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Chapter 1

Water and Flamingo Feces Bacterial Communities from High-Altitude Andean Lakes under Selective Antibiotic Pressure Studied by PCR-DGGE Analyses

María V. Fernández-Zenoff, Julián R. Dib, Anna Neumann, María C. Estévez and María E. Farías

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Abstract

High-altitude Andean lakes are exposed to extreme environmental factors like high salinity, ultraviolet radiation, heavy metals, among others. As it was previously shown, these lakes are not only the habitat of a high diversity of bacteria with multiple resistances; they also support an enormous population of flamingos, which migrate among these wetlands, and they could play a role as disseminators and/or reservoirs of pathogenic bacteria.

The aim of this work was to analyze, by Denaturant Gradient Gel Electrophoresis, the bacterial population under selective antibiotic pressure from bacterioplankton and flamingo feces from three lakes, placed between altitudes 4,200 and 4,560 m. Almost all bands were present in antibiotic-enriched cultures. Several bands identified in water were found in feces as well, presenting mainly correspondence with *Gamma-proteobacteria*. Few bands were exclusively identified in water, and those presenting correspondence with *Alfa-proteobacteria* and Actinobacteria were only identified in Laguna Azul.

This study established that flamingos' enteric biota is in close interaction with lake water and demonstrated that bacteria with the ability to grow in antibiotics are abundant and diverse in the microbiota of Andean lakes. Additionally, flamingos could be considered as vectors of pathogenic organisms, since *Stenotrophomonas* seem to be the widest spread bacteria in the studied lakes.

Keywords: Andean Lakes, Antibiotics, DGGE, Flamingos, Feces, Resistance

1. Introduction

High-Altitude Andean Lakes (HAAL) are a system of shallow oligotrophic lakes originated in the tertiary age distributed across the *Puna,* the highest plateau of the South American Andes

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that includes Peru, Bolivia, Chile, and Argentina. These lakes are scattered throughout the region at altitudes varying from 3,600 to 6,000 m above sea level. As it was described in previous works done by our group, HAAL are almost unexplored environments, most of them with no access roads; they are distant from each other (more than 500–700 km) and the unique connection among them would be flamingo migration. Furthermore, they are exposed to extreme conditions, such as high salinity, UV radiation, oligotrophy with low phosphor availability, daily temperature changes (-15ºC to 20ºC), and heavy metal content [1-6]. HAAL are home to large flocks of aquatic birds, mainly flamingos. Three of the six extant flamingo species coexist in HAAL: the James's Flamingo (*Phoenicopterus jamesi*), the Chilean Flamingo (*Phoenicopterus chilensis*), and the Andean Flamingo (*Phoenicopterus andinus*). James's flamingos are distributed primarily in the Andean wetlands, during the breeding season, and dispersed through the lowland wetlands in Argentina in winter, during the nonbreeding season, when some of the high-Andes lakes freeze [7-9]. The Chilean flamingo inhabits most of the Pampean lowland wetlands throughout the year. Both flamingo species have specialized beaks that allow them to filter and feed on many planktonic and benthic organisms [10]. Migratory birds are known to play a role as long-distance vectors for many microorganisms, and antimicrobial drug resistance has also been described in bacteria isolated from wild birds [11-15]. In previous publications carried out in our laboratory we demonstrated that bacteria isolated from water and flamingo feces from HAAL were resistant to at least three or more antibiotics (ATBs) [2, 3]. However, the response of the whole community to antibiotic pressure using molecular methods has never been studied.

In this chapter, we compare bacterial diversity using Denaturant Gradient Gel Electrophoresis (DGGE) under antibiotic pressure conditions in water and flamingo feces from three HAAL: Laguna Aparejos, Laguna Negra, and Laguna Azul.

2. Materials and methods

2.1. Description of environments and sampling

Aparejos, Negra, and Azul lakes are located in the Andes Mountains in the northwest of Argentina; their physic and chemical characteristics are described by Dib et al. [3]. They are a group of lakes and salar pads called Salar de la Laguna Verde in the Andean region of Catamarca province, Argentina (27º 34′ S; 68º 32′ W). Some of the highest mountains of the Andean system are located in this area: Ojos del Salado (6,885 m) and Nevado Pissis (6,779 m). The water temperature was 5ºC at the sampling time (1 pm local hour) and the maximal UV-B irradiance reached 3.3 Wm^{-2} for 312 nm (half band with 300–325 nm).

