en muestras de agua de río y muestras de tejido obtenidas de peces (n = 128) capturados en el medio silvestre de tres sitios en el río Ayuquila, en el centro oeste de México. El río es afectado por numerosas

actividades humanas. Identificamos 11 taxa bacterianos de los cuales *Pseudomonas stutzeri*, *Shigella sonnei*, *Escherichia coli* y *Bacillus subtilis* se aislaron de la mayoría de los peces. *Citrobacter freundii* y *E. coli* estuvieron presentes en muestras de agua de todos los sitios. La riqueza promedio de los taxa fue similar entre temporadas de muestreo. Las enterobacteriáceas dominan la microbiota bacteriana del agua y los peces del río Ayuquila, lo que sugiere que existe una influencia significativa de las actividades antrópicas en la cuenca. Nuestros resultados proporcionan la línea base sobre la cual mejorar nuestra comprensión de la microbiología del río Ayuquila, especialmente relacionada con los riesgos para la biota y las poblaciones humanas que dependen y subsisten del río.

INTRODUCTION

An important proportion of the rural population in Mexico relies on subsistence riverine fisheries for protein intake (Periago et al. 2005, Rincón-Rodríguez et al. 2013, Urquía-Fernández 2014). Subsistence fisheries usually complement agriculture and cattle rearing as main sources of food and wealth in many watersheds in west central Mexico (Elías-Fernández and Navarrete-Salgado 1998, Lyons et al. 1998). Unfortunately, rivers in this area suffer from multiple human stressors, including organic urban and agricultural pollution (Hernández-Antonio and Hansen 2011, Torres-Beristáin et al. 2013). Freshwater fisheries in the Ayuquila-Armería river (hereafter the Ayuquila river) in the states of Jalisco and Colima, Mexico, have experienced important declines but remain a key resource for the local population (Lyons et al. 1998, Mercado-Silva et al. 2011). The Ayuquila river has long been locally known for the quality of its fishery and as a biodiversity hotspot (Lyons et al. 1998, Arellano-Ríos and Rivera-Pahua 2011). However, agricultural activities and urban development have negatively affected various segments of the river. Importantly, urban centers with populations of > 40 000 people dispose of untreated wastewaters directly onto channels connected to the river and little control exists for disposing of feces from > 20 000 cattle in the basin (Graf-Montero et al. 2006). Such high levels of organic pollutants in the Ayuquila river represent a potential microbiological hazard for humans utilizing river resources.

Microbiological analyses of freshwater fish utilized in subsistence fisheries are relatively rare; most microbiological information to date comes from fish farms, aquaculture, and canning operations (Lyhs et al. 2001, Arvanitoyannis et al. 2008), but little is known about wild fish microbiota and how environmental conditions of rivers affect their microbial diversity (Lazado et al. 2015). Information is also scarce in Mexico about the potential health risks pathogenic bacteria from wild freshwater fish pose to fish handlers and consumers, especially when fish come from systems affected by urban or agricultural wastewaters (Cahill 1990). Here we present the results of genetically-based microbiological analyses of water and tissue samples obtained from fish specimens captured in the wild in three different Ayuquila river sections exposed to differential wastewater loads. We describe differences among sections and draw conclusions that should alert local health officials about the potential for health risks to humans in the basin.

MATERIAL AND METHODS

Study sites

The Ayuquila river is located in western Mexico, in the northwestern portion of the Sierra Madre del Sur ~50 km inland from the Pacific Ocean (**Fig. 1**). The Ayuquila runs along a series of gorges and valleys and has a total length of approximately 294 km. Urban centers and agricultural activities are concentrated in the valleys. The river is important from a conservation perspective, as it is the northern boundary of the Sierra de Manantlán Biosphere Reserve (SMBR). The SMBR is a protected area dedicated to biological conservation where low impact human activities are permitted, and where some human populations still rely on natural resource use and consumption (Graf-Montero et al. 2006). Fishing is an important activity for humans inside the SMBR, in particular those living in the margins of the Ayuquila (Mercado-Silva et al. 2011). This study focuses on a ~20 km segment of the Ayuquila that runs from the town of El Corcovado, ~1 km upstream from the valley of the cities of Autlán and El Grullo, through the valley, to a section of river located upstream from where the river becomes part of the SMBR boundary. Prior to entering the valley,

