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Microbiota sampled from a polluted stream in Recife-PE, Brazil and its importance to public health

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Pollution of water bodies can cause environmental and public health problems. The Cavouco stream is a tributary of the Capibaribe River, one of the main rivers in the state of Pernambuco, Brazil, and receives a high pollution load from residential, laboratory and hospital effluents. The aim of the present study was to perform phenotypic and molecular characterization in this stream, and evaluate the water quality using microbiological parameters. Water was collected from five sampling points, and bacterial species were identified using biochemical and molecular methods through *16S rRNA* gene sequence analysis. Total and thermotolerant coliforms were also quantified. Fermenting Gram-negative bacilli from the family Enterobacteriaceae (*Escherichia coli, Klebsiella pneumonia*e and *Proteus mirabilis*), non-fermenting bacilli (*Pseudomonas aeruginosa* and *Pseudomonas putida*) and Gram-positive bacilli (*Bacillus cereus, Bacillus licheniformis, Bacillus pumilus* and *Staphylococcus hominis*) were identified. A total of 25 bacterial isolates were phenotypically identified. All phenotypic identifications were confirmed by molecular analysis, except for *S. hominis*, which was molecularly identified as *Exiguobacterium*. Regarding water quality, all analyzed samples were positive for total and thermotolerant coliforms. The results obtained suggest that the Cavouco stream presents a potential risk for transmission of water-borne diseases, because of the presence of pathogenic bacteria. In addition, the current state of the stream also threatens the conservation of its native species.

Keywords: Public health, Enterobacteriaceae, thermotolerant, Bacilli.

INTRODUCTION

Aquatic ecosystems have suffered significant changes due to multiple environmental effects, resulting from the release of large quantities of effluent without prior treatment (International Joint Commission, 2015; USGS, 2015). This discharge can cause physical, chemical and biological deterioration, and endangers both the resident aquatic organisms and public health. Scientific, technological and epidemiological advances have provided new tools for the assessment of water quality, both for human consumption and environmental

purposes (Tanchou, 2014).

In 2015, the Centers for Disease Control and Prevention (CDC) reported that approximately 780 million people had no access to drinking water around the world. The consumption of contaminated water and lack of basic sanitation is estimated to cause 842,000 deaths per year worldwide, and 1,000 children under the age of five years die every day (WHO, 2014; WHO and UNICEF, 2015). Contaminated water carries pathogens that cause diarrhea, gastrointestinal disorders and systemic diseases, approximately 70% of diarrheal diseases could be avoided by improving basic sanitation (WHO, 2014). According to the Brazilian Ministry of Health, 6,715 deaths caused by diarrhea or gastroenteritis, presumably resulting from infection, were recorded between 2010 and 2015 (BRASIL, 2015).

Some studies have used traditional methods of selective isolation and cultivation to characterize the microbial communities of the affected environments (Skariyachan et al., 2013). However, taxonomic classification by these methods can be difficult because of variations in phenotypic characteristics (Woo et al., 2008). For this reason, molecular methods that allow fast and reliable confirmation of microbial identity have been developed (Ramírez-Castillo et al., 2015). Among these, methods using *16S rRNA* gene sequencing are predominant. This gene is used as a phylogenetic marker because its sequences are highly conserved (Srinivasan et al., 2015).

The aim of the present study was to evaluate the water quality by the isolation and identification of representative bacterial species present in this environment, using biochemical and molecular methods.

METHODS

Study area

The Cavouco stream, located at latitude 8°2'52.05"S and longitude 34°57'10.33" W, state of Pernambuco (UFPE), Brazil, is approximately 6 km long, and flows into the right margin of the Capibaribe River, one of the main rivers of state. Along its course, it receives pollutants from residential, laboratory and hospital waste, which reduces the water quality and threatens the aquatic life (Araujo and Oliveira, 2013; Freitas et al., 2016). Water samples (200 mL) were collected from five points (Figure 1) along the stream, according to the methods of Araújo and Oliveira (2013), and stored between 1 and 4°C until subsequent bacteriological analysis.

Isolation and phenotypic identification of bacterial isolates

For bacterial isolation, 50 µL of water was inoculated onto eosin methylene blue (EMB) agar and 5% bovine blood agar (to count colony forming units), and incubated at 37°C for 24 to 48 h. Gram staining was then performed to make presumptive identifications of the bacteria found according to the technique described by Koneman and Winn (2006).