Two types of samples were considered: water and flamingo feces. Surface water samples were collected during summer 2009 (near the beginning of austral spring) in 10 L sterile polyethylene bottles. Water samples were stored at 4ºC until further processing in the laboratory (within 24 h after collection), which is located 600 km away from the sampling site. Flamingo feces were taken near the lake and conserved in sterile bags at 4ºC until processing. Once in a sterile environment in the lab, core feces samples were extracted for cultivation.

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2.2. Antibiotics-resistant bacteria-enrichment cultures

To determine bacterial diversity under selective pressure, water samples from Laguna Aparejos, Laguna Negra, Laguna Azul, and four flamingo feces samples from each lake were analyzed. Samples were inoculated in 20 mL of R2A medium (yeast extract 0.5 g L^{-1}, peptone 0.5 g L^{-1}, casamino acids 0.5 g L^{-1}, glucose 0.5 g L^{-1}, soluble starch 0.5 g L^{-1}, sodium pyruvate 0.3 g L^{-1}, K_2HPO_4 0.3 g L^{-1}, $MgSO_4 \times 7H_2O$ 0.05 g L^{-1}; pH 7.2), with different ATBs. Control cultures without ATBs were also performed. Five ATBs were used: ampicillin (Amp), 100 μg mL^{-1}; chloramphenicol (Cm), 170 μg mL^{-1}; colistin (Col), 20 μg mL^{-1}; erythromycin (Ery), 50 μg mL^{-1}; and tetracycline (Tet) 50 μg mL^{-1}. After five days of incubation at 30ºC and 150 rpm, the cells were pelleted by centrifugation and total DNA was extracted from the ATB enriched cultures. Afterward, DGGE profiles of total community cultured without or with different ATBs were determined.

2.3. PCR amplification DGGE and sequencing

DNA extraction from total community cultures was performed using a CTAB method [16]. The variable V3 region of 16S rRNA gene was amplified by PCR [17]. The nucleotide sequences of the primers are as follows: primer 1 F341: 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3′, primer 2 R518: 5′-CGT ATT ACC GCG GCT GCT GG-3′, primer 3 F357: 5′-TTA CTG ATA GAA TGT GGA GC-3′[18].

PCR amplification was performed with a Biometra Termocycler as follows: 100 ηg of purified genomic DNA, 20 pmol of each primers (Genbiotech), 200 μmol of each deoxyribonucleoside triphosphate, 10 μL of 10× PCR buffer ($MgCl_2$) and 0.25 U of Go Taq polymerase (Promega) were added to a 0.2 mL volume microtube, which was filled up to a volume of 25 μL with sterile Milli-Q-water. PCR was performed using the following conditions: initial denaturing step of 15 min at 95°C, followed by 30 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min 30 s. A touchdown program was performed in order to down one grade at each cycle, until 55ºC. At this last temperature, 15 additional cycles were programmed, with a final extension at 72°C for 5 min. DGGE was performed with the Bio-Rad Protean II system, essentially as described previously [19]. PCR products were applied directly onto 8% (wt/vol) polyacrylamide gels in 1X TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA) and a linear gradient consisting of the denaturants urea and formamide; the concentration of the denaturants increased from 40% at the top of the gel to 60% at the bottom. Electrophoresis was performed at a constant voltage of 120 V and a temperature of 60ºC during 5 h. After electrophoresis, the gel was stained for 10 min with SYBR® Gold (Molecular Probes, Eugene, OR), rinsed with TAE buffer, and visualized with a Bio-Rad UV Gel Doc 2000 transilluminator. Distinguishable bands were excised from the gel; the eluted DNA was reamplified using the primers 2 and 3, and PCR products were sequenced.

2.4. Nucleotide sequence accession numbers and data analysis

Fifty-nine selected 16S rRNA sequences from DGGE bands in this paper have been deposited in GenBank database under the following accession numbers: AM712052–66, AM711573–79, AM711878–90, and AM889064–87.