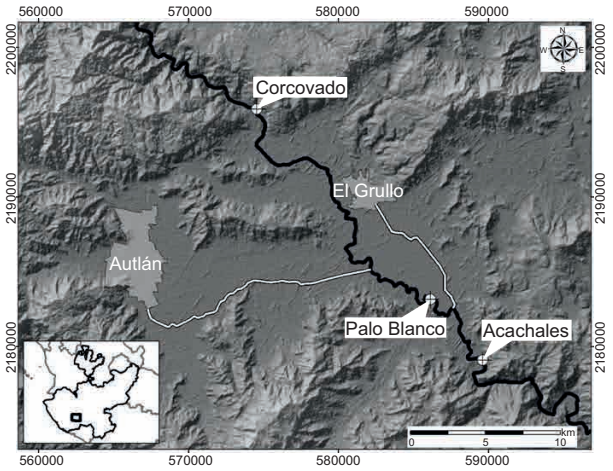

Fig. 1. Three sampling sites in Ayuquila river (west central Mexico) (black line). Cities of Autlán and El Grullo, and the main outlet for their sewage systems are shown (white lines) Coordinates in UTM (zone 13Q). The inset shows a map of the State of Jalisco, and the location of the study area

the river flows through a gorge with little human influence; as it runs through the valley and before entering the SMBR the river receives numerous canals carrying municipal and agricultural wastewater (**Fig. 1**). Within the river segment, fish and water samples were collected from three sites: (1) Corcovado, (2) Pablo Blanco and (3) Achacales. Site 1 was located just downstream (2 km) from the gorge in a section with comparatively little human influence, site 2 was located just downstream (0.5 km) from where municipal wastewater canals empty into the river, and site 3 was located about 1.5 km downstream from site 2, as the river enters the SMBR (**Fig. 1**). Fishing activities have been recorded from all three sites (Mercado-Silva et al. 2011). Water physical-chemical parameters (obtained with a Hanna 9828 multimeter in triplicate in each sampling event) varied from site to site and among wet (July-October) and dry (November-June) seasons at the time of sampling (**Table I**).

Sampling

Water and fish samples were obtained diurnally during 2015 in February, March and April (dry season) and in July, September and October (wet season), from each site. Fish (n = 128) were collected using traditional fishing methods (e.g., cast netting) and kept alive in river water as they were transported to the laboratory (Centro Universitario de la Costa Sur, Universidad de Guadalajara in Autlán, Jalisco, Mexico). Tilapia (*Oreochromis* sp.), bluegill (*Lepomis macrochirus*), carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*), all non-native fishes, were collected. Tilapia comprised the majority of examined fish (95 %); we do not examine microbiological differences among species. Fish were sacrificed within 24 hours of their capture, measured, weighed and dissected to obtain a 10 g tissue sample comprising a mix of intestine, branchiae, liver, muscle and eggs (when available). The sample was then preserved in 90 mL of enriched peptone water.

Water samples were collected using Moore's swab technique (Barrett et al. 1980). After an hour of contact time with river water, swabs were placed in sterilized glass containers with one of two liquid culture media (peptone water with 0.5 % W/V NaCl and thioglycolate broth). A separate 350 mL sample of river water was obtained in the field. Swabs and water samples were obtained in triplicate at each sampling event; within each site, locations where samples were obtained were all within 20 m from each other. All water samples were transported to the lab in ice chests at a temperature of 4 ºC.

TABLE I. PHYSICAL CHEMICAL PARAMETERS (AVERAGE) FROM WATER IN THREE (1-3) SITES (SEE METHODS FOR SITE DESCRIPTION) AND TWO SEASONS (WET = W; DRY = D) IN THE AYUQUILA RIVER, WEST CENTRAL MEXICO.

Season	Site 1		Site 2		Site 3	
	W	D	W	D	W	D
Temperature (º Celsius)	20.3	18.9	23.8	22.6	22.7	21.8
pH	8.26	7.75	7.60	7.40	7.90	8.48
Dissolved oxygen (mg/L)	5.70	5.90	2.98	2.10	2.09	4.90
Conductivity (µS/cm)	97.5	151.0	449.3	517.5	226.5	532.0
Total dissolved solids (ppm)	49.0	75.5	224.0	259.0	113.0	266.0
Salinity (ppt)	0.04	0.07	0.21	0.25	0.11	0.26

