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Article

Bifidobacteria serve as indicators of fecal contamination in tropical water bodies

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Abstract: Human fecal contamination poses a significant public health concern in water sources, yet standard indicator microorganisms for detecting such contamination fail to pinpoint the exact source. The genus *Bifidobacterium*, particularly species like *B. adolescentis* and *B. dentium*, has been suggested as a potential marker for identifying human fecal pollution, though this proposal has yet to be tested in tropical settings. This study aimed to assess the presence of bifidobacteria in a water sample from the Mesolandia swamp in the Colombian Caribbean, as well as in 260 human fecal samples and 94 samples from domestic animals in a nearby settlement. DNA was extracted from each sample and subjected to PCR amplification with gender-specific primers targeting the 16S rRNA gene, followed by DGGE (Denaturing Gradient Gel Electrophoresis) separation. DGGE bands were then excised, re-amplified, sequenced, and compared to the GenBank database. The DGGE profiling revealed the presence of eight Bifidobacteria species in the water sample, matching those found in human feces. The proposed markers *B. adolescentis* and *B. dentium* were also detected in domestic animal feces. Despite the efforts, the study was unable to identify a unique Bifidobacteria species that could serve as a reliable marker for human fecal contamination in tropical environments under the evaluated conditions. Nevertheless, the methodology employed provided a more precise approximation to the source of fecal contamination than traditional cultural methods, as identical DNA sequences were found in both water and fecal samples.

Keywords: water contamination; biomarkers; feces; denaturing gradient gel electrophoresis; *Bifidobacterium*; polymerase chain reaction

1. Introduction

The consumption of contaminated water is the cause of approximately 842,000 deaths per year, mainly in developing countries where pathologies such as dysentery and intestinal parasitism are associated with inadequate management of water sources. This not only happens due to deficiencies in sanitation and drinking water potabilization systems, but also due to the discharge of untreated wastewater into surface water sources that are subsequently used for recreational or agricultural activities [1]. The presence in a water source in permanent contact with the community of microorganisms of fecal origin generates an important impact for public health, even more so when the fecal contamination is of human origin, since it may contain pathogens such as hepatitis A viruses, enteroviruses causing diarrheal disease, pathogenic bacteria, infecting parasitic forms, among others. Traditional culture methods to determine fecal contamination in water bodies and other matrices such as Total Coliform (TC) and Thermo Tolerant Coliform (TTC) counts, among

others commonly used, do not allow specifying whether the contamination is of human or animal origin, a differentiation that would make it possible to improve sanitary control and prevent the transmission of species-specific diseases through water [2].

In recent decades, there has been great interest in the development of new methods for determining human fecal contamination, including the investigation of chemicals and drugs unique to human use (such as caffeine), or the presence of species-specific enteric viruses [3,4]. Numerous bacterial genetic markers have been designed for molecular detection of human fecal contamination in environmental waters. However, the widespread use of these markers has been hampered by lack of knowledge regarding their stability and geographical validity, being restrictively designed and tested in a small number of well-characterized regions [5]. Determining in water bodies, the presence of bifidobacteria species that exclusively inhabit the human gut, such as *Bifidobacterium adolescentis* and *B. dentium* has been one of the best accepted proposed markers of human fecal contamination in recent years [6–8]. However, this marker has been developed and tested in regions with different temperatures, biological conditions, and socioeconomic characteristics than those present in tropical regions. To date, there is no evidence that, in tropical environments, the presence of these bifidobacteria species could be an adequate indicator of human fecal contamination, highlighting that in these environments high temperatures and high relative humidity predominate, as well as precarious sanitary conditions and the coexistence between humans and domestic animals that favor cross-contamination.

In this study, carried out in a human settlement adjacent to a contaminated water body in the Colombian Caribbean Region, the presence of bifidobacteria as potential markers of human fecal contamination was evaluated in water and feces of humans and animals. Molecular techniques such as PCR-DGGE (denaturing gradient gel electrophoresis) were used.