The similarity in DGGE bands in each lake was assessed by Cluster Analysis using the Jaccard's index, applying the UPGMA (unweight pair-group method using averages) algorithm with software MVSP 3.2.

3. Results

3.1. Diversity of ATB-resistant bacteria in water and feces

The affiliation of the prominent reamplified bands from DGGE gels from major bacterial community members obtained from ATB enrichment cultures, from water and feces, in all studied lakes is shown in Table 1. 16S rRNA gene sequence comparisons revealed that most of the water and feces DGGE bands were represented mainly by *Proteobacteria*, particularly, *Gamma-proteobacteria*, which is grouped among typical planktonic bacteria, but also *Alpha-proteobacteria* and *Beta-proteobacteria*. *Gamma-proteobacteria* group is represented by *Pseudomonadales*, *Aeromonadales*, and *Xanthomonadales* members in Aparejos, Negra, and Azul lakes, in both water and feces samples.

Band sequences related to *Beta-proteobacteria*, belonged mainly to *Burkholderiales* (*Comamonas* sp., *Curvibacter* sp., and *Duganella* sp.) members, were recovered from Aparejos and Azul samples. Bands sequences related to *Alpha-proteobacteria* group were only recovered from Azul samples. The presence of Firmicutes was indicated by several DGGE bands in the three studied lakes, most of them grouped among *Bacillales*, *Clostridiales*, and *Lactobacillales* members.

In Laguna Aparejos, two bands sequences (A15 and A18) were exclusively recovered from water and they presented similarities with members of the genera *Enterobacter* and *Commamonas*.

The sequence related to *Stenotrophomonas* was the most widespread among all lakes, in both water and bird feces samples, since sixteen band sequences were matched to this genus.

Phylogenetic Group	DGGE Bands Closest Identified Relative (Accession Number)	% Similarity	Source	ATBs Resistances
Laguna Aparejos				
Beta-proteobacteria				
A16	*Duganella* sp. (AM711889)	90	F, W	Col
A18	*Comamonas* sp. (AM711890)	98	W	Amp
Gamma-proteobacteria				
A1	*Pantoea* sp. (AM711573)	100	W, F	Amp, Col, Ery Cm, Tet
A2; A3; A4A; A16a, A5A	*Stenotrophomonas* sp. (AM711575; AM711576; AM711577; AM711888; AM711577)	96-100	W, F	Amp, Ery, Cm, Tet
A9; A10; A14; A8A	*Pseudomonas* sp. (AM711878;AM711879; AM711886; AM711579)	98-100	W, F	Amp, Ery, Col, Tet
A2A	*Pseudomonas* sp. (AM711574)	98	F	Amp
A13	*Klebsiella* sp. (AM711884)	95	W, F	Amp, Ery

Phylogenetic Group	DGGE Bands Closest Identified Relative (Accession Number)	% Similarity	Source	ATBs Resistances
A15	*Enterobacter* sp. (AM711887)	98	W	Amp, Col, Tet
A13a	*Acinetobacter* sp. (AM711883)	98	F	Amp, Ery, Cm, Tet
Firmicutes				
A11a	*Carnobacterium* sp. (AM711880)	96	F	
A11	*Enterococcus* sp. (AM711881)	98	F	Col
A14a	*Clostridium* sp. (AM711885)	99	F	
A12a	*Bacillus* sp. (AM711882)	97	F	
Laguna Negra				
Gamma-proteobacteria				
N1	*Aeromonas* sp. (AM712052)	97	W, F	Amp, Col
N2	*Enterobacter* sp. (AM712053)	99	W, F	Amp, Col
N8; N5	*Escherichia* sp. (AM712056; AM712054)	96-99	F	Amp
N10; N11; N11a; N12	*Rahnella* sp. (AM712058; AM712060; AM712059; AM712061)	99	W, F	Amp, Col
N13; N14	*Aeromonas* sp. (AM712062; AM712063)	95-99	W, F	Amp, Col, Tet
N16; N19	*Stenotrophomonas* sp. (AM712065; AM712066)	99-100	W	Col, Ery, Tet
Firmicutes				
N6	*Clostridium* sp. (AM712055)	99	F	Amp
N9; N15	*Bacillus* sp. (AM712057; AM712064)	99-100	F	Amp, Col
Laguna Azul				
Alpha-proteobacteria				
Az1; Az14	*Sphingomonas* sp. (AM889064; AM889077)	92-95	W	Amp, Col, Tet
Beta-proteobacteria				
Az11	*Curvibacter* sp. (AM889074)	98	F	Cm, Tet
Az9	*Delftia* sp.(AM889072)	99	F	Col
Az16; Az18	*Variovorax* sp. (AM889079; AM889081)	96	F	Col, Tet
Gamma-proteobacteria				
Az4	*Pseudomonas* sp.(AM889067)	83	W	Col
Az19; Az20; Az2;	*Pseudomonas* sp. (AM889082; AM889083; AM889065;	96-99	W, F	Amp
Az6; Az15; Az10; Az21; Az8; Az25; Az23; Az7; Az17	*Stenotrophomonas* sp.(AM889069; AM889078; AM889073; AM889084; AM889071; AM889087; AM889085; AM889070; AM889080)	96-99	F	Amp, Ery, Tet
Firmicutes				
Az24	*Bacillus* sp.(AM889086)	96	F	Ery
Az13; Az12; Az3	*Bacillus* sp.(AM889076; AM889075; AM889066)	98-100	W, F	Ery, Amp, Col
Actinobacteria				
Az5	*Arthrobacter* sp. (AM889068)	96	F	