Sample processing

Water and fish tissue samples were planted in culture media to obtain bacterial strains. Media used were thiosulfate-citrate-bile salts-sucrose (TCBS) agar, bismuth sulfite agar, *Salmonella-Shigella* agar, MacConkey agar and nutritive agar (Bioxon, Mexico). Fish tissue samples were planted in petri dishes with each media. A 1 mL sample from Moore swabs was obtained and planted into separate Petri dishes with media. A 1 mL sample of river water was obtained and planted in culture media upon arrival at the laboratory. Bacterial strains from fish tissue and water samples obtained after 24±2 h incubation were isolated and purified in tryptic soy agar (Becton, Dickinson and Company, France). Twenty three isolates were obtained from both water and fish samples. From each isolate, a subsample was obtained for polymerase chain reaction (PCR) analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS) was used for nucleic acid extraction for each isolated strain (Kabir et al. 2006). Each sample was diluted (1:20), and we used 25 µL for PCR amplification of fD1 and rD1 16S ribosomal genes. PCR products were purified in Bio-Rad chromatography columns (Quantum Prep PCR Kleen Spin columns, Bio-Rad ®). Non-specific bands in chromatography columns were recovered using Promega ® Kit Wizard SV Gel and PCR Clean Up System. Purified products were verified using agarose gel electrophoresis (Lee et al. 2012). Recovered PCR products were sequenced at Macrogen Inc. (Seoul, Korea). Sequences were analyzed using BLAST algorithms from databases at the National Center of Biotechnology Information (NCBI BLAST 2017) and the Kyoto Encyclopedia of Genes and Genome (KEGG 2017).

Using the taxonomic identity of collected bacteria from water samples we compared species presence and species richness (Student t-tests) between sites and between seasons (wet vs. dry). Presence of bacteria isolated from fish tissue samples were also compared among sites and sampling seasons. For bacteria isolated from fish samples, we further analyzed the prevalence (i.e., number of fish containing a certain taxa) among sites and seasons.

RESULTS

We identified 11 taxa of bacteria from water and fish tissue samples from the Ayuquila river (**Table II**). Isolates from six taxa were identified to genus level only (*Alcaligenes* sp., *Bacillus* sp., *Methanobacterium* sp., *Proteus* sp., *Providencia* sp., and *Pseudomonas* sp.). Species in three of these genera were also identified from isolates (exceptions being *Methanobacterium* sp. and *Alcaligenes* sp.). Ten isolates were identified to species level, and only one was identified to subspecies (**Table II**).

Most taxa were identified from both water and fish tissue samples. *Bacillus subtilis* was the only species present in fish tissue samples but absent from water samples. *Citrobacter freundii* and *Escherichia coli* were present in water samples from both wet and dry seasons from all sites. *Pseudomonas stutzeri*, *Serratia liquefasciens* and *Shigella sonnei* were recovered from all dry season water samples, but one of them was missing from at least one site sampled during the wet season (**Table II**). No taxon was exclusive of a site. Average taxa richness was similar among seasons (Student $t = 2.64$, $p = 0.11$) but slightly higher richness was observed during the wet season (11-13 in the dry season vs 11-15 in the wet season). Water samples obtained in the wet season from site 2 had the lowest taxa richness.

Five taxa isolated from fish tissue samples were absent from at least one site; *Bacillus* sp. was absent from site 1, although *B. subtilis* and *B. subtilis inaquosorum* were present in the site during both seasons. *Enterobacter cloaceae* was only present in site 3. *Methanobacterium* sp. and *Providencia alcalifasciens* were absent from sites 2 and 1, respectively, but present in other sites during at least one sampling season. *Pseudomonas stutzeri* and *Shigella sonnei* were isolated from the most fish (64) across all sites, followed by *E. coli* (57) and *B. subtilis* (50). Least prevalent taxa found in fish samples were *E. cloaceae*, *P. alcalifasciens* (in five fish only each) and *Methanobacterium* sp. (in six fish). Site 3 had the highest richness of all sites; all taxa were found in fish captured from the site. Three bacterial taxa –*Serratia liquefasciens*, *Proteus* sp. and *Providencia* sp. – were absent from fish samples in the dry season and only *P. fragi* was absent from fish samples in the wet season at site 3 (**Table II**).

Shigella sonnei, *P. stutzeri*, *E. coli* and *B. subtilis* were present in the highest percentage of examined fish tissue samples; *S. sonnei* and *B. subtilis* were recovered from 87.5 % of samples from site 2 during the dry season. A high proportion of samples from the dry season in site 2 also tested positive for *P. stutzeri*. *Escherichia coli* was identified from a relatively high percentage of fish tissue samples from most sites and seasons; *E. coli* was recovered from a relatively high proportion of fishes from site 1 in the dry season (68.4 %), and between 20-50 % of all other fishes collected on other sites and seasons were positive for this species (**Table II**).