Gram-negative isolates were preliminarily identified using the following biochemical tests: glucose, lactose and sucrose fermentation, hydrogen sulfide production, motility, indole production and citrate, lysine and urea degradation. Species identification was confirmed using the Kit API 20E (Biomérieux), according to the manufacturer's instructions.

Gram-positive isolates were preliminarily identified through colony morphology, hemolysis in blood agar, and presence/absence and position of spores, visualized through Gram staining. Species identification was confirmed using an automated system (BD Phoenix™ Automated Microbiology System).

Analysis of total and thermotolerant coliforms

For analysis of total and thermotolerant coliforms, water samples were collected from the water surface at a depth of 30 cm, from each of the five sampling points. The samples were stored between 1 and 4°C until analysis.

The presence and number of total and thermotolerant coliforms were determined using the multiple-tube fermentation method, and the results were expressed as most probable number (MPN) per 100 mL of sample, according to APHA (2015).

Molecular identification using *16S rRNA* gene sequence analysis

The bacterial samples were inoculated into 5 mL brain heart infusion (BHI) broth for 24 h at 37°C for to DNA extraction. Chromosomal DNA was extracted using the phenol-chloroform method (Sambrook and Russell, 2001). DNA quality was evaluated by electrophoresis in a 0.8% agarose gel using 0.5× TBE buffer and run at 100 V for 1 h; gels were analyzed using a UV transilluminator and photographed. DNA concentration was quantified using a Nanodrop spectrophotometer (Thermo Scientific).

The *16S rRNA* gene was amplified by polymerase chain reaction (PCR), using the primers fD1 (5´-AGAGTTTGATCCTGGCTCAG-3´) and rD1 (5´-AAGGAGGTGATCCAGCC-3´) (Weisburg et al., 1991). PCR was performed in a final volume of 25 µL, containing 1× buffer, 200 µM dNTPs, 1.5 mM $MgCl_2$, Taq DNA-polymerase (1 U/µL; Invitrogen), 10 pmol of each primer, and 10 ng of DNA template. The PCR was performed using a thermocycler (C1000 Thermal Cycler – BioRad), and the PCR program consisted of 95°C for 5 min, 30 cycles at 95°C for 45 s, primer annealing at 54°C for 45 s, extension at 72°C for 2 min, and a final extension at 72°C for 5 min.

PCR products were purified using the Pure Link purification kit (Invitrogen) according to the manufacturer's instructions, and sequenced using the Big Dye Kit (Applied Biosystems) on an automated DNA sequencer (ABI 3100). The *16S rRNA* gene sequences obtained was compared with sequences deposited in the GenBank database (NCBI). The dendrogram was constructed using multiple sequence alignment, based on genetic distances, maximum parsimony, and maximum likelihood, using Molecular Evolutionary Genetic Analysis 5.2 software (MEGA5).