2. Materials and methods

The sampling site corresponds to the Ciénaga de Mesolandia, a body of water that forms part of the delta at the mouth of the Magdalena River to the Caribbean Sea in northern Colombia. This swamp in 2015 presented high levels of microbiological contamination [9]. The contamination comes mainly from water discharged from the surrounding municipalities (Malambo and Soledad) and from marginal urban settlements on the shore of the swamp, such as Barrio Mesolandia, with poor sanitary conditions. A punctual sampling was carried out to obtain fecal samples from humans and animals, according to the procedures established by the Institute of Hydrology, Meteorology and Environmental Studies [10] and the National Institute of Health of Colombia [11]. The geoposition (10°53′30″ N & 74°45′53″ W) corresponds to approximately 3 m from the eastern side of the marsh, in the area bordering the Mesolandia neighborhood.

The in situ physicochemical parameters: pH, temperature, conductivity, turbidity, dissolved oxygen and salinity were determined using the Multiparametric WTW Multi 3420 equipment, duly calibrated. The sample (in duplicate) was

transported at 5 ℃ to the Metropolitan University laboratory for processing.

In Barrio Mesolandia, 260 human fecal samples were collected (simple sampling with a margin of error of 6.0% and confidence level of 95% in relation to the population of the Barrio) and 94 fecal samples from domestic animals as follows: 6 samples coming from pigs (Sus scrofá), 5 from roosters (Gallus gallus), 5 from cats (Felis silvestris), 4 from birds (Order Passeriformes), and 74 from dogs (Canis lupus). The animal samples were collected by the owners or caretakers from fresh stool, extracting only the material from the top without touching the ground to avoid contamination. Samples were transported at 5 ℃ to the Metropolitan University laboratory and stored at −80 ℃ until use.

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Microbiological analysis of water by cultural methods: Total Coliform (TC) and Thermotolerant Coliform (TTC) counts were performed by the multiple tube technique that determines the most probable number of microorganisms in 100 mL of water (MPN/100 mL) according to the protocols of the American Public Health Association (APHA) [12].

Extraction of bacterial DNA from water and feces: From the water sample, 100 mL were centrifuged at 13,000 g for 10 min, the sediment was subjected to a 1 h pre-lysis treatment at 37 ℃ in 100 mM phosphate buffered saline pH 7.0 with 1 mg/mL lysozyme, and then DNA was extracted (NucleoSpin Kit from Macherey Nagel). From each human and animal stool sample, 200 mg were diluted in 2 mL of saline phosphate buffer with lysozyme, centrifuged and used to extract bacterial DNA (Isolate II Genomic DNA Kit from Bioline, USA). All DNAs were quantified spectrophotometrically by measuring absorbance at 260 nm.

PCR amplification of *Bifidobacterium* species in water and feces: 100 ng of DNA extracted from water or feces were PCR amplified using the species-specific primers Bif164F (5'-GGGGGTGGTAATGCCGGGGATG-3') and Bif662R-GC (5'-CGCCCGCCGCGCGCGGGCGGCGGGGCGGGGGGGCACGGGGGGGGCCAC CGT T ACACCGGGAA-3') which amplify 523 bp of 16S rDNA [13,14]. The PCR reaction consisted of 3 min at 98 ℃, 34 cycles of 98 ℃ 30 s, 62 ℃ 30 s and 72 ℃ 1 min, with a final cycle of 72 ℃ for 5 min, using Bioline Velocity polymerase. PCR reactions were verified on 2% agarose gels using Syber Green to visualize the amplified DNA.

DGGE of PCR amplified samples: 8% acrylamide gels of size 16 × 17.5 cm were used with a denaturing gradient of urea and formamide increasing in the direction of electrophoresis between 30 to 50% (100% denaturation corresponds to 7 M Urea and 40% formamide) and using the Cleaver Cientific DGGE equipment. Electrophoresis was performed for 6 h at a constant 130 V and a temperature of

62 °C, in TAE 1X Buffer (TAE 50X: TRIS base 2 M, Acetic Acid 1 M and EDTA pH 8.0 50 mM). The gels were then stained for 15 min in a solution of Syber Green in 0.5X TAE Buffer and the fluorescence produced was recorded with the ChemiDoc XRS+ System photodocumenter from BIO-RAD.

