Analysis of the 16S rRNA gene for the characterization of the bacterial community of the Lambro river (Italy)

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Characterization of the microbial community of a river can provide various indications, such as its general state of health or the presence of contamination. Furthermore, the study of *Bacteroidetes*, which have a high degree of host specificity, can provide information on the species involved in any fecal contamination. The analysis of the 16S rRNA was used to characterize the bacterial community of the Lambro river (Italy) through. The results, which were obtained by analyzing water from 15 sampling points, show a reduction in the complexity of the bacterial community as the river enters a densely populated region. The cause could be a source of chemical or physical contamination that carries out a positive selection toward some bacterial species and negative toward others. In addition, a notable increase in the percentage of *Bacteroidetes was reported, especially* when the river enters regions characterized by high agricultural and livestock activity, such as cattle and pig farming. However, in the samples taken from this area, no *Bacteroidetes* ascribable to these two species or to the other species considered (i.e., human, dog, and cat) were found. Surprisingly, suspected bacterial contamination of swine origin was identified in a sparsely populated region characterized by small family farms. Finally, the efficient treatment of urban wastewater was confirmed as no markers of fecal pollution of human origin were identified.

KEYWORDS

PCR, microbial communities, fecal contamination, Lambro river, 16S rRNA

Introduction

Water is a crucial element for the life of plants and animals, and the availability of water sources free of microorganisms is a fundamental condition for community development as it reduces the likelihood of bacterial infections. The analysis of the microbial communities present along a river can therefore represent a useful tool to determine its state of health, identify any sources of contamination, learn more about the origin of the contamination, and prepare any necessary remediation plans (Hwang et al., 2012; Kwon et al., 2018). Water can be polluted both from natural causes, for example volcanic eruptions, as well as from those related to human activities,

including fecal contamination caused by non-compliant treatment of urban and livestock waste. However, it is important to define the origin of fecal contamination (human vs. animal). *Escherichia coli* has always been used as a marker of fecal contamination, but its high genetic diversity, unrelated to a specific host and associated with the ability to replicate outside of a host, limits the possibility to define the source of contamination (Simpson et al., 2002). Bacteria belonging to the *Bacteroidetes phylum* represent a better alternative to *E. coli*, as they make up a large part of the fecal bacterial population (Madigan et al., 2003). Specifically, they have a poor ability to replicate in the environment (Fiksdal et al., 1985), and they exhibit a very high degree of specificity for the host (Dionisi et al., 2003). For this reason, *Bacteroidetes* have often been used in the past to identify the species involved in fecal contamination (Bernhard and Field, 2000; Layton et al., 2006; Villemur et al., 2015; Staley et al., 2018; Cruz et al., 2021; Yasar et al., 2021).

The Lambro is a river with a total length of about 130 km. The source of the river is located in Alpe del Piano Rancio (942 m a.s.l., 45°55′05.26″N, 9°14′24.06″E), and it ends near Orio Litta (51 m a.s.l., 45°08′07.86″N, 9°32′45.55″E). During its relatively short path, it crosses six Lombardy provinces, which include Como, Lecco, Monza/Brianza, Milano, Pavia, and Lodi (Supplementary Figure 1). It also spans different geographical regions, including mountainous and densely populated areas as well as those ones where zootechnical activity is intensively represented. Therefore, it represents an interesting model for investigating the relationships between the environment and microbial communities. In this work the bacterial communities of the Lambro along its path was analyzed and this fact represents a novelty: until now no river had been characterized in all its length, from source to mouth. The supervisory authorities have declared the complete isolation of urban and livestock waste so fecal contamination, whether of animal or human origin, was expected to be excluded from any Lambro sample. Moreover, a detailed analysis of *Bacteroidetes* was conducted to identify the eventual origin of any fecal contamination.

Materials and methods

Sample collection

Water samples (400 ml) were taken from 15 different sampling points (Supplementary Figures 1, 2). Sampling was carried out as close as possible to the central part of the river in order to avoid collecting material from the banks. All samples were obtained on the same day. The samples were collected in sterile plastic bottles, transported to the laboratory at 4°C and subsequently stored at −80°C. Finally, animal and Human fecal samples were also used.