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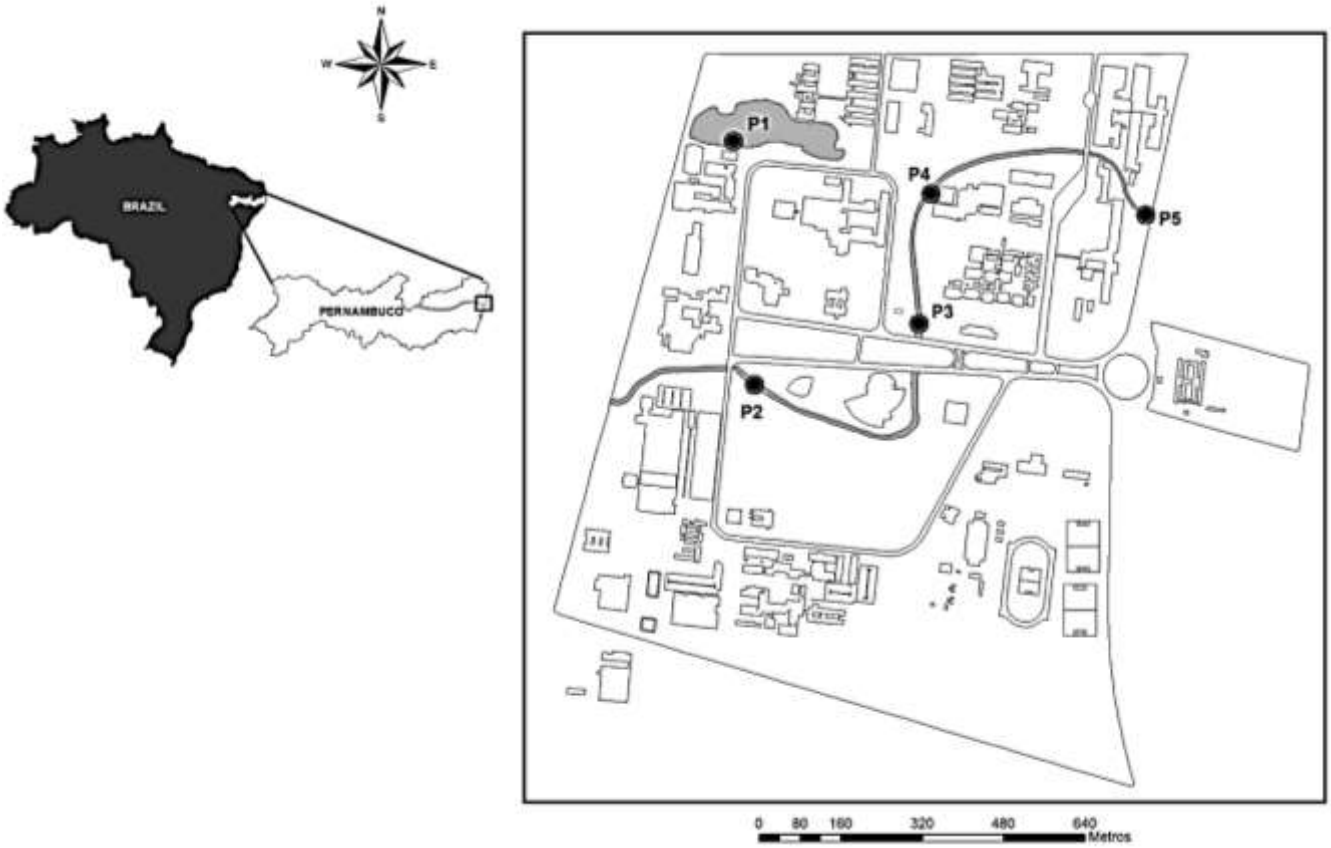

Figure 1. Representation of Cavouco creek area, Brazil, showing the five collection points (P1-P5) and microbial representatives obtained from each point.

Bacterial library of Cavouco stream – UFPE

Identified bacteria were stored in 80% glycerol (150 µL of glycerol and 850 µL of bacterial culture) at -80°C, and under pure mineral oil (Isofar) at ambient temperature. The isolates were labeled according to: C (Cavouco), P (sampling point), the number of sampling points, and the number of isolations.

RESULTS

Microscopic and biochemical identification of bacterial colonies

Inoculated blood agar presented innumerable colony forming units (CFU), except for water samples collected at the stream source (Point 1), for which only a few colonies were observed. Due to EMB medium selectivity, these plates presented an average of 200 CFU, with a lower number of CFUs observed for Point 1, and higher number for point 5 EMB plates presented colonies with metallic green sheen, dark center, some with bright or pink edges, and mucoid appearance. In blood agar, some colonies were shiny or gray, with or without beta-hemolysis, and with irregular edges. Microscopic examination of Gram-stained colonies revealed the presence of Gram-negative and positive bacilli. Some Gram-positive bacilli were observed to be arranged in chains, filaments and spherical bodies. Approximately, 22 and 25 colonies were selected from the most frequent colonies present on EMB medium, and from blood agar, respectively.

A total of 25 isolates were identified phenotypically, belonging to seven genera (*Bacillus, Enterobacter, Escherichia, Klebsiella, Proteus, Pseudomonas* and *Staphylococcus*). Enterobacteria were present in all sampling points. *Proteus mirabilis* was highly prevalent among Gram-negative species (with eight isolates mostly from point 5), followed by *Escherichia coli* (six isolates), and *Klebsiella pneumoniae* (five isolates, from points 2, 3, and 4). Among the Gram-positive bacilli, the genus *Bacillus* was prevalent, with two species (*Bacillus licheniformis* and *Bacillus pumilus*) identified from point 4, and one (*Bacillus cereus*) from point 1. Another Gram-positive species, *S. hominis,* was identified from point 4 (Table 1).