Identification of the bifidobacteria species present in the water: The resulting bands in DGGE of the water sample were cut and the DNA extracted (ISOLATE II PCR and Gel Kit from Bioline); reamplified by PCR using the primers Bif164F and Bif662R without guanine-cytosine tail (5'-CCACCGTTACACCACCGGGAA-3') under the PCR conditions described previously. The identity of the isolates was performed by sequencing of the two amplified DNA strands (Sanger method, at Corpogen Bogotá-Colombia) and subsequent comparison in the Gen Bank database using the Blast program. The PCR re-amplifications of the identified bifidobacteria species were used as a control in DGGE of human and animal fecal samples.

Ethical aspects of the research: In compliance with decree 1376 of 21 June 2013, which regulates the collection of specimens of biological diversity for scientific research purposes, permit Auto No. 00000021 of 2015 was requested before the Corporación Autónoma Regional del Atlántico (CRA). Human fecal samples were collected after approval of the ethics committee and signature of informed consent.

3. Results

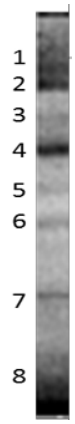
Water sample analysis: The physicochemical parameters determined directly in the water body were as follows: pH 8.3; temperature 31 °C; dissolved oxygen 10.13 mg/lt; salinity 1.3; conductivity 2.5 ms/cm. The results of the CT and CTT counts were higher than 160,000 NMP/100 mL, exceeding the limits allowed for primary and secondary contact activities [15]. The PCR-DGGE analysis showed a profile of bands that, when re-amplified and sequenced, allowed the identification of eight species of bifidobacteria present in the water (**Table 1**).

Profiling of bifidobacterial bands in PCR-DGGE of the water sample. The identity of the bifibobacteria represented in each band was determined by sequencing the re-amplified DNA of the bands and comparing them in the GenBank database.

Band profile in PCR-DGGE of the water sample. The *Bifidobacterium* identity was determined by DNA sequencing, reamplification and comparison in the GenBank database.

Analysis of human and animal fecal samples: PCR amplification of the species of the genus *Bifidobacterium* under the experimental conditions described was possible in 31.54% of the human fecal samples and 36.17% of the animal samples. In DGGE it was observed that the eight bands corresponding to the bifidobacteria identified in the swamp water were present in human feces (**Figure 1**), being the bands corresponding to *B. lactis* and *B. adolescentis* the most frequent in the population with 19.23% and 11.92% respectively, *B. dentium* was present in 4.61% of the human samples.

Table 1. Identification of Bifidobacteria by PCR-DGGE from a water sample from the Mesolandia swamp.

	Band Number	Number bp	% Identity	GEN BANK
PCR-DGG E	B 1	514	99	*Bifidobacterium longum* ZGP-Blo.40 16S rDNA: KT222150.1
	B 2	518		*Bifidobacterium adolescentis* 16S rDNA: JCM 1275. LC071806.1
	B 3	519		*Bifidobacterium dentium* 16S rDNA: JN004065.1
	B 4	526		*Bifidobacterium pseudolongum subsp. globosum* 16S rDNA: AB507140.1
	B 5	524	99	*Bifidobacterium lactis* 16S rDNA: X89513.1
	B 6	523	99	*Bifidobacterium animalis subsp. lactis* 16S rDNA: JCM 10602
	B 7	519		*Bifidobacterium ruminantium* 16S rDNA, JCM 8221: AB507150.1
	B 8	518		Non-culturable *Bifidobacterium* sp. HM14 16S rDNA:FJ441227.1

