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Bacterial microbiota management in free-living amoebae (Heterolobosea lineage) isolated from water: The impact of amoebae identity, grazing conditions, and passage number

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ABSTRACT

Free-living amoebae (FLA) are ubiquitous protozoa mainly found in aquatic environments. They are well-known reservoirs and vectors for the transmission of amoeba-resistant bacteria (ARB), most of which are pathogenic to humans. Yet, the natural bacterial microbiota associated with FLA remains largely unknown. Herein, we characterized the natural bacterial microbiota of different FLA species isolated from recreational waters in Guadeloupe.

Monoxenic cultures of *Naegleria australiensis*, *Naegleria* sp. WTP3, *Paravahlkampfia ustiana* and *Vahlkampfia* sp. AK-2007 (Heterolobosea lineage) were cultivated under different grazing conditions, during successive passages. The whole bacterial microbiota of the waters and the amoebal cysts was characterized using 16S rRNA gene metabarcoding. The culturable subset of ARB was analyzed by mass spectrometry (MALDI-TOF MS), conventional 16S PCR, and disk diffusion method (to assess bacterial antibiotic resistance). Transmission electron microscopy was used to locate the ARB inside the amoebae.

According to alpha and beta-diversity analyses, FLA bacterial microbiota were significantly different from the ones of their habitat. While *Vogesella* and *Aquabacterium* genera were detected in water, the most common ARB belonged to *Pseudomonas*, *Bosea*, and *Escherichia/Shigella* genera. The different FLA species showed both temporary and permanent associations with differentially bacterial taxa, suggesting host specificity. These associations depend on the number of passages and grazing conditions. Additionally, *Naegleria*, *Vahlkampfia* and *Paravahlkampfia* cysts were shown to naturally harbor viable bacteria of the *Acinetobacter*, *Escherichia*, *Enterobacter*, *Pseudomonas* and *Microbacterium* genera, all being pathogenic to humans. To our knowledge, this is the first time *Paravahlkampfia* and *Vahlkampfia* have been demonstrated as hosts of pathogenic ARB in water.

Globally, the persistence of these ARB inside resistant cysts represents a potential health risk. To ensure the continued safety of recreational waters, it is crucial to (i) regularly control both the amoebae and their ARB and (ii) improve knowledge on amoebae-bacteria interactions to establish better water management protocols.

1. Background

Free-living amoebae (FLA) are ubiquitous unicellular eukaryotes that can be detected in soils, air, biofilms and natural or anthropogenic water sources. They can take two or three different forms depending on the species: the trophozoite, the cyst and the flagellate. The trophozoite form corresponds to the active stage of amoeba with a metabolic activity allowing multiplication (by binary fission), locomotion and nutrition. A cystic form (which is dormant and stress-resistant form) is observed in cases of food deprivation, overpopulation, pollution, lack of oxygen, pH change or increase in ionic force, and an unfavorable temperature; however, when the conditions become favorable, they can transform back to trophozoites (Anderson, 2018; Delafont et al., 2016; Miller et al., 2018; Rodríguez-Zaragoza, 1994; Stahl and Olson, 2020; Thomas et al., 2010). In many FLA species from the Heterolobosea lineage, a flagellated form can also be observed, and it represents the transient form

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between the trophozoite and the cyst (De Jonckheere, 2014; Pánek et al., 2016). Most FLA are non-pathogenic but some can be involved in human diseases: *Naegleria fowleri* (which causes primary amoebic meningoencephalitis, PAM), amoebae of the genus *Acanthamoeba* (which are involved in amoebic keratitis, granulomatous amoebic encephalitis (EGM) and skin lesions), *Balamuthia mandrillaris* (responsible for granulomatous amoebic encephalitis (EGM) and skin lesions) and *Sappinia pedata* (causative agent of amoebic encephalitis) (Qvarnstrom et al., 2009; Sarink et al., 2022).

FLA are important environmental predators feeding on populations of multiple micro-organisms (bacteria, fungi, and algae) (Rodríguez-Zaragoza, 1994). While predatory interactions are ecologically relevant to control bacterial population level, the relationship between amoeba and associated bacteria are complex, ranging from antagonism to mutualism (Shi et al., 2021). The impact of such symbiotic amoeba-bacteria relationships on human health has raised increased interest because some pathogenic bacteria have evolved survival strategies to avoid amoebal digestion and remain protected from hostile environmental conditions, especially when they reside inside the cysts that provide a physical barrier against disinfection methods (Cateau et al., 2014; Thomas and Ashbolt, 2011). These amoebae-resistant bacteria (ARB) also take advantage of their amoebal host as training ground for increased virulence and antimicrobial resistance (as reviewed by (Bornier et al., 2021; Greub and Raoult, 2004; Molmeret et al., 2005)).

The general ecology of many FLA remains largely unknown. To our knowledge, there is a limited number of reports describing the natural microbiome of FLA in water: the whole bacterial microbiota of FLA isolated from drinking water samples was determined by Delafont et al. (2013) while Moreno-Mesonero and co-workers (Moreno-Mesonero et al., 2020) characterized the bacterial microbiota of FLA isolated from wastewater. In fact, most of the research performed on amoebae-bacteria interactions has been conducted in laboratory conditions using amoebae cultivated in axenic conditions and experimentally co-cultured with specific bacteria. These studies are being performed, for instance, with *Acanthamoeba* and bacteria pathogenic to humans such as *Legionella pneumophila* or *Mycobacterium* sp. (Balczun and Scheid, 2017; Hall and Voelz, 1985; Henriquez et al., 2021). Other ARBs such as *Legionella, Listeria, Pseudomonas, Salmonella* have also been largely studied in FLA (Greub and Raoult, 2004; Horn and Wagner, 2004; Nisar et al., 2022). As FLA have been proposed to play an important role in hosting and disseminating various human pathogens (Thomas and Ashbolt, 2011), it is imperious to increase knowledge on which FLA can act as a natural host in water, what can control them, or which conditions promote amoebae-resistant bacteria.

Guadeloupe is an archipelago in the southern Caribbean Sea composed of five islands where, on average, the climate is warm, and humid all year round. The territory of Basse-Terre is a volcanic island with many recreational baths with warm waters (with temperatures ranging from 25 to 40 °C) around the Soufrière volcano. Guadeloupean people and tourists frequently come in contact with raw water at recreational springs. In 2008, a 9-year-old child with PAM (caused by *N. fowleri*) died a few days after bathing in the hot spring of Dolé (Nicolas et al., 2010). After this accident, follow-up measures and treatments of the baths were implemented by the local Regional Health Agency (ARS Guadeloupe) to better manage the risk of *Naegleria* sp. in these raw waters. Further analyses revealed that *Naegleria* sp. and other Heteroloboasean amoebae are abundant in recreational waters (Moussa et al., 2013) and in soil in Guadeloupe (Reynaud et al., 2020). In fact, the recreational baths are contaminated with *Naegleria* when the water runs over the soil (Moussa et al., 2015). The aim of our work was to characterize the natural bacterial microbiota of FLA (specifically from the Heterolobosea lineage) isolated from raw waters in Basse Terre (used for recreational purposes) to further evaluate and understand the relationships and interactions between FLA and the surrounding bacterial community. For this, we combined complementary molecular and culture-based approaches and explored the effects of amoebae identity and grazing conditions upon bacterial microbiota composition.