Bacterial DNA extraction

First, the samples were filtered through a paper filter to eliminate soil and/or plant contaminants, and subsequently, the bacteria were collected on a 0.22 μm membrane. The bacteria were then resuspended by stirring the filters in 3 ml of sterile H_2O for 1 h, and a subsequent centrifugation at 12,000 rpm for 30 min collected a bacterial pellet. Bacterial DNA was extracted using the NucleoSpin Tissue kit (Qiagen) following the supplier's directions. The DNA was initially resuspended in a volume of 40 μl of sterile H_2O and subsequently diluted to obtain a final concentration of 5 ng/μl. To create a reference of the microbial communities of fecal samples, samples of human, dog, cat, pig, and bovine feces were analyzed. In this case, the bacterial DNA was obtained using the NucleoSpin Soil kit (Macherey-Nagel) starting from 25 mg of material.

16S gene amplification and sequencing

A portion of the 16S rRNA was amplified using 5 μl of the extracted DNA in a final reaction volume of 25 μl using Platinum Taq DNA Polymerase High Fidelity (Thermofisher) in accordance with the manufacturer's instructions. The amplifications were performed for 26 cycles of the following amplification profile: 95°C-60 s (denaturation), 55°C-60 s (annealing) and 72°C-60 s (extension). The following primers were used: Pro341F: 5′-CCTACGGGNBGCASCAG-3′ and Pro805R: Rev 5′-GACTACNVGGGTATCTAATCC-3′ (Takahashi et al., 2014). These primers produce an amplicon of ∼445 bp (depending on the amplified bacterial species). The overhang adapter (forward overhang: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-locus specific sequence and reverse overhang: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-locus specific sequence; from BMR Genomics srl-Italy) was added and followed by library construction. The libraries were purified with Beads Amplure XP 0.8X, amplified with Indexes Nextera XT Illumina, and then normalized, mixed, and loaded on Miseq with 2 × 300 bp (paired-end).

Bioinformatics analyses

The raw sequences were verified and filtered by quality, trimmed by the primers, and fused with Qiime2 v. 8 software (Bolyen et al., 2019). DADA2 (Qiime2) software isolated the OTUs (Operational Taxonomic Unit), whose sequences were compared against Greengenes v. 13-8 database to obtain the taxonomic assignment. A similarity analysis between the species detected in the feces and those ones in the water was performed using the BLAST program, available at NCBI (Altschul et al., 1990).

Statistical analysis

The interpretation of the results regarding the percentage of the different bacterial species was carried out using Excel 365. PCA (Principal Components Analysis) analysis was performed using StataMp v. 17 software. The statistical evaluation of the different Shannon–Weaver index obtained (SWi) was calculated with the two sample Mann–Whitney test.

Results and discussion

Richness estimation

The complexity of the bacterial populations was evaluated using the Shannon–Weaver index (SWi; Figure 1A). A higher value was identified at sampling D (8.06), while a lower value was identified at sampling R (4.37). These values are consistent with what has been observed in other watercourses (Cruz et al., 2021; Tee et al., 2021). The trend of the SWi at the different sampling points indicates that the maximum values were near the source of the river, while the minimum values were near the end of the path. The greatest complexity change occurred between points H and I. This fact could be explained by considering that between those two sampling points, the Lambro river passes from a sparsely inhabited area to a highly inhabited area (municipality of Milan) and then crosses an area densely characterized by farms. The median of the SWi values obtained from the A–H samplings, 7.48, was significantly higher ($p < 0.001$) than the median of the values obtained at the I–R sampling points, which

was 4.94 (Figure 1B). A great variety in the bacterial populations of a watercourse is an indication of good health and represents also a main factor that allows the ecosystem to react with adverse phenomena (Loreau et al., 2001; Cardinale, 2011; Zinger et al., 2012). What has been observed supports the hypothesis that the quality of the water of Lambro worsens significantly when it crosses areas with high anthropic activities.

The deterioration of the water quality was also confirmed by observing the number of OTUs identified (Figure 1A). The number of OTUs, standardized on 15,000 sequences, varied from 126 (Q and R sample points) to 607 (G sample point). The average number of OTUs in the first seven withdrawals (from A to G) was approximately double of the average of the last eight withdrawals (from I to R) at 423 compared to 220, respectively.