Table 1. Phylogenetic affiliation of sequences obtained from DGGE bands from water (W) and feces (F).

3.2. Antibiotics resistances

The microbial diversity by DGGE in water and feces after cultivation under antimicrobial pressure could be explained by the presence of ATB-resistant traits or the acquisition of resistant traits by horizontal gene transfer events during cultivation.

In Laguna Aparejos, there was a band sequence detected in the five enrichment cultures conditions. It was the case of a band sequence related to *Pantoea* sp. This band sequence was found in water as well as feces samples in all ATB tested. Another example of an extensive ATB resistance to tested ATB was those of band sequences (A13a) matched with *Acinetobacter* sp.

In Laguna Negra, most of the DGGE-detected bands were found in Col- and Amp-enriched cultures. Two band sequences (N13, N14) matched with *Aeromonas* sp. were detected in enrichment cultures with Amp, Col, and Tet. Two band sequences related to *Stenotrophomonas* (N16, N19) were the only ones that depicted resistance to Ery in this lake. In Laguna Azul, there were bands detected in all of the ATB enrichment culture tested. However, there was one band sequence that matched with *Arthrobacter* sp. (Az5) that was not visible in any ATB-enriched culture but only in control culture for feces.

DGGE band sequences matched with *Stenotrophomonas* were present in all studied lakes; however, their "ATB resistance profile" was different among them. While in Aparejos it was recovered from Amp, Ery, Cm, and Tet enrichment culture, in Negra, resistances included Col, Ery and Tet, while in Azul Lake, the ATB profile included Amp, Ery, and Tet.

3.3. DGGE analyses

Figure 1 shows the dendrogram resulting from the Cluster Analysis performed among samples taking into account the presence or absence of individual bands obtained by DGGE profiles of Laguna Aparejos. The analysis evidenced that water and flamingo feces without any antimicrobial pressure clustered together conforming a subgroup.

In Laguna Negra, cluster analysis indicates that water, feces, and feces with Amp clustered within the same subgroup (Figure 2).

In Laguna Azul, two clear groups can be observed, one for feces samples and the other for water samples (Figure 3).

4. Discussion

It was proposed that landscape ecology, which links the biotic and abiotic factors of an ecosystem, might help to untangle the complexity of antibiotic resistance and improve the interpretation of ecological studies [20]. Continuing that idea, we have previously demonstrated that water in high-irradiated pristine environments was a source for isolating bacteria able to grow in the presence of antibiotics, and that the bacteria were also present in flamingos' enteric biota, probably taken from the water where they feed [3]. In addition, we have found