2. Materials and methods

2.1. Water sampling and filtration

Water samples were collected from three recreational baths (Bain des Amours, Grosse Corde, and Morphy), all of them being located on the island of Basse-Terre (Supplementary Table 1). From each site, multiple samples were taken according to previously described methods (Moussa et al., 2020) in 2020, 2021 and 2022. Briefly, from each sampling point, the water samples (2 × 1 L, one liter for the identification of the bacteria in the bath and the other to isolate Heteroloboasean amoebae) were collected by submerging 1 L sterile bottles underneath the surface of the water (30 cm). A median of temperature and pH measured on site for each sample taken is presented in Supplementary Table 1.

The water samples were filtered either using 0.2 μm (for characterization of the bacterial microbiota of water) or 1.2 μm pore size filters (for FLA culture). Filters for bacteria detection in water were placed in Eppendorfs containing 1 mL of T1 buffer (from NucleoSpin® Tissue DNA extraction kit, Macherey-Nagel, Germany), vortexed and kept at −20 °C until DNA extraction. Filters for FLA culture were processed as described below.

2.2. Amoebae culture and molecular identification

Each filter was cut into 10 parts, then placed, on a grid side, on a Petri dish containing non-nutrient agar (NNA, Oxoid Ltd.) coated with an *E. coli* suspension (*Escherichia coli* American Type Culture Collection (ATCC®) 25922), as previously described (Moussa et al., 2020; Moussa et al., 2013). The plate was then incubated under aerobic conditions at 37 °C (to promote the growth of thermophilic amoebae) and examined daily during one week under an inverted light microscope (Motic IB, Leica). To subculture isolates, a section showing amoebae growth was excised and placed onto a freshly prepared *E. coli*-NNA plate and incubated at 37 °C for 7 days. This subculturing procedure was repeated during several weeks until an amoeba monoxenic culture was obtained. FLA were then cryopreserved in inactivated fetal calf serum (FCS, Eurobio, France) and DMSO (90:10 V/V) at −80 °C until use. During this process, amoebae samples were collected from NNA-plates at different passage number using 180 μL of T1 buffer (from NucleoSpin® Tissue DNA extraction kit, Macherey-Nagel, Germany) and DNA extraction was performed according to the manufacturers' instructions (Macherey-Nagel). Amoebae identification was achieved by PCR using ITS primers, as described elsewhere (Moussa et al., 2013). ITS amplicons Sanger sequencing was performed at Eurofins Genomics (Germany) and the homology search was performed with BLASTn software from the National Center for Biotechnology Information homepage (http://www.ncbi.nlm.nih.gov/).

2.3. Amoebae grazing conditions

As mentioned above, FLA are normally fed on gram-negative bacilli *E. coli* ATCC 25922. Before analyzing the ARB of the isolated FLA, we first evaluated the ability of the newly isolated amoebae to survive and replicate using 4 different culture media to mimic environmental grazing conditions: (i) Non-nutritious Agar (NNA) + suspension of *E. coli* ATCC 25922 (control culture conditions, FLA feeding on bacteria), (ii) NNA + Water (Nuclease-free, Molecular Biology Reagent Water, Sigma, France; "Water", nutrient starvation condition), (iii) NNA + *Saccharomyces cerevisiae* in Water ("Yeast", SIGMA, France; FLA feeding on unicellular eukaryotes) and (iv) NNA + Water supplemented with FCS (10 % V/V, "FCS + Water", FLA feeding on lipid and protein-rich media). All media were readily sterile or were sterilized by autoclaving (121 °C, 15 min) and were supplemented with a solution of penicillin/streptomycin (1 % V/V, Eurobio, France). All amoebae cultures were maintained at

37 °C, and were left to encyst before each passage; one passage corresponds to a full cycle of inoculated cysts-replicative trophozoites-cysts).

2.4. Bacterial microbiota analysis

To characterize the whole and culturable ARB, we developed an alternative protocol for amoebae cysts washing (to remove external bacteria, Supplementary Fig. 1) and lysis (to recover intra-amoebal bacteria) based on (Dietersdorfer et al., 2016; Thomas et al., 2008). Briefly, mature cysts were harvested from NNA plate cultures using 10 mL of Water (Nuclease-free, Molecular Biology Reagent Water) and kept at room temperature for up to 10 min (depending on the FLA species) to gently detach the cysts from the plate. Amoebae were then removed from the agar using a cell scraper, and the amoeba solution was transferred to 15 mL tubes and centrifuged for 10 min at 1300*g* at room temperature (RT); the supernatant was discarded, and the pellet resuspended in 5 mL of Water. This washing process was repeated 4 times for *E. coli*-containing media and 3 times for the feeding condition based on Water supplemented with FCS, yeast-containing media and Water only. Following the last centrifugation, the pellet containing intact cysts was resuspended in 2 mL of Water and split in two tubes (1 mL each) and centrifuged for 10 min at 2000*g* at RT. Supernatant was discarded and pellet was resuspended either in T1 lysis buffer (for DNA extraction and metabarcoding analysis) or Water. This water suspension with FLA cysts was lysed using 10 strokes using a 10 mL syringe and a 26G needle. Few drops of this whole extract were used to inoculate a petri dish with BCP agar. Whenever necessary, alternative bacterial culture media such as CCA, TSA and Chromagar Acinetobacter Agar were used to isolate culturable ARB.

2.4.1. Whole bacterial microbiota (16S rRNA gene metabarcoding)

To analyze the whole bacterial microbiota (culturable and non-culturable bacterial subsets) of recreational baths and their indigenous isolated FLA, we used DNA metabarcoding. First, DNA extraction was performed on T1-membrane and T1-amoebae suspensions (obtained as described above) using the NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel) following the manufacturer's recommendations. DNA was stored at −20 °C until use. For the identification of the whole bacterial microbiota, DNA samples were amplified using 16 primers (16S-illu: 5'-CCTACGGGNGGCWGCAG-3′ and 16S-R-illu: 5'-GACTACHVGGGTATCTAATCC-3′) targeting an amplicon size of 464 bp covering the V3-V4 regions (Herlemann et al., 2011; Klindworth et al., 2013). The amplicons were sent to the BIOMICS platform of the Institut Pasteur in Paris, for Illumina sequencing. FASTQ files obtained from Illumina sequencing of the 16S amplicons were first analyzed using the platform Galaxy IPG (Couvin et al., 2022). The data were treated using DADA2 (Callahan et al., 2016) for raw data pre-processing and clustering, chimera removal, data filtering and affiliation attribution for each ASV using SILVA 16S database (Quast et al., 2013). Metabarcoding differential analyses (including alpha and beta diversity assessment) were performed using the phyloseq pipeline (McMurdie and Holmes, 2013).

2.4.2. Culturable bacterial microbiota (isolation, identification and antibiotic susceptibility testing)

Bacteria previously isolated from the amoebae at different passages were grown on selective or non-selective agar plates for 24 h at 37 °C. After visual inspection of the bacterial colonies, each colony was selected and put on the MALDI plate. The matrix HCCA (α-Cyano- 4-hydroxycinnamic acid) was added on the top of the sample and air-dried, and the analyses were carried out by the MALDI Biotyper ™ (Bruker). Bacteria identification was also performed by PCR using the universal primers 16S 27f (5'–AGAGTTTGATYMTGGCTCAG-3′) and 1492r (5'–GGTTACCTTGTTAYGACTT- 3′) targeting a fragment around 1500 bp, as previously described by (Sagar et al., 2014). 16S amplicons Sanger sequencing was performed at Eurofins Genomics (Germany) and the homology search was performed with BLASTn software from the National Center for Biotechnology Information homepage (http://www.ncbi.nlm.nih.gov/).