Presence of total and thermotolerant coliforms

All analyzed samples were positive to total and thermotolerant coliforms, presenting an MPN > 1.4 ×

Table 1. Phenotypic and molecular identification of isolated bacteria from Cavouco stream collection points. ID: Identity. *Access number refer to sequences deposited in GenBank.

Points	Isolates	Phenotypic Identification		16S rRNA gene sequencing		
		API 20E (ID%)	BD Phoenix	Species	Similarity (%)	Accession*
P1	$CP_1 1_S$	-	*Bacillus cereus*	*Bacillus cereus*	99	KT719668.1
P2	$CP_2 3_P$	*Escherichia coli* (99.8%)	-	*Escherichia coli*	99	CP014225.1
	$CP_2 4_P$	*Escherichia coli* (94.8%)	-	*Escherichia coli*	99	CP014225.1
	$CP_2 2_P$	*Klebsiella pneumoniae* (97.8%)	-	*Klebsiella pneumoniae*	99	KM233642.1
P3	$CP_3 5_S$	*Proteus mirabilis* (99.9%)	-	*Proteus mirabilis*	99	KR150991.1
	$CP_3 6_S$	*Pseudomonas putida* (44.5%)	-	*Pseudomonassp.*	99	JQ994361.1
	$CP_3 8_S$	*Klebsiella pneumoniae* (97.9)	-	*Klebsiella pneumoniae*	99	AB680212.1
	$CP_3 9_P$	*Klebsiella pneumoniae* (98.1%)	-	*Klebsiella pneumoniae*	99	AB680212.1
	$CP_3 13_P$	*Klebsiella pneumoniae* (97.7%)	-	*Klebsiella pneumoniae*	99	AB680212.11
	$CP_3 7_S$	*Escherichia coli* (99.8%)	-	*Escherichia coli*	99	CP014225.1
	$CP_3 10_P$	*Escherichia coli* (99.8%)	-	*Escherichia coli*	99	CP014225.1
	$CP_3 11_P$	*Escherichia coli* (99.8%)	-	*Escherichia coli*	99	CP014225.1
P4	$CP_4 14_S$	*Escherichia coli* (99.9%)	-	*Escherichia coli*	98	CP013837.1
	$CP_4 16_S$	*Klebsiella pneumoniae* (97.7%)	-	*Klebsiella pneumoniae*	99	KC524425.1
	$CP_4 15_S$	*Enterobacter clocae* (99.4%)	-	*Enterobacter clocae*	99	GU191924c.1
	$CP_4 18_S$	-	*Bacillus licheniformis*	*Bacillus licheniformis*	99	KJ26873.1
	$CP_4 19_S$	-	*Bacillus pumilis*	*Bacillus pumilis*	99	KJ526890.1
	$CP_4 20_P$		*Staphylococcus hominis*	*Exiquobacterium* spp.	99	KT074375.1
P5	$CP_5 22_S$	*Proteus mirabilis* (99.9%)	-	*Proteus mirabilis*	99	KF811051.1
	$CP_5 23_S$	*Proteus mirabilis* (99.9%)	-	*Proteus mirabilis*	99	KR150991.1
	$CP_5 25_S$	*Proteus mirabilis* (99.9%)	-	*Proteus mirabilis*	99	KR150991.1
	$CP_5 26_P$	*Proteus mirabilis* (99.9%)	-	*Proteus mirabilis*	99	KR150991.1
	$CP_5 27_P$	*Proteus mirabilis* (99.9%)	-	*Proteus mirabilis*	99	KR150991.1
	$CP_5 28_P$	*Proteus mirabilis* (99.9%)	-	*Proteus mirabilis*	99	HQ169118.1
	$CP_5 27_P$	*Proteus mirabilis* (99.9%)	-	*Proteus mirabilis*	99	KR150991.1

10^3/100 mL.