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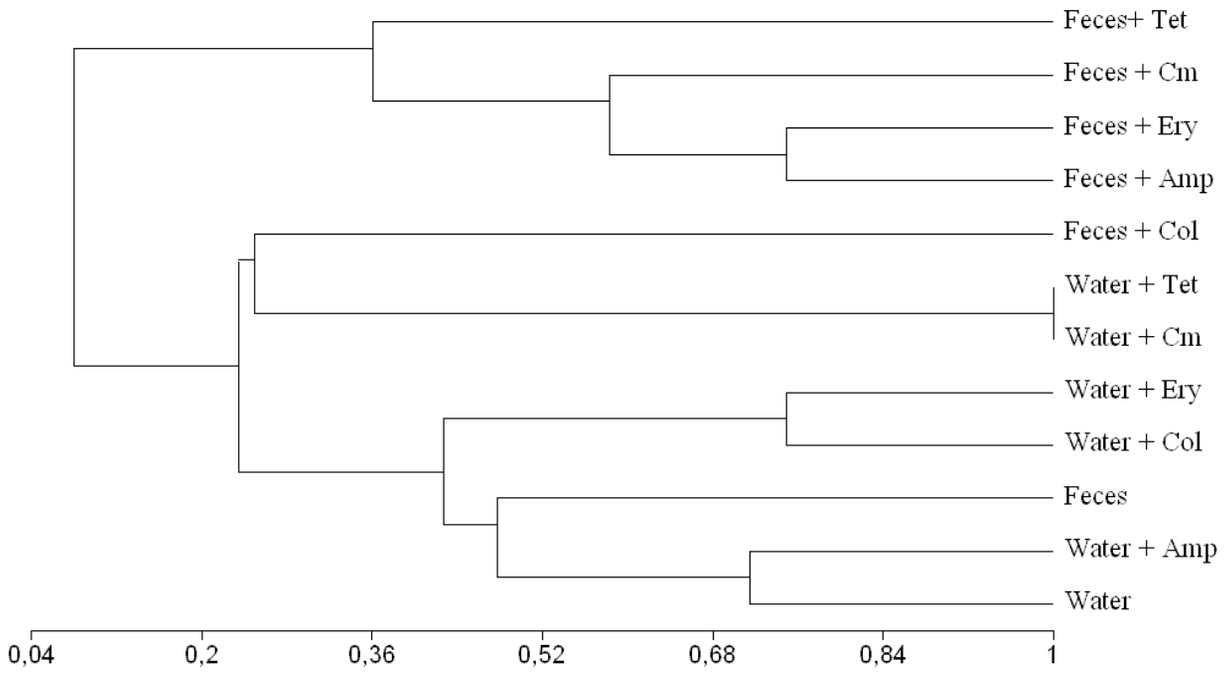


Figure 1. Clustering using band-based Jaccard coefficient for Laguna Aparejos samples.