TABLE II. BACTERIA IDENTIFIED FROM WATER AND FREE LIVING FRESHWATER FISH SAMPLES FROM THREE (1-3) SITES (SEE METHODS FOR SITE DESCRIPTION) IN THE AYUQUILA RIVER, WEST CENTRAL MEXICO. LIST ORGANIZED ALPHABETICALLY. SAMPLES WERE TAKEN FROM WET (W) AND DRY (D) SEASONS RESPECTIVELY. PRESENCE/ABSENCE IS INDICATED FOR WATER SAMPLES. FOR FISH TISSUE SAMPLES THE NUMBER OF FISH TESTING POSITIVE FOR A TAXON IS INDICATED; THE TOTAL NUMBER OF FISH CAPTURED IN A GIVEN SAMPLING EFFORT IS INDICATED ADJACENT TO D OR W IN COLUMN HEADERS. IN PARENTHESES, THE % OF FISH WHERE A TAXON WAS IDENTIFIED. TOTAL NUMBER OF TAXA IDENTIFIED FROM SAMPLING EVENTS IS PRESENTED (TAXA SUM)

Sample Source	Water						Fish					
Site	1		2		3		1		2		3	
	D	W	D	W	D	W	D (19)	W (22)	D (16)	W (17)	D (26)	W (28)
Species ID												
Alcaligenes sp.	-	1	-	1	-	1	8 (42.1)	1 (4.5)	1 (6.2)	8 (47.0)	3 (11.5)	11 (39.2)
Bacillus sp.	1	-	1	-	1	-	-	-	7 (43.7)	-	1 (3.8)	1 (3.5)
Bacillus subtilis	-	-	-	-	-	-	6 (31.5)	7 (31.8)	14 (87.5)	8 (47.0)	4 (15.3)	11 (39.2)
B. subtilis inaquosorum	-	1	1	1	1	-	5 (26.3)	10 (45.4)	2 (12.5)	7 (41.4)	11 (42.3)	6 (21.4)
Citrobacter freundii	1	1	1	1	1	1	3 (15.8)	1 (4.5)	3 (18.7)	2 (11.7)	8 (30.7)	5 (17.8)
Enterobacter cloaceae	-	1	1	-	1	1	-	-	-	-	4 (15.3)	1 (3.5)
Escherichia coli	1	1	1	1	1	1	13 (68.4)	5 (22.7)	4 (25.0)	8 (47.0)	13 (50.0)	14 (50.0)
Methanobacterium sp.	1	1	-	-	-	1	-	2 (9.0)	-	-	3 (11.5)	1 (3.5)
Proteus sp.	-	-	1	-	-	1	1 (5.2)	3 (13.6)	-	2 (11.7)	-	6 (21.4)
Providencia alcalifaciens	1	1	-	-	1	1	-	-	2 (12.5)	-	2 (7.6)	1 (3.5)
Providencia sp.	1	-	1	-	1	1	4 (21.0)	-	-	4 (23.5)	-	7 (25.0)
Pseudomonas fragi	1	-	-	-	1	-	-	5 (22.7)	1 (6.2)	1 (5.8)	1 (3.8)	-
Pseudomonas sp.	1	-	1	1	-	-	2 (10.5)	4 (18.1)	6 (37.5)	4 (23.5)	7 (26.9)	7 (25.0)
Pseudomonas stutzeri	1	1	1	1	1	-	7 (36.8)	13 (59.0)	12 (75.0)	4 (23.5)	19 (73.0)	9 (32.1)
Serratia liquefaciens	1	1	1	-	1	1	6 (31.5)	-	-	7 (41.4)	-	10 (35.7)
Shigella sonnei	1	-	1	1	1	1	9 (47.3)	13 (59.0)	14 (87.5)	6 (35.2)	13 (50.0)	9 (32.1)
Taxa sum	11	9	11	7	11	10	11	11	11	12	13	15

DISCUSSION AND CONCLUSIONS

Microbiological information from freshwater systems can help our understanding of biological diversity, the functional role of microorganisms in natural processes (i.e., nutrient cycling), how biota is affected by anthropic stressors, and their interactions with other organisms, including humans (Cahill 1990, Barrera-Escorcia et al. 2013, García-Pérez and Aguilar 2013). These areas of knowledge rely on studies that, as a starting point, identify the suite of species present in the environment or those associated with organisms utilized as resources. Our results provide one of the first accounts of the microbiota from free living fishes in Mexico. Some studies have reported on microbiological conditions of the Gulf of Mexico (Godoy-Lozano et al. 2018) and of river waters in Mexico (Arellano-Ríos and Rivera-Pahua 2011, Barrera-Escorcia et al. 2013), or have analyzed fish destined for consumption in urban markets (Constantino-Casas et al. 1997) but no reports exist on the microbiology of fishes utilized in subsistence fisheries, especially related to natural protected areas.