Antimicrobial resistance phenotyping was assessed in the standard disk diffusion method on Mueller-Hinton agar. The antimicrobial panel was chosen according to the tested bacteria, as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), Breakpoint table 2022 (https://www.eucast.org/). The antimicrobials tested were as follows: Ampicillin (10 μg), Amoxicillin-clavulanic acid (20–10 μg), Temocillin (30 μg), Ticarcillin (75 μg), Cefotaxime (5 μg), Cefoxitin (30 μg), Ceftazidime (10 μg), Cefepime (30 μg), Ertapenem (10 μg), Imipenem (10 μg), Meropenem (10 μg), Gentamicin (10 μg), Amikacin (30 μg), Tobramycin (10 μg), Nalidixic acid (30 μg), Levofloxacin (50 μg), Ciprofloxacin (50 μg), Tigecycline (15 μg), Trimethoprim/sulfamethoxazole (25 μg), Fosfomycin (200 μg), Aztreonam (30 μg). The results were interpreted referring to the breakpoint table proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (https://www.eucast.org/; 2022).

2.4.3. Transmission electron microscopy

The amoebae cultures were resuspended with 5 mL of phosphate buffer saline (PBS, pH 7.2) and gently recovered with a scraper after 5 min. The amoebae suspensions were then collected in 15 mL Falcon tubes, centrifuged at 1300*g* for 10 min at RT; after supernatant removal, this washing step was repeated once again. The final pellet was resuspended in 1 mL of cacodylate buffer (0.1 M, pH 7.2) adjusted to 400 milliosmoles and placed at 4 °C for 20–30 min in a horizontal position. Glutaraldehyde (25 % V/V, Sigma) was added to a final concentration of 2.5 % (V/V) and amoebae suspensions were kept at 4 °C for 4 h to fix the proteins. Amoeba cells were then centrifuged as mentioned above and the resulting pellet was resuspended in 10 μL of 1 % (W/V) Low Melting Point agar solution to get a sphere full of amoebae. This sample was then washed three times (5 min each) with the cacodylate buffer (0.1 M, pH 7.2) to remove aldehyde before fixation for 45 min at room temperature in 1 % osmium tetroxide-1 % potassium ferrocyanide in the same buffer. Samples were then rinsed three times in distilled water for 5 min before dehydration through a graded acetone series at room temperature. Once totally dehydrated, samples were then embedded in epoxy resin as described before (Yong et al., 1985). Thin sections (60 nm thick) were observed using a transmission electron microscope (JEOL 1230) at 80 kV.

3. Results

3.1. Identification of the amoebae isolates

According to the sequencing results, the cleaned sequence of each amoeba sample confidently resolved to taxonomically distinct species from the Heterolobosea clade: *Naegleria australiensis*, *Naegleria* sp. WTP3, *Paravahlkampfia ustiana* and *Vahlkampfia* sp. AK-2007 (Supplementary Table S2).

3.2. Comparison between recreational baths and amoebae bacterial microbiota

To determine whether the amoebae bacterial microbiota were different from the bacteria detected in their water substrate, we explored the sample complexity (richness, evenness, and diversity) of recovered ASVs by the Illumina sequencing. Rarefaction curves of 16S samples analyzed attained the saturation plateau, indicating that the sequencing depth was sufficient (Supplementary Fig. 2). We then analyzed the alpha- and beta-diversity between amoebae and recreational baths samples. Fig. 1A reveals that the alpha-diversity (Shannon index) of the four amoebae *N. australiensis*, *Naegleria* sp. WTP3, *P. ustiana, and Vahlkampfia* sp. AK-2007 (Fig. 1A(a)) was significantly different than the alpha-diversity of the recreational bath samples (Fig. 1A(b)) (ANOVA, p-

value = 3.4 × 10^{-4}). Beta-diversity was visualized using Principal coordinates analysis (PCoA) (Fig. 1B) and the results also revealed that the amoebae and water bacterial microbiota are significantly different (PERMANOVA, R2 = 0.31, p-value = 0.001).

3.3. The impact of amoebal host, grazing conditions and passage number on bacterial microbiota composition

3.3.1. Impact of grazing conditions of the amoebae growth

The testing conditions presented above showed that *Vahlkampfia* sp. AK-2007 and *P. ustiana* can replicate in all tested culture media, while *Naegleria* sp. WTP3 was able to grow in all tested conditions except in Yeast suspension. *N. australiensis* growth was only observed with an *E. coli* suspension and in Water supplemented with FCS (Supplementary Fig. 3).

3.3.2. Whole FLA bacterial microbiota characterization

Fig. 2 shows the variation in the bacterial microbiota composition for each amoeba and for the recreational baths from where they were isolated. The results presented in Fig. 2A reveal that when *N. australiensis* feeds on *E. coli*, the highly abundant taxa *Pseudomonas* detected at passages #1 and #2 is replaced by the *Bosea* genus at passage #3. When this amoeba is cultivated in the FCS-supplemented Water, *Pseudomonas* is the most abundant genus detected, throughout the successive passages. Interestingly, while many bacterial genera such as *Acinetobacter*, *Aquacterium*, *Ideonella*, *Novosphingobium*, *Rheinheimera*, *Sphaerotilus*, *Vogesella* and *Zoogloea* were detected in Grosse Corde recreational bath, *Pseudomonas* genus was only detected in the amoeba (Fig. 2A, Recreational Bath).

For *Naegleria* sp. WTP3 (Fig. 2B), the diversity and relative abundance of bacteria genera varied greatly depending on the culture medium and the number of amoeba passages. When the amoeba feeds on *E. coli*, at passage #1, the amoeba bacterial microbiota was mainly composed of *Acinetobacter* (86.6 %); then at passage #2, the bacterial genera *Bosea* were detected and the relative abundance of *Escherichia/Shigella* bacteria increases. After 4 successive passages, *Bosea* was the main bacterial genus detected. After 5 passages, *Pseudomonas* became the most abundant bacterial genera. *Microbacterium* is detected in all passages except at passage #5. When the amoeba is cultivated in FCS-supplemented Water, the genus *Escherichia/Shigella* is replaced by the genus *Acinetobacter* at passage #3, while at passage #4, the genus *Bosea* is the most abundant. Under this culture condition, we also observed that the genus *Massilia* is detected at passages #4 and #5 and the genus *Kinneretia* is detected at passage #5. When this amoeba is grown under Water (nutrient starvation), a significant variation in predominant taxa from *Escherichia/Shigella* to *Bosea* is observed at passage #3; the genus *Kinneretia* is detected at passage #5. Our results also show that while the genera *Acinetobacter*, *Escherichia/Shigella* and *Pseudomonas* were both detected in the amoebal host and in Morphy recreational bath, the genera *Aquabacterium, Comamonas, Polynucleobacter* and *Vogesella* were mainly detected in the recreational bath. Interestingly, although the genera *Bosea, Massilia and Kinneretia* were clearly detected in *Naegleria* sp. WTP3 cysts (at some passages), they were detected at very low abundance in the recreational bath.