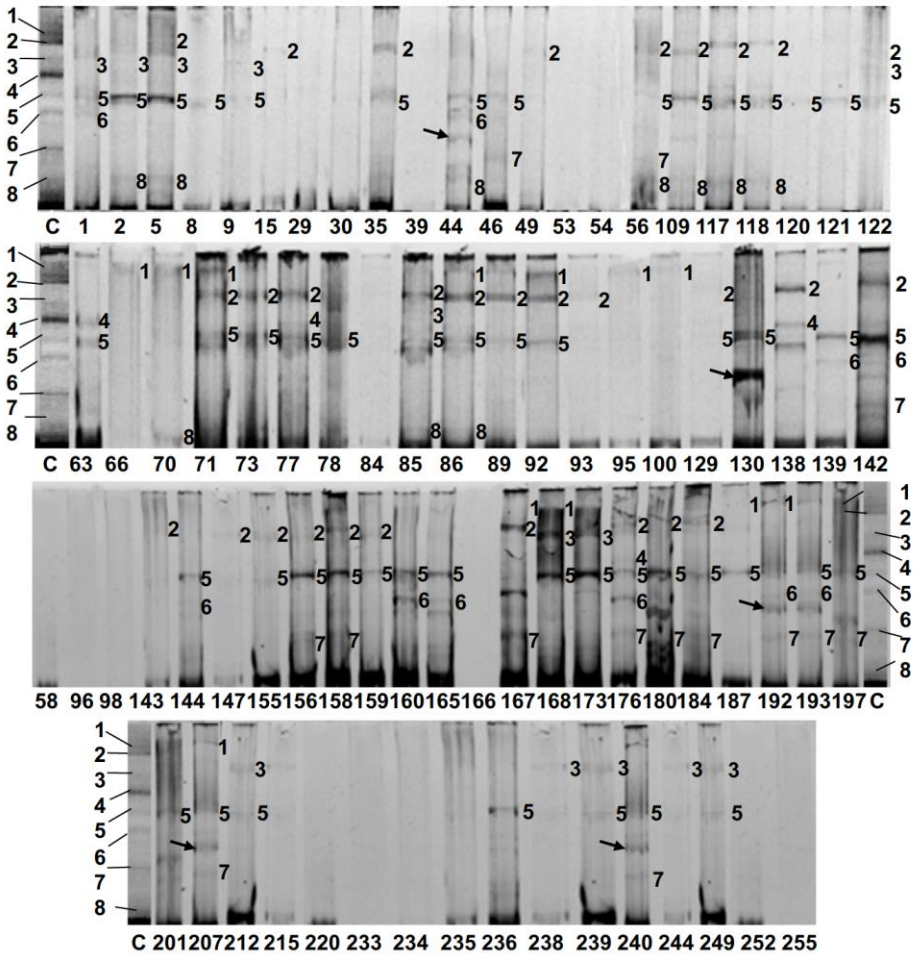


Figure 1. PCR-DGGE banding profiles of Bifidobactene species present in human

fecal samples. C: Banding pattern where 1: *Bifidobacterium* longum, 2: B adolescentis, 3: *B. dentium*, 4: B. pseudolongum subsp. globosum, 5: B. lactis, 6: B. animalis subsp. lactis, 7: B. ruminantium and 8: *Bifidobacterium* sp. The numbers in each profile correspond to its homologous band in the pattern. Arrows indicate bands with marked intensity in some samples that are not in the standard.

In animals, the percentage of PCR positivity was variable according to the species: pig 50% (*n* = 3), rooster 60% (*n* = 3), cat 40% (*n* = 2), bird 25% (*n* = 1) and dog 33.78% (*n* = 25). In DGGE (**Figure 2**) the bands corresponding to B. lactis and *B. pseudolongum* were the most frequent in dogs. In pigs and cats, the intensity of the bands in DGGE was very low, with *B. animalis* identified in one pig; in birds, more bands were observed in samples taken from birds (parrots living in captivity) than from samples taken from roosters. Bifidobacteria species that are considered exclusive inhabitants of the human intestine, such as *B. adolescentis* and *B. dentium*, were found in fecal samples from animals, with *B. adolescentis* in 4.05% of dogs (*n* = 3) and *B. dentium* in one bird. All bifidobacterial species identified in water were also present in human samples and in at least one animal species.