Bacteria community analysis

Considering all 15 sampling points, bacteria belonging to 37 different *phyla* were identified, even if only 16 of them were present at a percentage >0.5% in at least one sample (Figure 2A). The most represented *phyla* were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. They all represent from a minimum of 79.05% (point C) to a maximum of 99.99% (point R) of the entire bacterial community; furthermore, after sampling point D, their percentage was always higher than 90% (Figure 2B). *Proteobacteria* represented the majority *phylum* at all sampling points, and their percentage never dropped below 50%. The bacteria belonging to the *Bacteroidetes*, in
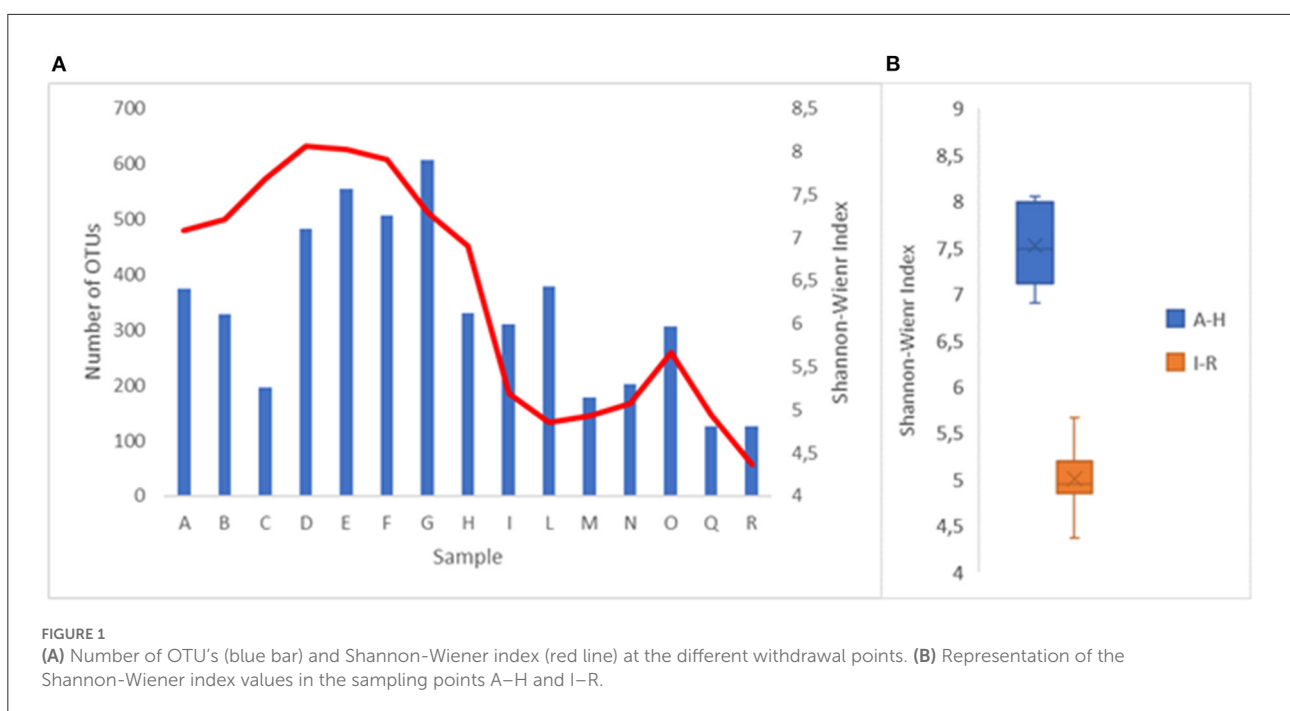

FIGURE 1
(A) Number of OTU's (blue bar) and Shannon-Wiener index (red line) at the different withdrawal points. **(B)** Representation of the Shannon-Wiener index values in the sampling points A−H and I−R.

FIGURE 2

(A) Distribution of the 16 *phyla* identified in at least a percentage higher than 0.5% in at least one sampling point. *Phyla* with a lower percentage are grouped under the category "others." **(B)** Distribution of the presence of the four main phyla (*Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*) in the 15 sampling points.

contrast, showed an almost constant trend upward to sampling point L and then an increase to the last sampling point. Sampling point L marks the entrance of the Lambro in the region largely characterized by the presence of farms. The *Firmicutes* exhibited an opposite trend, in fact their presence was appreciable up to point I and then disappeared in the

subsequent sampling points, while the *Actinobacteria* showed a constant presence in all sampling points, except points I and L, where they reached higher percentage. Finally, regarding the other *phyla* observed (always among those present in at least 0.5% of at least one sampling point), the data are shown in Supplementary Figure 3.

The analysis of the bacterial species identified in the 15 samples revealed that 87 of them showed a presence higher than 1% in at least one single sample (the list of these species is reported in Supplementary Figure 4). Considering the total percentage of these species at sampling points, it is possible to note an increasing trend from the source (60%) to the end of the path (92%; Supplementary Figure 5). An opposite trend could be observed by considering the number of species that possessed a presence higher than 1% at the individual sampling points, which included 15/20 near the source to 8/10 near the last sampling points. In both cases, the variations were not progressive but occurred abruptly between the withdrawal points H and I. The observed result confirms what has already been seen observed previously regarding the sudden change in the quality of the Lambro when crossing it crosses the most populated area (municipality of Milan).