When *P. ustiana* feeds on *E. coli* and Yeast suspensions, the genus *Bosea* becomes highly abundant, at passages #3 and #2, respectively (Fig. 2C). When grown in FCS-supplemented Water, we observed that the abundant genus *Pseudomonas* initially detected in *P. ustiana* bacterial microbiota slightly decreases, with the appearance of the genera *Bosea*, *Kinneretia,* and *Paucibacter*. When compared to the Bain des Amours bath bacterial content (Fig. 2C, Recreational Bath), we observed that the genus *Pseudomonas* is highly abundant in both the amoeba *P. ustiana* and the Bain des Amours recreational bath. Fig. 2C also reveals that while *Escherichia/Shigella* genus is present in the bath (20 %), its relative abundance greatly fluctuates in the amoebal host. On the other hand, *Bosea* is relatively abundant in the amoebal host (at some passages) while present at low levels in the recreational bath.

The results presented in Fig. 2D reveal that *Vahlkampfia* sp. AK-2007 bacterial microbiota is composed of multiple genera of bacteria (*Bosea, Enterobacter, Kinneretia, Paucibacter, and Pseudomonas*) and, independently of the grazing conditions, the same genera were identified with different relative abundances. Interestingly, at passage #5, in all culture media tested, we observed a shift in bacteria abundance; for instance, when the amoeba is feeding on Yeast, at passage #5, the bacteria from the genus *Pseudomonas* became the most abundant (87.8 %). When globally comparing the diversity of bacteria in the amoeba *Vahlkampfia* sp. AK-2007 and its water bath of origin (Morphy), we observed a large difference between the 2 bacterial communities (amoeba vs recreational

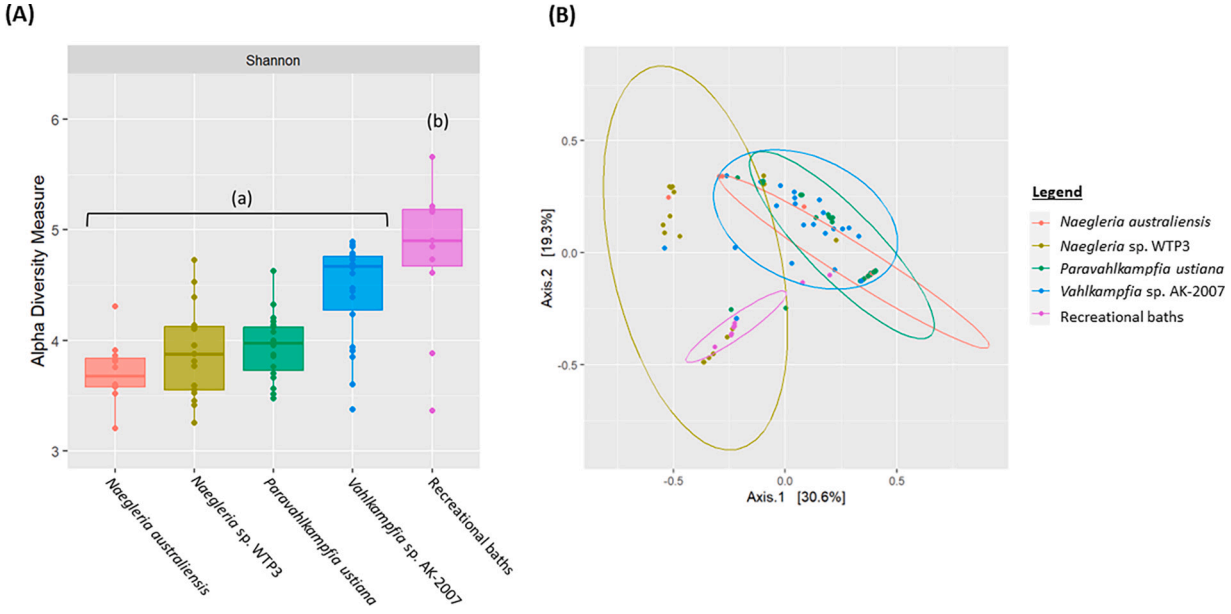

Fig. 1. Recreational baths versus amoebae bacterial microbiota diversity based on high-throughput sequencing of 16S rRNA gene (A) Alpha diversity (letters denote 2 groups, (a) for FLA and (b) for recreational baths identified through ANOVA with significant difference at p-value = 3.4 × 10^{-4}) and (B) Beta diversity PCoA (p-value =0.001).