Molecular identification using *16S rRNA* gene analysis

The *16S rRNA* gene sequences (approximately 1.500 bp) for the 25 selected isolates were aligned, and compared with sequences deposited in the GenBank database. The obtained sequences were found to have a high degree of genetic similarity (98-99%) with deposited sequences for the same species, confirming their phenotypic identification. Of the 25 isolates, only *S. hominis* did not display concordance between the phenotypic and molecular identification. This isolate was identified phenotypically as belonging to the genus *Staphylococcus*, but through molecular analysis, it revealed 99% similarity with the genus *Exiguobacterium* (Table 1). The phylogenetic tree showed clustering of *16S rRNA* gene sequences obtained from the studied isolates with those from GenBank, confirming molecular identification (Figure 2).

Construction of the bacterial library of Cavouco

The bacterial library of Cavouco currently includes 21 Gram-negative and four Gram-positive species isolated from five sampling points, stored in frozen stocks under mineral oil. These are the first bacterial isolates of this environment, and the first representatives of the Bacterial Library of Impacted Environments of UFPE.

DISCUSSION

The analyses showed high water contamination of the Cavouco stream, located in Recife-PE, Brazil, with all samples containing thermotolerant coliforms. The presence of coliforms is a parameter evaluated for water quality monitoring programs and indicates the presence of potentially pathogenic microorganisms (WHO, 2014).

In the present study, the quantification of microbiological parameters indicated that all samples were unfit for human consumption and recreational use. The observed coliform levels were higher than those considered safe by

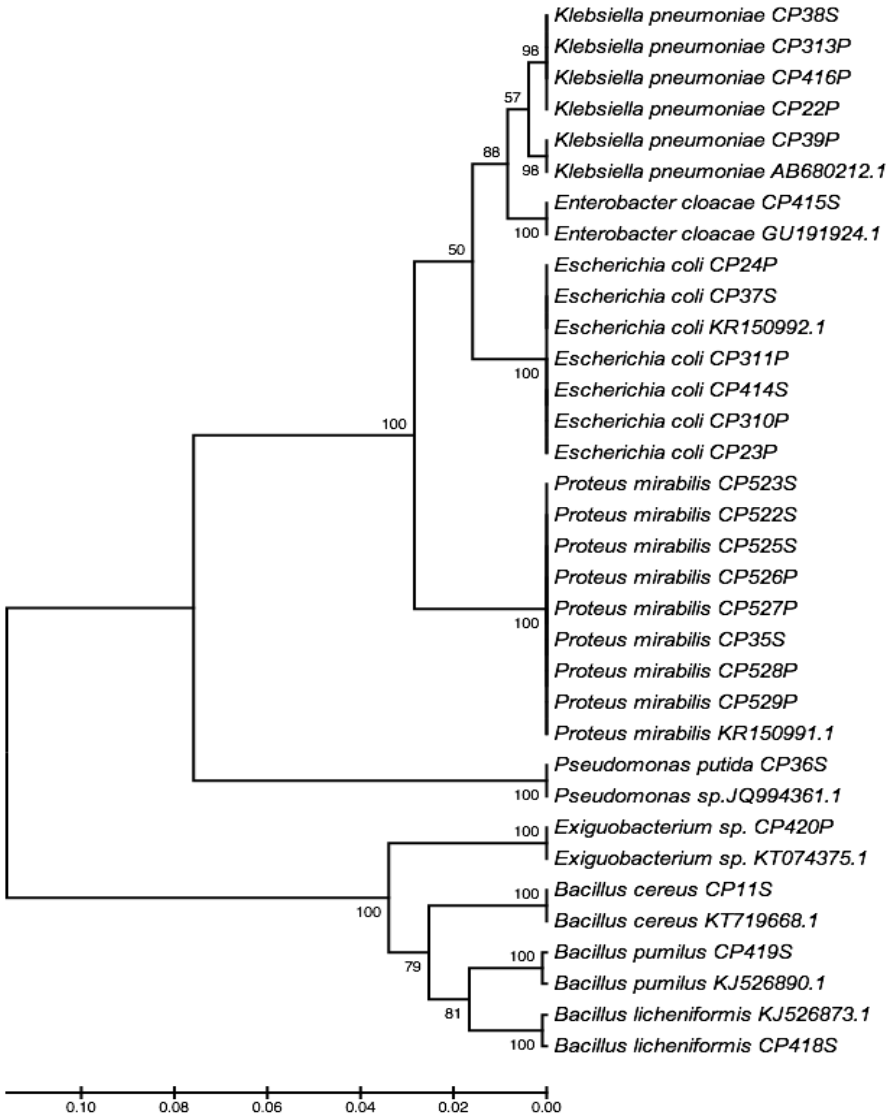

Figure 2. Similarity dendrogram generated from the comparison between the sequences of *16S rRNA* strains isolated from collection points at Cavouco and the deposited ones in GenBank database.