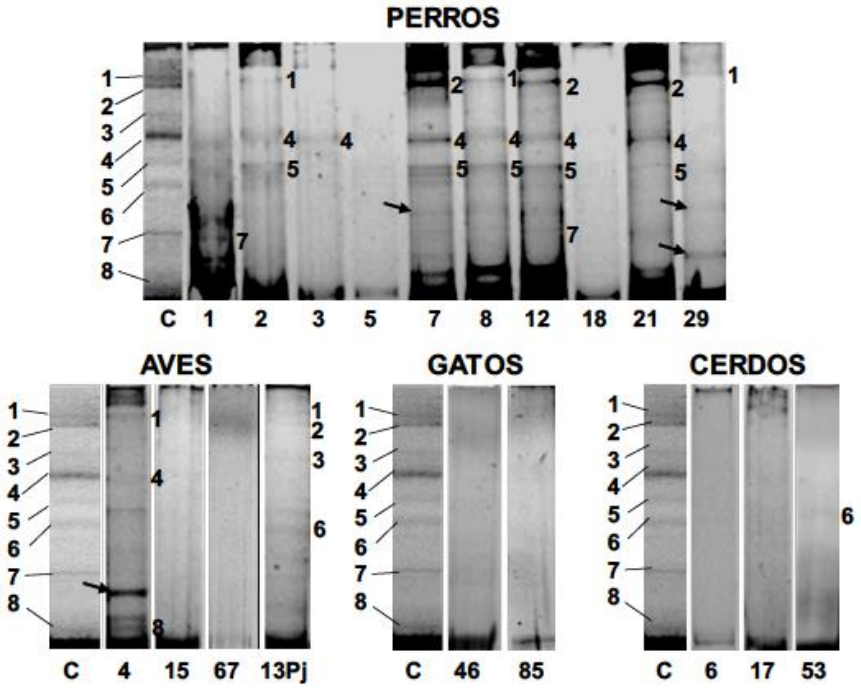


Figure 2. PCR-DGGE banding profiles of Bifidobacteria species present in fecal samples of domestic animals. C: Banding pattern where 1: *Bifidobacterium* longum, 2: B adolescentis, 3: *B. dentium*, 4: B.pseudolongum subsp. globosum, 5: B. lactis, 6: B. animalissubsp. lactis, 7: B. ruminantium and 8: *Bifidobacterium* sp. The numbers in each profile correspond to its homologous band in the pattern.

4. Discussion

In the Mesolandia marsh, high levels of microbiological contamination were found through CT and CTT counts, a result consistent with what was previously reported by the Corporación Autónoma Regional de Atlántico [9]. This is evidence

of a recurrent problem in this body of water with implications for public health, even more so when there are human settlements in constant interaction with the water in the surrounding area. Regarding the physicochemical parameters found in this study, they showed to be similar to those reported by the CRA and the Institute of Marine and Coastal Research (INVEMAR) [16], which are considered compatible with the conservation of life in the aquatic ecosystem of the marsh.

Eight different species of bifidobacteria were identified by PCR-DGGE in the bog water. This finding shows that, despite the conditions of temperature, solar radiation, high microbial presence and even chemical contamination from companies near the swamp, it is possible to identify bifidobacteria species by the molecular methods described, demonstrating sufficient permanence in the water to allow their identification. Although the scope of this study does not allow us to evaluate the permanence time of bifidobacteria in the water body after their entry through fecal matter, some species of bacteria identified in this study could serve as indicators of fecal contamination. In this regard it should be noted that microorganisms such as Escherichia coli and other fecal coliforms commonly used to define fecal contamination can be very susceptible to environmental factors, which affects their usefulness as an indicator [17,18]. Therefore, it would be of great interest to raise new studies that allow comparing the susceptibility to environmental factors of traditional indicators of fecal contamination with bifidobacteria. In order to avoid false negatives in the investigation of fecal contamination in tropical waters, due to the susceptibility of microorganisms to environmental conditions, some authors propose the combined use of the identification of microorganisms with the evaluation of substances such as coprosterol [19].