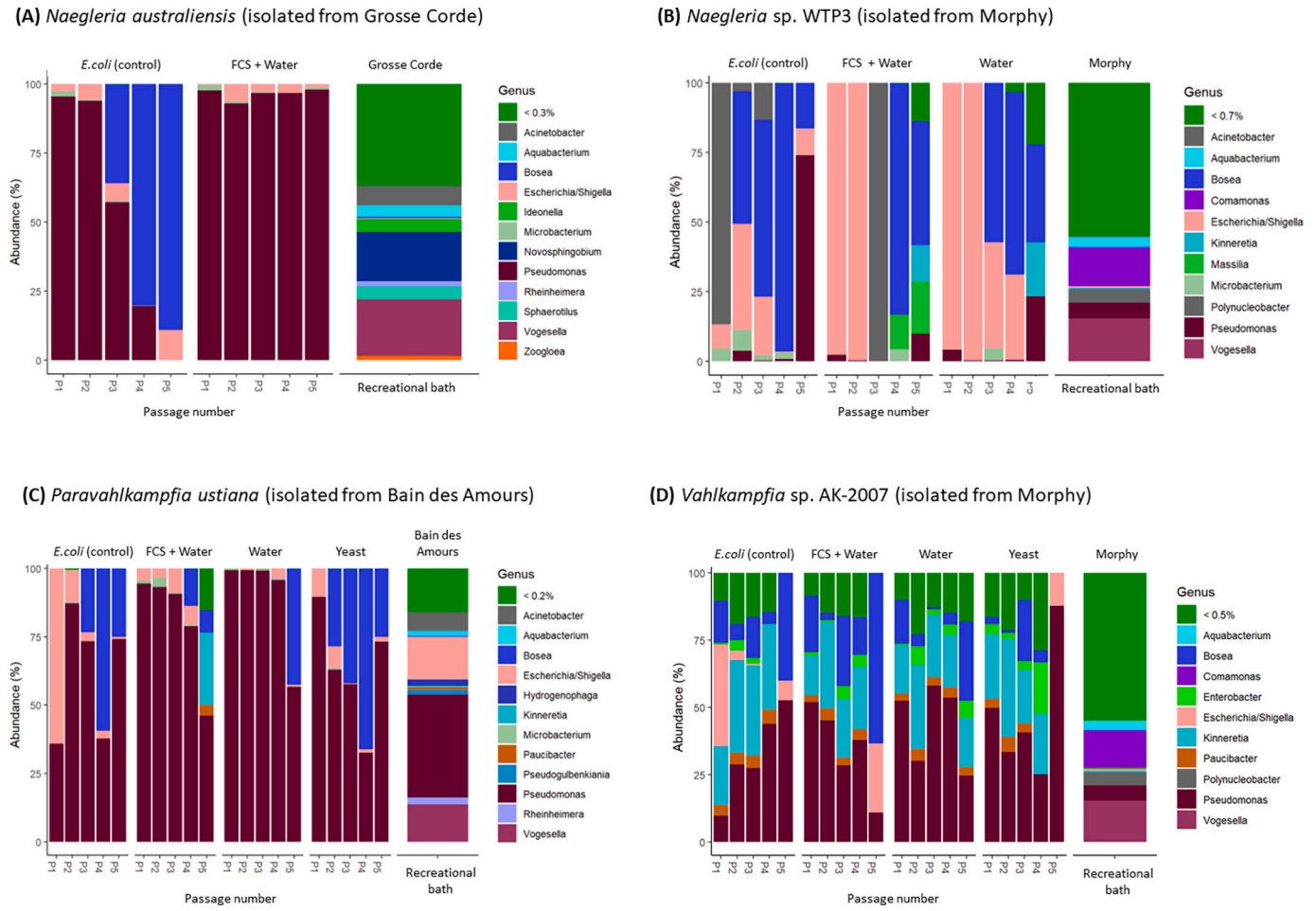

Fig. 2. Bacterial microbiota composition in different amoeba species (*Naegleria australiensis* (A), *Naegleria* sp. WTP3 (B), *Paravahlkampfia ustiana* (C) and *Vahlkampfia* sp. AK-2007*)* according to the passage number and grazing conditions and their respective recreational bath, based on high-throughput sequencing of 16S rRNA gene.

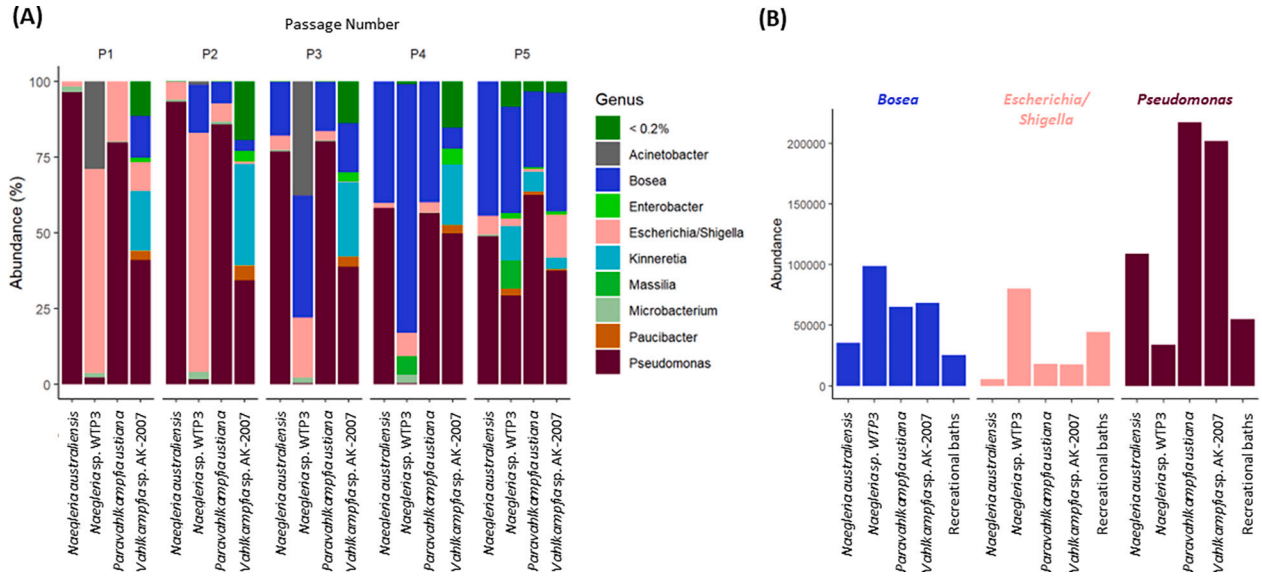

Fig. 3. Variation in bacterial microbiota composition for the different amoeba species at each passage independently of the culture medium used (A) and relative abundance of *Bosea*, *Escherichia/Shigella* and *Pseudomonas* genera in amoebae host and their respective recreational bath (B) based on high-throughput sequencing of 16S rRNA gene.

bath).

The results presented in Fig. 3A clearly show that, independently of the grazing conditions, the amoeba bacterial microbiota varies with the passage number. For certain amoeba species, a significant shift in bacterial microbiota composition is observed after 3 passages (namely for *Naegleria* sp. WTP3), while other bacteria taxa are maintained independently of the culture conditions (namely *Pseudomonas*). Fig. 3B highlights that some bacteria genera are more prone to be found in certain amoebae. Indeed, while *Pseudomonas* is highly abundant in *P. ustiana*, *Vahlkampfia* sp. AK-2007 and *N. australiensis*, the genera *Bosea* and *Escherichia/Shigella* genera are rather detected in *Naegleria* sp. WTP3.

3.3.3. Culturable ARB characterization and antimicrobial susceptibility pattern

The results obtained by MALDI-TOF MS (Supplementary Table 3) combined with sequencing of 16S rDNA PCR products (Supplementary Table 4) clearly showed that we could isolate and identify, from different amoeba cysts, during successive passages, the species *Acinetobacter baumannii, Enterobacter cloacae*, *Escherichia coli, Ideonella dechloratans, Microbacterium paraoxydans,* and *Pseudomonas otitidis.*

As above mentioned, ARB can use their amoebal host to increase their antimicrobial resistance; as such, we assessed the antimicrobial susceptibility pattern for the species *A. baumannii*, *E. cloacae, E. coli*, and *P. otitidis*, since these are known to be pathogenic to humans. According to EUCAST breakpoint table and our results presented in Supplementary Table 5, all strains tested had a wild-type phenotype and presented only natural resistances to the antibiotics tested for each species.

3.3.4. Bacteria localization inside amoeba cysts and trophozoites

Figs. 4 and 5 are representative examples of the different amoebal hosts studied herein and aimed to show the bacterial localization within large amoeba (such as *P. ustiana*) and small ones like *N. australiensis*. Transmission electron micrographs (TEM) of *P. ustiana* trophozoites show intact bacteria (which we infer to be Gram-negative) surrounded by multi-lamellar bodies and bacteria located inside food vacuoles (Fig. 4B and D). The bacterial intact appearance suggests that they are resistant to amoeba phagocytic digestion. We also observe empty multi-