the US Environmental Protection Agency (EPA), 2011 and the Brazilian Environmental Council (Normative Resolution N° 357/2005) (CONAMA, 2005), representing risk for human health.

Indications of significant pollution in the studied regions of the Cavouco stream had been previously observed. Araujo and Oliveira (2013) analyzed Cavouco stream water, and found changes in the levels of dissolved oxygen, ions and ammonia, as well as values of the Index of Water Quality for the Protection of Aquatic Life (IQAPVA) indicating low capacity for the maintenance of aquatic life.

Enterobacteria were present at all sampling points in the present study. Several studies have shown that most enterobacteria can cause infections because of their ability to survive in hostile environments and their ability to develop resistance to antimicrobial drugs (Irenge et al., 2015; Tajbakhsh et al., 2015; Patel et al., 2016).

Several pipes located along the margins of the Cavouco stream discharge residential, hospital, and laboratory effluents into the stream, which explains the presence of bacteria of fecal origin. In addition, the existence of pastures for animal grazing and the development of other activities, together with the poor conservation state of the local riparian forest, could contribute to this microbiological water pollution.

Previous studies of affected environments have reported similar results. Rodrigues et al. (2009), evaluated the water quality of the Piracuama River, located in the state of São Paulo (SP), Brazil, and identified enterobacteria of

genera *Citrobacter, Edwardsiella, Enterobacter, Escherichia, Klebsiella, Morganella*, *Salmonella* and *Shigella.* A study performed on a contaminated river in India also identified the presence of important pathogens for public health, namely *Edwardsiella* spp.*, Enterobacter* spp.*, E. coli, Morganella* spp.*, Proteus* spp.*, Pseudomonas* spp.*, Serratia* spp. and *Staphylococcus* spp. (Skariyachan et al., 2013), with fecal coliforms being the most common pathogens in contaminated rivers and streams (Kim et al., 2013; Liu et al., 2015).

Escherichia coli was isolated from several sampling points of stream, is used as a specific indicator of fecal contamination in tropical and temperate regions (Páll et al., 2013). This species is considered one of the main causes of diarrhea in adults and children in developing countries, infection most often occurring through contact with contaminated water (Walker et al., 2007; Isidean et al., 2011) and is an important cause of urinary tract and wound infections and pneumonia in immunosuppressed hospitalized patients, and meningitis in newborn children (Wijetunge et al., 2015; Martelius et al., 2016).

Proteus spp. has been described as an important infectious agent in hospital environments (Chen et al., 2014; Murray et al., 2015). Bacteria from the genus, are commonly found in the environment, especially in locations with water pollution and soils with degraded material (Drzewiecka, 2016). A study performed in hospitals located in northeast Brazil, the same region as the present study, isolated *P. mirabilis*, which produces extended spectrum beta-lactamase (ESBL), have been reported to be an important cause of nosocomial infection in worldwide (Abreu et al., 2011). In the present study, this species was isolated in an area where there is discharge of hospital waste.

The genus *Klebsiella* is widely distributed in nature and in the gastro intestinal tract of humans and animals.. *K. pneumonia* can also be found in the oropharynx of hospitalized patients, constituting a source of pulmonary infections, and usually occurring in patients with debilitating conditions such as alcoholism and diabetes (Distel et al., 2013). This species can also infect the urinary tract, cutaneous wounds, and blood, causing bacteremia, meningitis in infants, hepatic abscess, and urinary tract infections (Siu et al., 2012). This species has gained importance due to the development and inter-species and intra-species dis-semination of several antimicrobial resistance mechanisms, namely, the production of beta-lactamases such as ESBLs and KPC (*K. pneumoniae* Carbapenemase), which degrade beta-lactam antibiotics, frequently detected in hospitalized patients in Recife, PE, Brazil (Lopes et al., 2010; Cabral et al., 2012; Melo et al., 2014).