In addition to its resistance to environmental factors, the ideal characteristics that a microorganism should meet to be considered a suitable indicator of fecal contamination are: to be present in the intestinal tract, not to proliferate in the environment, not to be pathogenic and to be easily detectable by relatively inexpensive methods [20]. These characteristics are present in specific strains of bifidobacteria such as *B. adolescentis* and *B. dentium*, recognized as possible indicators of fecal contamination of human origin in different studies [6–8]. In this study some of these characteristics were ratified by the finding of bifidobacteria in the fecal samples and in the water. Moreover, taking into account that bifidobacteria are not facially culturable microorganisms, their identification by molecular techniques (currently widespread technology) can be rapid, reliable and relatively inexpensive.

It is known that bifidobacteria are generally the first microorganisms that colonize the human intestine from birth and that they are an important part of the intestinal microbiota until adulthood, with modifications in their population associated with diet, interaction with other intestinal microorganisms and under various environmental conditions [21]. In this work, it is striking that only in 31.54% of the human fecal samples it was possible to amplify by PCR with specific primers for that genus, which merits raising new studies to explain the low frequency of bifidobacteria in the studied population. It is possible that it is necessary to modify the experimental conditions, since although the primers used in this study have been successfully used by other authors in the evaluation of bifidobacteria in intestinal or

complex environmental ecosystems, there are no precedents of their use in the environmental and population conditions of the Colombian Caribbean. On the other hand, the type of diet and poor sanitary conditions such as those found in the marginal population studied, may be reasons that explain the low frequency of bifidobacteria in human fecal samples, since the intestinal microbiota depends directly on the quality and quantity of carbohydrates, proteins and other nutrients in the diet [22]. The presence of *B. adolescentis* and *B. dentium* was relatively low in the evaluated human samples, an unfavorable condition for a specific fecal contamination indicator.

There are few studies that evaluate the presence of bifidobacteria in animals. However, Gavini et al. [23] isolated bifidobacteria in 122 of 145 fecal samples from domestic animals: cattle (*Bos taurus*); pigs (*S. scrofa*); sheep (*Ovis orientals'*); goats (*Capra aegagrus*); horses (*Equus caballus*); rabbits (*βryctolagus cuniculus*); chickens (*G. gallus*); geese (*Anser anser*) and pigeons (*Columba livia*) on farms in France and in 92 of 955 animals in Austria (*B. taurus* and *S. scrofa*), and found *B. pseudolongum* to be the most frequent species in animals. In a recent study it was found that in dogs (*C. lupus*), *B. animalis* and *B. pseudolongum* are the most frequently found bifidobacteria species, suggesting that dietary changes may modify the intestinal population of these [24]. In this work, mainly dogs were included because they were the most frequent animal species in the slum evaluated, also *B. pseudolongum* was the most found bifidobacteria species in animals. However, it should be noted that all eight bifidobacteria identified by PCR-DGGE in the swamp water were present in the feces of at least one of the animal samples evaluated.

The microbiological contamination of the Ciénaga de Mesolandia with animal and human fecal remains indicates the presence of a significant number of microorganisms in the water, many of which have pathogenic potential for river dwellers. This also constitutes a public health problem as it is a potential reservoir for various microbial resistance mechanisms. This contamination, which was evidenced by cultural methods such as CT and CTT counts, was corroborated by the finding of specific sequences of bifidobacteria, which allowed a closer approximation to the real origin of the fecal contamination, given the presence of these sequences directly in the water and in the feces of humans and animals. These results allow us to propose that the identification of specific bifidobacteria sequences could be an effective method to determine fecal contamination.

The mechanisms of gene transfer and epigenetic inheritance that occur in the complex intestinal ecosystem allow the adaptation and constant change of microbial populations [25]. Under this proposition, microorganisms such as *B. adolescentis* and *B. dentium* considered exclusive inhabitants of the human intestine, could also adapt to intestinal environmental conditions of other warm-blooded animals, even more so when the coexistence between humans and domestic animals results very close and in hygienic and environmental conditions that could favor coprophagy in animals. In this work it was found that the study of bifidobacteria by molecular methods such as PCR-DGGE in contaminated surface water in tropical regions, allows the identification of fecal contamination, but it is not very reliable to discriminate whether its origin is human or animal. There is a need for new studies to create indicators of fecal contamination in contaminated tropical environments to

specifically identify human fecal contamination.

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