Among all the species observed, only one, *Acinetobacter johnsonii*, was present in quantities higher than 1% in all 15 samples. This bacterium made up 4.5% of the bacterial community in sample A and 60.7% in sample R; however, in this case, the growth was not gradual and constant but there was a sudden growth between withdrawal H (7.7%) and withdrawal I (54.8). The same trend, albeit with lower percentages, could be observed for an unidentified bacterium belonging to the genus *Acinetobacter*. Finally, in addition to the two bacteria previously mentioned, only four other bacteria had a presence higher than 10% in at least one of the sampling points: a bacterium of the genus *Enhydrobacter* (32.2% in sample B), a bacterium of the genus *Delftia* (24.1% in sample A), a bacterium belonging to the genus *Flavobacterium* (20.9% in sample R), and, finally, a bacterium of the genus *Arcobacter* (12.9% in sample H). *Acinetobacter johnsonii* is a very common bacterium in the environment, so it is not surprising that it was the most common
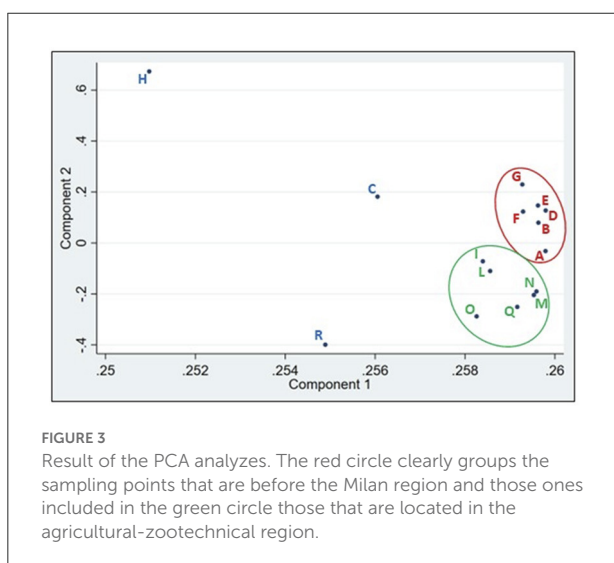
microorganism found (Guardabassi et al., 1999). Moreover, it rarely poses a health hazard. Bacteria of the genus *Deftia*, and in particular *Deftia acidovorans*, prefers an environment with

TABLE 1 Species of *Bacteroidetes* identified in the feces.

HS[a]	CF[b]	SS[c]	BT[d]	FC[e]	OTU
0.304[f]					HS1
0.068					HS2
0.062					HS3
0.048					HS4
0.048				0.049	HS-CF
0.045	0.338				HS-CF
0.045					HS7
0.044					HS8
0.030					HS9
0.030					HS10
0.027	0.035			0.117	HS-CF
0.026				0.168	HS-CF
	0.288				CF2
	0.096				CF3
	0.076				CF4
	0.066				CF5
	0.033	0.037			CF-SS
	0.032				CF7
	0.026				CF8
		0.084			SS1
		0.082			SS2
		0.059			SS3
		0.038			SS4
		0.037			SS5
		0.037			SS6
		0.036			SS8
		0.032			SS9
		0.028			SS10
		0.025			SS11
			0.030		BT1
			0.025		BT2
				0.151	FC2
				0.137	FC3
				0.083	FC5
				0.065	FC6
				0.058	FC7
				0.050	FC8
				0.036	FC10

Only species with a relative abundance >2.5% are indicated.
[a]HS, Homo sapiens; [b]CF, Canis familiaris; [c]SS, Sus scrofa; [d]BT, Bos taurus; [e]FC, Felix catus.
[f]Relative abundance values are shown.
The absence of the number indicates either a presence of <2.5% or the absence of bacterium in the sample.

FIGURE 3
Result of the PCA analyzes. The red circle clearly groups the sampling points that are before the Milan region and those ones included in the green circle those that are located in the agricultural–zootechnical region.

TABLE 2 OTUs identified in stool samples (with a relative abundance >2.5%) that showed >99% identity are reported.