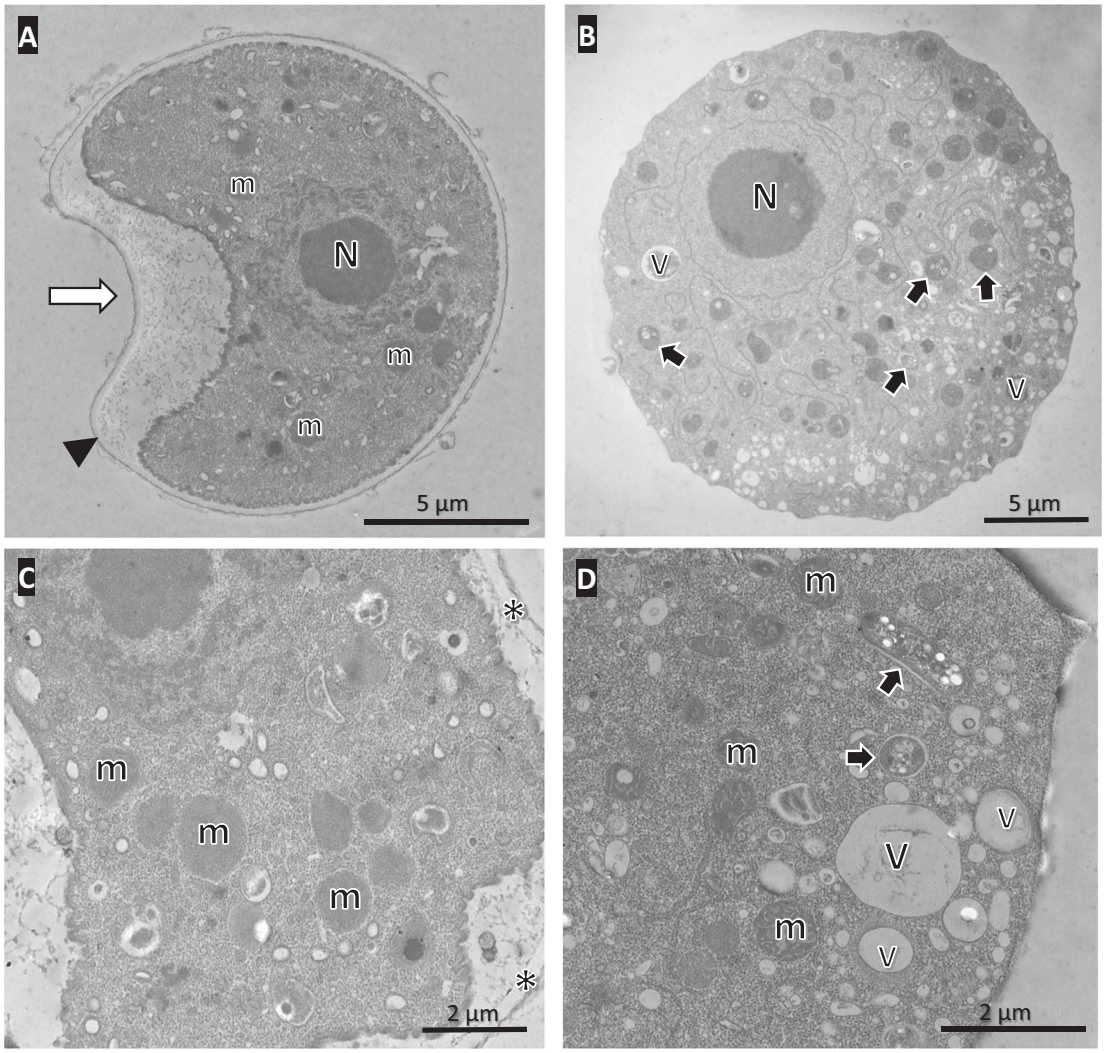

Fig. 4. Transversal sections of *Paravahlkampfia ustiana* cysts (A, C) and trophozoites (B, D) with TEM. The cysts (A) from this species are quite spherical and covered with an envelope (arrowhead) characterized by an obvious depletion (white arrow) as observed using light stereomicroscope directly from the culture plates. According to higher magnification (C), the cytoplasm of the cyst cells contains numerous mitochondria and small vacuoles characterized by a heterogeneous content, but no obvious intracellular bacteria. From both cysts and trophozoites, the nucleus (N) is quite large with a large central nucleolus (A, B). The cytoplasm of the trophozoite cells (B, D) contains numerous food vacuoles appearing quite empty (V) and envacuolated Gram negative bacteria (black arrows) representing putative intracellular bacterial symbionts. The bacteria outside the eukaryotic cells are *E. coli* used for food during cultivation (see M&M). (m: Mitochondria, N: Nucleus, asterisk: envelope of the cyst).

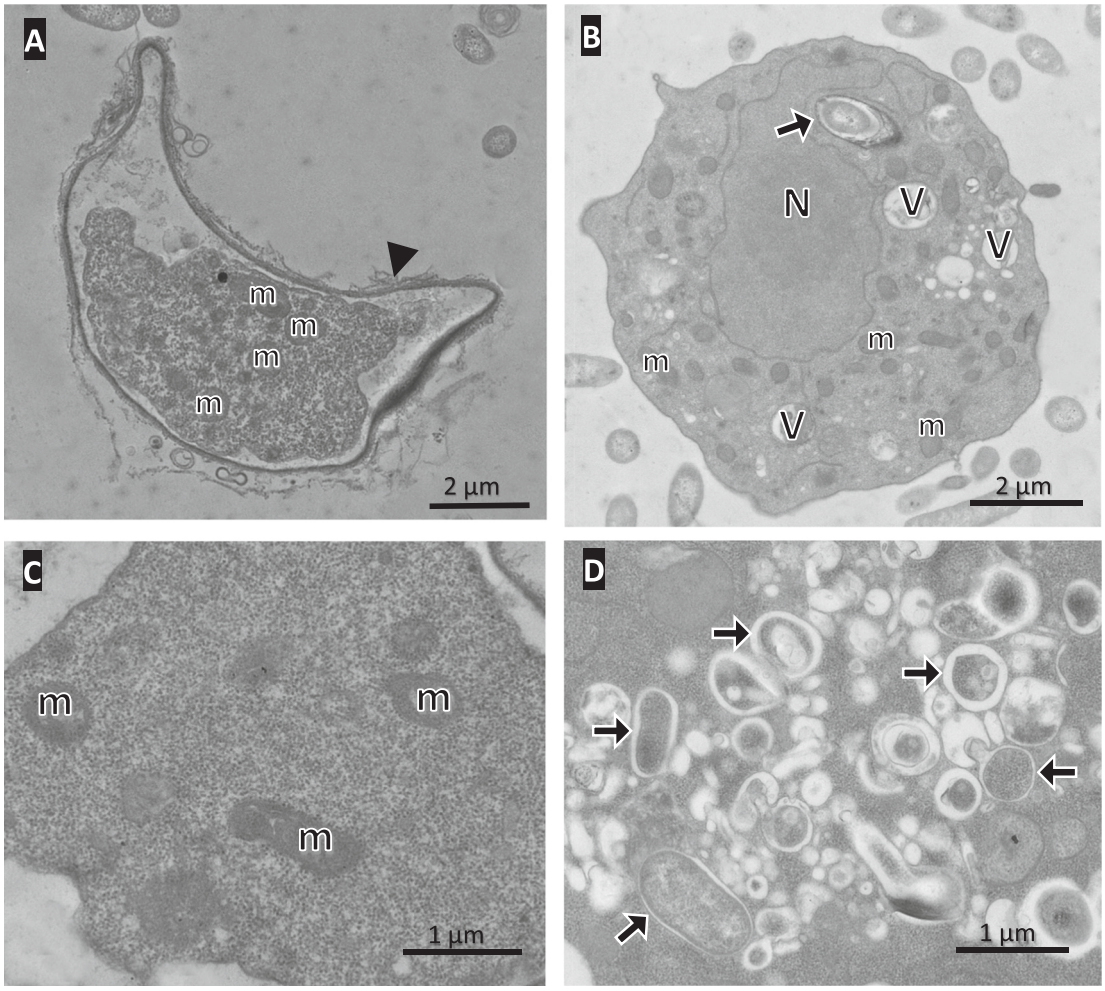

Fig. 5. Ultrathin sections of *Naegleria australiensis* cysts (A, C) and trophozoites (B, D) analyzed by TEM. The cysts (A) of *N. australiensis* possess a cell covered with an envelope (arrowhead) and no organelles within the cytoplasm excepted poorly preserved mitochondria (m) and ribosomes (A, C). The cytoplasm of the trophozoites (B, D) contains numerous food vacuoles (V) and numerous envacuolated Gram negative bacteria (black arrows) representing putative intracellular bacterial symbionts. The bacteria outside the eukaryotic cells are *E. coli* used for food during cultivation of trophozoites (see M&M). (N: Nucleus).