Our finding that Enterobacteriaceae dominate the bacterial microbiota of water and fishes of the Ayuquila river provides evidence of the influence anthropic impact is having on the river (García-Pérez and Aguilar 2013). This is important both from a public health perspective and considering human effects to aquatic ecosystems in the SMBR. Presence of *E. coli*, a well-known microbiological water quality indicator, which we found in all sites and in relatively high number of fishes, can be related to constant fecal (animal or human) matter influx to the river (Eckner 1998, Somarelli et al. 2007). *Escherichia coli* can generate opportunistic pulmonary, gastric and skin infections in humans consuming, swimming or bathing in river waters (Periago et al. 2005, Ishii and Sadowsky 2008). It could also generate infections in wildlife of the Ayuquila, such as the neotropical river otter *Lontra longicaudis*. Bacterial diseases

are known to affect otters elsewhere (Moore et al. 2002, Lalucat et al. 2006) and could further hinder *L. longicaudis* population conservation in the Ayuquila.

We carried out this study under the hypothesis that wastewaters from cities in the Autlán valley would generate an increase in the number of potentially pathogenic bacteria downstream from the areas where sewage canals empty into the river. Thus, we expected site 1 to be relatively poor in potentially pathogenic strains both in water and fish tissues. While site 1 generally had indeed a lower bacterial species richness than sites 2 and 3, pathogenic bacteria (i.e., *E. coli* and *Pseudomonas* sp.) were present throughout the river and in a high proportion of tissue samples. This was not a surprise because studies of marine microbiota in the Gulf of Mexico have also found potentially pathogenic strains even in very deep waters where there is little anthropic influence (Escobedo-Hinojosa and Pardo-López 2017). Areas upstream from site 1 in the watershed, though relatively distant (> 25 km), are also used for agriculture, free range cattle use and are sparsely inhabited (Gerritsen and van der Ploeg 2006). These could be sources for potentially pathogenic microbes in what we considered the relatively unaffected site 1.

We identified at least three species in the genus *Pseudomonas*. While *P. fragi* and *P. stutzeri* have no or low virulence, respectively (Lazado et al. 2015), further research is required to better identify *Pseudomonas* varieties in the Ayuquila. In particular, *P. aeruginosa* could be a potentially pathogenic strain that should be of concern for humans utilizing the Ayuquila river (Lyhs et al. 2001, Centeno and Rodríguez 2005).

Our findings provide a baseline upon which to improve our understanding of the microbiology of the Ayuquila. To surpass some of the limitations of our study, future approaches should 1) incorporate other culture media to aid in increasing the diversity of bacteria recovered from fish or water samples, 2) increase the number of samples obtained and analyzed across both, space and time, 3) use culture-independent techniques such as 16S rRNA gene taxonomic profiling methods and high-throughput sequencing to analyze water and fish samples, and 4) include as many species of fish and fauna as possible from the Ayuquila, including native species important as food or under conservation status. Improving on these limitations should allow a better understanding of the dynamics of the microbiological community in the river and provide information that could be used to prevent pathogenic bacteria in the Ayuquila river from affecting the local human population or resources in the SMBR. Further, a future study should make use of Rep-PCR molecular tools to identify the specific sources of bacteria in the watershed; other studies have found that wildlife and cattle are more pervasive sources of some bacterial species in the Ayuquila than human populations (Torres-Beristáin et al. 2013).

Subsistence freshwater fisheries in the Ayuquila and other rivers in west-central Mexico will continue to provide an important, inexpensive source of animal protein for numerous communities as long as efforts continue to abate and control pollution. Multiagency efforts have achieved many goals in controlling degradation of the Ayuquila river (Graf-Montero et al. 2006). Our results, alongside public health studies in the SMBR and urban centers in the basin, should provide guidance to reduce microbiological risks for biota and human populations dependent on the river.

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