The presence of the genus *Bacillus* in the Cavouco stream is worrisome. The presence of *Bacillus* in food in amounts higher than 10ϖ cells per gram indicates multiplication, and indicates a high health risk (Germano and Germano, 2003). Another interesting aspect was the observed diversity of *Bacillus* species. This might indicate that the environmental conditions (nutrients, temperature, humidity, oxygen concentration, and pH) were favorable for the multiplication and maintenance of these species at the studied site.

Bacillus species can sporulate, and the resistance conferred by these spores constitutes an important problem for the epidemiology of associated infections. Because the Cavouco stream is a tributary of the Capibaribe River, which is used as a water source for local agriculture and fishing, the presence of *Bacillus* could contribute to food contamination.

Of the five sampling points, points 3 and 4 presented the highest microbial diversity, whereas point 5 yielded only one species, *P. mirabilis*. The absence of diversity at point 5, which receives the discharge of effluents from a morgue and hospital, may have been due to difficulty of isolating Gram-positive bacteria and the lack of selectivity of the blood agar used for bacterial cultures. Another possible explanation for the difficulty in isolating Gram-positive bacteria was the presence of high concentrations of toxic substances in hospital effluents, such as antibiotics, cytostatic agents, heavy metals, disinfectants and hormones, which could have a genotoxic effect on these bacteria (Jean et al., 2012; Devarajan et al., 2015).

Molecular tools were used in the present study to confirm the phenotypic identification of the 25 isolates. The *16S rRNA* gene is widely used as phylogenetic marker, and it has been sequenced for a large number of bacterial lineages (Srinivasan et al., 2015). Most of these sequences are deposited in the GenBank database (Benson et al., 2012), and can therefore be compared with sequences of new isolates.

For isolates belonging to different species, their *16S rRNA* gene sequences must share less than 97% similarity (Goebel and Stackebrant, 1994). The sequences obtained in the present study shared 98% or higher similarity with sequences deposited in the GenBank database, confirming the phenotypic identification of all isolates, except for *S. hominis*, which presented 98% similarity with genus *Exiguobacterium.* This can be explained by the fact that both genera have very similar morphological and biochemical characteristics, such as motility, positive catalase and urease activity, negative phosphatase and coagulase activity, presence or absence of nitrate reduction, and acid production under aerobic conditions, and both contain aerobic and facultative anaerobic species (Stieglmeier et al., 2009). These colonies are spherical, opaque, butyrous or yellow-orange, growing at temperatures varying between 20 and 45°C (Schleifer et al., 1979; Collins et al., 1983). The similar morphological and biochemical characteristics of the two genera might have contributed to its initial identification as *Staphylococcus*. Similar results were reported by Elmaci et al. (2015), who observed a divergence in identification at the species level for 53.3% (81/152) of the tested lactic

acid bacteria isolates, identified phenotypically using the API CHL method and *16S rRNA* gene sequence analysis. Bosshard et al. (2006) observed a discrepancy at the genus and species level for 20.6% (12/58) of the tested non-fermenting Gram-negative bacterial isolates, identified using API 20 NE and *16S rRNA* gene sequence analysis. Both studies attributed this divergence to the similarity of morphological and biochemical characteristics of different taxonomic groups, making identification using phenotypical methods difficult, and confirming the specificity of molecular analysis.

The present results indicate that the Cavouco stream could significantly contribute to an increase in microbial pollution, presenting a potential risk of waterborne disease transmission, as genera of pathogenic bacteria were identified. In addition, the current state the Cavouco stream also threatens the conservation of its native species. Environmental cleaning actions such as the establishment of a sewage collection and treatment systems are urgently needed to improve the water quality of this and other affected aquatic environments, such as rivers, lakes and coastal areas. The obtained data enabled the establishment of a bacteria library, which will help to understand the evolution of impacted environment, in terms of environmental quality, overtime. The present study characterized a sample of cultured bacteria isolated from the studied area. In spite of the importance and relevance of this data for public health, it does not represent the totality of organisms present in the studied area.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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