lamellar bodies inside food vacuoles of amoebae (Fig. 4D). In the host cysts, only very few endosymbionts can be detected within the cytoplasm (Fig. 4A and C), probably due to the lower number and/or as the thickness of the *P. ustiana* envelope which probably prevents the penetration of the fixative solution leading to the poorest preservation of these bacterial cells. For *N. australiensis,* no intracellular bacteria were observed in *N. australiensis* cysts (Fig. 5A and C) while the cytoplasm of the trophozoites contain numerous intracellular bacterial symbionts not envacuolated within the food vacuoles (Figs. 5B and D). The data obtained from the thin sections of trophozoites from these two distinct amoebae suggest the existence of a large and diverse symbiotic bacterial population, which confirms the molecular data.

4. Discussion

Amoebae have been grazing on bacteria for more than a billion years, forcing bacteria to evolve several survival strategies, namely, to escape phagocytosis (Sallinger et al., 2020; Shi et al., 2021). This amoeba-bacteria relationship provides protection to the bacteria from external interventions (namely the use chemicals for water treatment) and a dispersal mechanism across various habitats, posing a serious threat to water safety and human health (Balczun and Scheid, 2017; Samba-Louaka et al., 2019; Thomas and Ashbolt, 2011; Thomas et al., 2010). Yet, the natural bacterial microbiota of free-living amoebae isolated from water remains largely unknown. Herein, we investigated the range of non-culturable and culturable bacterial associations with wild FLA (from the Heterolobosea taxa) isolated from raw recreational waters in Guadeloupe (Lesser Antilles).

4.1. Recreational water versus amoebae bacterial microbiota

While it could be suggested that abundant bacteria detected in water could be found associated to amoebae, our metabarcoding analyses showed that the wild amoebae bacterial microbiota are distinct from the natural bacterial microbiota of the surrounding water. Indeed, while *Vogesella* and *Aquabacterium* genera were always detected in the recreational waters, the most abundant bacteria genera detected in amoebae include *Bosea*, *Escherichia/Shigella* and *Pseudomonas.* Differences in natural microbiome content between hosts and their respective environment have been previously reported for sponges (Thomas et al., 2016) and plant roots (Bonito et al., 2014) in soil, and tardigrades in water (Vecchi et al., 2018) while few information being available for amoebae (Delafont et al., 2013; Haselkorn et al., 2021; Moreno-Mesonero et al., 2020; Sallinger et al., 2020). It has been suggested that these differences could be due to (i) the small size of the amoebae, (ii) the incapacity of certain bacteria to adapt to the host environment of the amoeba (Horn, 1971; Sallinger et al., 2020; Shi et al., 2021) and/or, (iii) the fact that the amoebae used in this work retained these different ARB

while residing in soil before being isolated from water (Moussa et al., 2015; Sallinger et al., 2020). The factors possibly affecting the amoebae bacterial microbiota composition will be discussed in more details below.

While the metabarcoding strategy allowed us to identify a wide range of ARB, we could not detect strict intracellular bacterial endosymbionts such as *Chlamydiae*, *Dependentiae*, *Rickettsiales*, *Mycobacteria* and *Legionellae* in the four free-living amoebae species studied herein. Although the reasons for this are not yet clear, this could be due to the fact that the amoebae investigated here were collected from natural environments instead of highly anthropized areas. The above mentioned bacteria are known to hamper *Acanthamoeba* and *Vermamoeba* encystment (Delafont et al., 2018; Samba-Louaka, 2023). All the amoebae used in this work were able to encyst during several passages, and this may also contribute to support why no strict intracellular bacteria were recovered in the analyzed samples. From this, we used a culturable experimental strategy that enabled us to select and identify facultative intracellular or saprophytic bacteria species namely *A. baumanii, E. coli, E. cloacae, M. paraoxydans, P. otitidis* (all being pathogenic to humans (Caixinha et al., 2021; Chorost et al., 2018; Kyriakidis et al., 2021; Pot et al., 2021) and *I. dechloratans* (Malmqvist et al., 1994). While *Acinetobacter*, *Enterobacter*, *Ideonella*, and *Microbacterium* were considered as low abundant bacteria genera in our metabarcoding analyses, they were highly abundant in culture. This observation denotes the bias that can be seen between the culturable bacteria and the actual relative abundance of bacterial genera by metagenomic analysis. To our knowledge, this is the first time that *M. paraoxydans and I. dechloratans* were isolated from wild free-living amoebae. It is important to mention that we may have missed some culturable bacteria during our analyses (i) because *Pseudomonas* was the dominant genus in all the tested culture conditions and (ii) isolation of wild amoebae requires several laboratory subculturing passages, and some bacterial genera initially present in the FLA, may have been released from the amoebae in the meantime.

Bacteria can use their amoebal host to modify their resistance to antibiotics (Barker et al., 1995; Miltner and Bermudez, 2000; Nguyen et al., 2020) herewith influencing the efficacy of the prophylactic use of antimicrobials. When we analyzed the antibiotic resistance profile of *A. baumanii, E. coli, E. cloacae* and *P. otitidis,* isolated from amoebae cysts, the results showed that these ARB presented natural resistances profiles, suggesting that amoeba-bacteria interactions do not always result in increased bacterial antibiotic resistance. Antibiotic resistance is a natural phenomenon (that can arise from both spontaneous mutation and the acquisition of resistance traits) but biological interactions such as predation and competition are potential drivers of antibiotic resistance in natural environments with limited anthropogenic impact (Kamaruzzaman et al., 2017; Nguyen et al., 2020). Our results suggest that the ARB analyzed in this study, while residing within their respective FLA, did not present acquired resistance profiles possibly because they were protected by their eukaryotic host from numerous abiotic stressors and high levels of competition present in the water habitat. This phenomenon is often observed in obligate intracellular bacteria, as they are sheltered by their host (McOrist, 2000).

4.2. Factors affecting amoebae bacterial microbiota composition

Bacteria can evolve to resist amoeba phagocytosis and associate either transiently or stably within their amoebal hosts (Molmeret et al., 2005; Sallinger et al., 2020). However, resistance to phagocytosis is not the primary factor affecting amoeba bacterial microbiota composition (Sallinger et al., 2020). Our ability to detect different ARB in distinct FLA cultivated under various grazing conditions during several in vitro passages clearly suggest that environmental variability and host specificity are key factors for bacterial microbiota composition and its variation through time.