Feces specie-OTU[a]	Genus	% in feces[b]	Sample (%)[c]	% Identity	Alignement length	Mismatches	Gap opens
SS-4	Paludibacter	3.8	C (0.1)	100.00	422	0	0
SS-1	Paludibacter	8.4	C (80.1)	99.76	422	1	0
HS-3	Cloacibacterium	6.2	M (3.3) N (0.8) Q (0.4)	99.74	422	1	0
FC-10	Cloacibacterium	3.6	M (3.3) N (0.8) Q (0.4)	99.52	422	2	0
SS-4	Prevotella	3.8	H (0.1)	99.05	422	4	0
CF-4	Paludibacter	7.6	C (80.1)	99.05	422	4	0

[a]SS, Sus scrofa; HS, Homo sapiens; FC, Felix catus; CF, Canis familiaris.
[b]Indicates the relative abundance of the bacterium in the corresponding stool sample.
[c]Indicates the relative abundance of the bacterium in the corresponding water withdrawal.

low salt concentrations that are typical of high-altitude springs and known for their ability to aggregate gold and produce small nuggets (Johnston et al., 2013).

A further interesting fact emerged from the principal coordinates analysis (PCA; Figure 3). The results clearly revealed two clusters of withdrawal points. The first group was associated with the samples taken before the entrance of the Lambro into highly populated areas (red circle in Figure 3), while the second group included sample points belonging to the region with the highest agricultural density (green area in Figure 3). This result is very interesting as there is a close relationship between the geographic position of the sampling point and the composition of the microbial community. Observing the *phylums* identified in these two groups and the variations (Supplementary Figure 6) it can be observed that only three *phylum* undergo a percentage increase (*Proteobacteria*, *Bacteroidetes* and *Actinobacteria*), while all the others undergo a strong decrease. This observation is consistent with what was previously observed regarding the decrease in bacterial variability after sampling point H.

Analysis of Bacteroidetes

To obtain information about the Bacteroidetes present in feces, samples belonging to five different species were analyzed: human, cattle, pig, dog, and cat. The *phyla* identified in the five different types of feces are shown in Supplementary Figure 7A. In the samples, most of the microbial components were made up of *Firmicutes* and *Bacterioidetes*, which together never comprised less than 90% of the total microbial flora. With regard to the feces samples, the number of OTUs (standardized to 15,000 sequences) and the Shannon–Wiener index are indicated in Supplementary Figure 7B. The composition observed was consistent with what was previously published (Bermingham et al., 2018; Mehta et al., 2018; Lourenco et al., 2020; Pilla and Suchodolski, 2020; Wylensek et al., 2020). By analyzing the species of *Bacteroidetes* present at a percentage higher than 2.5% in the stool samples, it was possible to confirm the great

specificity of this *phylum* with the origin of the feces (Table 1). Of the 38 species considered, only five were present in the feces of at least two species, and four involved man, dog, and cat. In fact, the species present in pig and bovine feces were strictly specie-specific. The analysis of the similarities between these specie of Bacteroidetes and those identified in the water withdrawals indicated that there were only six similarities higher than 99% (Table 2). The results show that a porcine-specific *Bacteroidetes* (SS4, 3.8%) was identified at a percentage higher than 0.1% at sampling point C. Furthermore, at the same sampling point, a specie was found that had a high similarity with another specific specie of the pig (SS1, 8.4%). Finally, it was noted that at the sampling points M, N and Q, a bacterial specie very similar to *Bacteroidetes* specific to man (HS3, 6.2) and cat (FC10, 3.6) was identified. Sampling point C corresponds to a municipality where it is possible to hypothesize the presence of family-type pig farms.

Conclusions

The analyses carried out show a sudden change in the complexity of the bacterial population between the withdrawal points H (Brugherio) and I (Parco Lambro-Milano), which corresponds to the entrance of the Lambro into the area of competence of the municipality of Milan. This is an important observation, as a decrease in the complexity of a bacterial community has been associated with a deterioration in water quality. We can hypothesize that between these two sampling points the Lambro River undergoes chemical and/or physical contamination which favors the development of some bacterial species to the detriment of others. A further result emerged from the PCA analysis: in this case there is a correspondence between the microbial characteristics and the geographical location of the samples. Finally, the analyses have shown that the contamination of *Bactroidetes* in almost all cases cannot be attributed to the species considered, except at sampling point C, where contamination of pig origin could

be detected. In the future, it is recommended that further analyses be performed near sampling points C, H, and I to better understand the possible source of these changes in water quality or contamination.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.5061/dryad.95x69p8nv.

Author contributions

LD and PP made the withdrawals, managed the results of the analyzes, and wrote the paper. BC extracted the bacterial DNA from the samples, performed the PCRs, and prepared the samples for sequencing. The sequencing was carried out at an external service (BMR Genomics SRL, Padova, Italy). All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frwa.2022.1008838/full#supplementary-material

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