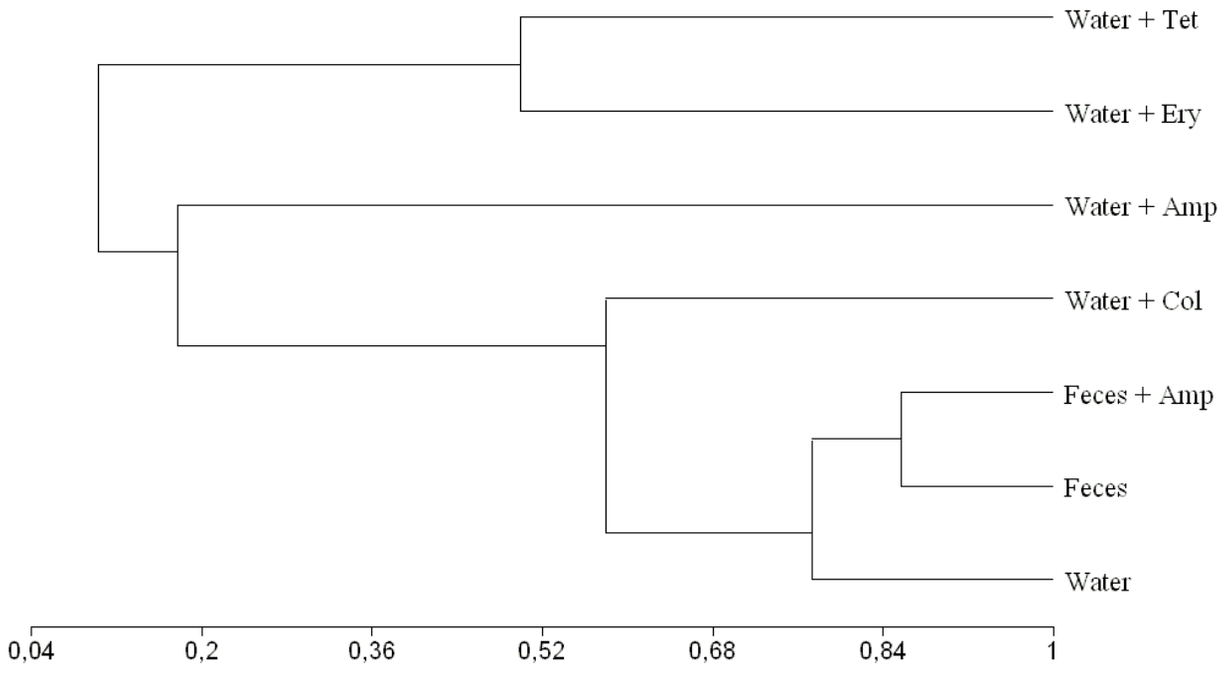


Figure 2. UPGMA dendrogram resulting from the Cluster Analysis performed among samples from Laguna Negra.

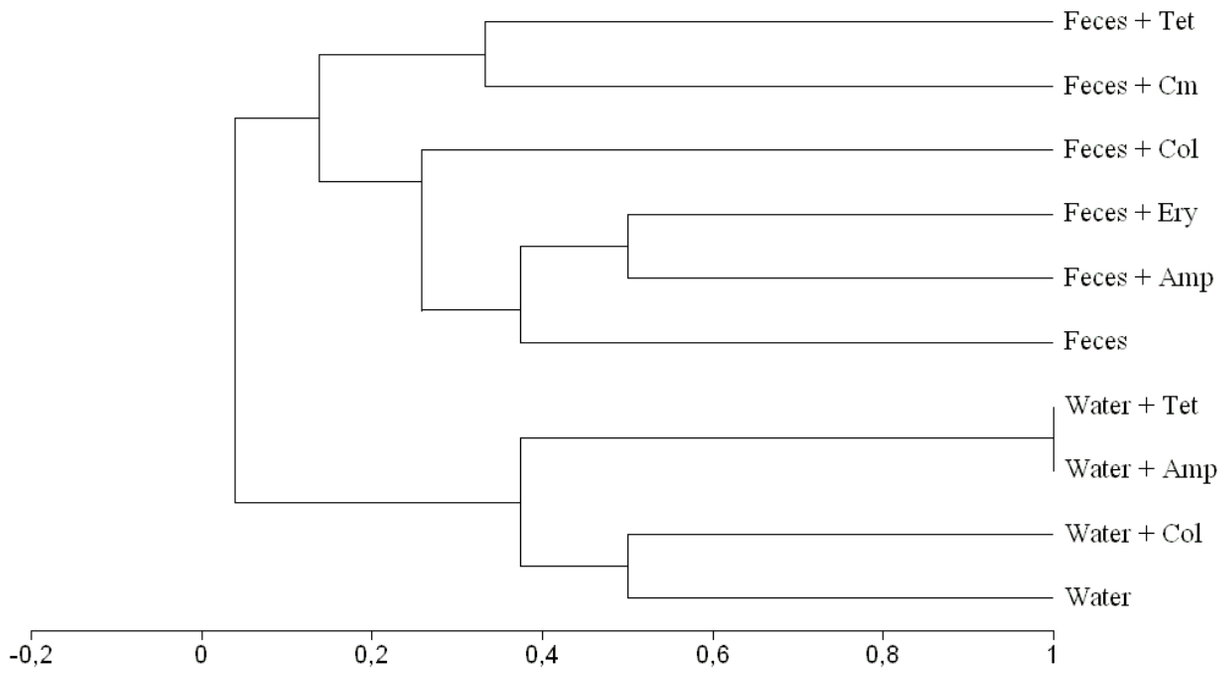


Figure 3. Dendrogram based in Jaccard coefficient showing the similarity coefficient of bacterial community from enrichment ATBs cultures from water and flamingo feces.

that several isolated bacteria present giant extra chromosomal linear elements, the so-called linear plasmids [21-23]. We found that the presence of linear plasmids might be related to the antibiotics-resistant dispersion. In this work, we attempt to study the total bacterial community under different selective pressures and the connection between the microbiota associated to lake water and flamingo feces.

4.1. Antibiotic-resistant bacteria is an spread phenomenon in high-altitude lakes

We showed that the ability to grow in ATB or the rapid spread of this ability was abundant, diverse, and widely distributed in the water and feces of the studied high-altitude environments. As it was postulated by our group in previous publications [2, 3], UV radiation would be in connection with ATB resistances since under extreme UV stress, bacteria are known to increase mutational events, through a resistance mechanism named error-prone repair [24]. In many cases, spontaneous resistance to ATB is known to emerge under such mutagenic conditions, as consequence of mutagenesis modified potential target genes. In addition, a possible connection of oxidative stress resistances and an association with ATB resistances were also established [25]. As it was largely established that UV radiation produces high oxidative stress, thus a high-irradiated environment is expected to select oxidative stress-resistant bacteria, and this could also be in connection with ATB resistances found in more irradiated environments.

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One the other hand, exposure of wild birds to human-generated wastewater presents a pathway for transfer of bacteria and the antibiotic resistance genes that they carry [26]. Water bodies of Pampean Lakes are threatened by many anthropic activities, resulting from land use, agriculture, and livestock, with the subsequent deposition of a significant amount of organic wastes, fertilizers, and pesticides [27-30]. Therefore, flamingos exposed to such sources could be colonized by microorganisms that are not typical of their natural habitats and are involved in the dissemination of multidrug-resistant bacteria since migration of flamingos, among lakes from Andean lakes in summer to Pampean lakes in winter, is an established phenomenon [31]. Our next challenge is subject to deeper studies the flamingo's role as disseminators and/or reservoir of multidrug-resistant bacteria.

4.2. Microbiota in water and birds feces

Mostly, band sequences identified in water samples were also found in feces. Thus we observed a connection between the bacterial community's inhabitant flamingos intestinal and those of the water lake, where these birds obtain their food: community structure harboring similar ATB resistances were similar in both water and feces samples, sampled from the same lake. Special attention should be given to *Stenotrophomonas*, since it seems to be the widest spread bacterium. It was detected in DGGE bands of water and feces of Aparejos, Negra, and Azul lakes and isolated from water and feces of Negra and Azul lakes and, in all the cases, it was the most resistant to multiple ATB [2]. This bacterium has been increasingly recognized as an important cause of nosocomial infection. Infection occurs principally, but not exclusively, in debilitated and immunosuppressed individuals. The management of *S. maltophilia* infections is often problematic because this pathogen is frequently inherently resistant to multiple antibiotics [32, 33].

A band corresponding to *Acinetobacter* sp. was also detected in Laguna Aparejos. In previous reports we showed *Acinetobacter* strains that offer high ATB and UV resistance [1, 2, 16, 34, 35] and also other related strains from water and feces of Negra, Azul, and Vilama with multiple ATB resistances [5].

As it was determined by our preview reports [2, 3], we confirm the idea that pathogenic organism resistant to multi-antibiotics are not a phenomena restricted to spoiled environments and that pristine environments could be considered as important reservoirs of bacteria like *Klebsiella pneumoniae*, *Staphylococcus* sp., *Aeromonas* sp., *S. maltophilia*, and a wide group of enteric bacteria resistant to multiple ATB. Birds with wide migration itineraries could indeed spread these bacteria. Thus, from an epidemiological point of view, pristine UV-irradiated environments should receive more attention as reservoirs of potential human pathogens as well as ATB resistances.

Nomenclatures

HAAL – High Altitude Andean Lakes; ATBs – Antibiotics; DGGE – Denaturant Gradient Gel Electrophoresis

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Author details

María V. Fernández-Zenoff[1,3*], Julián R. Dib[1,3], Anna Neumann[2], María C. Estévez[1] and María E. Farías[1]

*Address all correspondence to: verozenoff@hotmail.com

1 Planta Piloto de Procesos Industriales Microbiológicos (PROIMI -CONICET), Tucumán, Argentina

2 Technische Universität Berlin, Berlin, Germany

3 Instituto de Microbiología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

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