4.2.1. Food source available

FLA proliferation in water depends mainly on ecological factors (including nutrients levels and food sources) and on environmental parameters (such as water temperature and the presence of disinfectant residuals). However, not all food sources (bacteria, fungi and algae) seem to be equally suitable for amoebae (Delafont et al., 2016; Thomas et al., 2010). For instance, distinct *D. discoideum* species have different and consistent bacterial food preferences and these social amoebae can modulate their microbiome via food preferences (Horn, 1971). Our findings are in agreement with this observation, as our results indicate that amoeba growth and amoeba-bacteria interactions are affected by different food sources (bacteria, eukaryotes, lipid and protein rich, and no external food sources). While *Vahlkampfia* sp. AK-2007 and *P. ustiana* were able to replicate under all tested grazing conditions, *N. autraliensis* had a very restrictive diet. Moreover, depending on the availability and type of food and the passage number, we observed a variation in ARB content for each FLA species. The reasons for this are not yet clear but this suggests that amoebae isolated from water can establish transient and permanent associations with differentially abundant bacterial taxa; this was observed in particular for both *Naegleria* species and *P. ustiana*. The genera *Acinetobacter, Massillia*, and *Microbacterium* seemed to be "transient bacteria", as their presence fluctuates in FLA bacterial microbiota. Other bacterial genera (namely *Bosea, Escherichia/Shigella* and *Pseudomonas)* appear to be preserved within the amoebae independently of the food source and amoeba passage number, suggesting that these bacteria can form a permanent amoebae-resistant bacterial microbiota. Such bacteria could be located within the cytoplasm and outside of the vacuole foods. These bacteria genera have lifestyles ranging from free-living to symbiotic and can be pathogenic to human (Delafont et al., 2013; Garau and Gomez, 2003; La Scola et al., 2003; Skipper et al., 2020). Other ARB of free-living amoebae such as *Aeromonas*, *Arcobacter*, *Campylobacter, Helicobacter*, *Klebsiella*, *Legionella*, *Mycobacterium* and *Salmonella* have been already described in literature (Greub and Raoult, 2004; Haselkorn et al., 2021; Horn and Wagner, 2004; Thomas et al., 2010; Thomas et al., 2008). More specifically, it has long been known that *D. discoideum, Acanthamoeba* sp. and *Vermamoeba* sp. have bacterial endosymbionts that persisted through multiple life-cycles (Brock et al., 2020; Delafont et al., 2015; Horn and Wagner, 2004; König et al., 2019). Our results show that persistent bacterial endosymbionts can be observed in other less studied FLA species belonging to the genera *Paravahlkampfia* and *Vahlkampfia*, which are phylogenetically distinct from *Acanthamoeba*, *Dictyostelium* and *Vermamoeba* (all from the Amoebozoa taxa). However, some selectivity in amoebal bacterial microbiota composition can be observed even if the bacteria are not intracellular and instead reside within the extracellular matrix or on the surface of the free-living host (Sallinger et al., 2020).

4.2.2. Host-related factors

The comparatively low diversity of bacteria that associate with amoebae compared to the water bacterial microbiota indicates some different degrees of specialization to amoeba hosts. This becomes more obvious when comparing *Naegleria* sp. WTP3 and *Vahlkampfia* sp. AK-2007 that were isolated from the same recreational bath (Morphy) but showed different ARB and behavior in culture. Indeed, *Naegleria* sp. WTP3 has a more diverse bacterial microbiota than *Vahlkampfia* sp. AK-2007 and varies the most in different media and passages. We also noticed that *N. australiensis*, *P. ustiana* et *Vahlkampfia* sp. AK-2007 cysts had mainly *Pseudomonas* sp. in their bacterial microbiota, while *Naegleria* sp. WTP3 contained mainly *Bosea* and *Escherichia/Shigella*; the low frequency of *Pseudomonas* in *Naegleria* sp. WTP3 suggests that this amoeba is not a preferred host. Different levels of host specificity in the microbiome have been also seen in other organisms, such as sponges and *Dictyostelium* (Nasser et al., 2013; Thomas et al., 2016).

As above mentioned, we found that several bacterial genera were able to persist through successive passages, suggesting wherever these bacteria are located within the amoebal cyst they can be transmitted to

the next generation. While bacterial endosymbionts were difficult to observe in the cysts (probably due to the low permeability of the cyst wall to the fixing agent), we detected bacteria either inside and outside non-digestive vacuoles in the cytoplasm of all *P. ustiana* trophozoites. For *N. australiensis*, the bacteria were mainly located within vacuoles and contrary to *P. ustiana*, not all *N. australiensis* trophozoites were infected with bacterial endosymbionts (data not shown). The reasons for this are not yet clear, but we can hypothesize that, as observed for the social amoeba *Dictyostelium*, the asexual binary fission process would result in lineages that can be quite different with and without bacterial endosymbionts (Bloomfield et al., 2010). If an amoeba lineage that has acquired bacterial endosymbionts can evolve to establish long-term relationships, transmission of the symbionts can occur vertically upon host cell division. Whenever symbiont transmission is not linked to host reproduction, but instead to host cell lysis with symbionts to infect new host lineages, transmission occurs horizontally (Herrera et al., 2020; Molmeret et al., 2005; Shu et al., 2018).

Host genetic factors could also play a role in microbiome composition. *D. discoideum* has different mechanisms for dealing with different prey species (Nasser et al., 2013). *D. discoideum* is capable of ingesting, killing and digesting at least one bacterium per minute as long as the preys are available (Cosson and Soldati, 2008). However, upon depletion of available food, the amoeba can transform into a multicellular organism with cells capable of immune-like functions that can remove bacterial pathogens (Brock et al., 2018). Although the genomes of *Paravahlkampfia* and *Vahlkampfia* are not yet available, our group has recently revealed that *Naegleria* sp. possess genes coding for an immune system and to deal with toxic preys (Dereeper et al., 2023). Using transcriptomic studies to assess the effects of restrictive diet on *Naegleria* bacterial microbiota variation could help elucidate the role of amoeba–bacteria interactions.

4.2.3. Bacteria-associated factors

Bacteria developed diverse mechanisms ranging from modification of the cell surface to the production and delivery of bacterial effectors, to reside inside their host while escaping its immune system (Ribet and Cossart, 2010; Samba-Louaka, 2021; Simkovsky et al., 2012).

For instance, *Legionella pneumophila* is able to escape lysosomal digestion from its amoebal host *Acanthamoeba castellani* by shedding LPS outer membrane vesicles (Seeger et al., 2010). It has been shown that modifications in the production of O-antigen epitopes of *Salmonella enterica* (Wildschutte et al., 2004) and *Synechococcus elongatus* (Simkovsky et al., 2012) can result in different feeding strategies by their amoebal host. Herein, we noticed that while the *E. coli* ATCC 25922 is a preferential food source for the studied amoebae, other *E. coli* strains (such as the one isolated from the cyst) may resist to amoebal phagocytosis, possibly by modifying a surface glycoprotein. It would rather be interesting to compare the surface glycoproteomes of these two bacterial strains, namely using enrichment protocols (Marcelino et al., 2019).

Secretion systems are commonly used by bacteria to secrete effectors into a host cell after engulfment and target components of the host innate immune system such as Toll-like receptors and Nod-like receptors (Green and Mecsas, 2016). Proteobacteria are particularly rich in secretion systems (T1SS, T2SS, T3SS, T4SS, T5aSS, T5bSS, T5cSS, and T6SS) having more different types of secretion systems than all other phyla (Abby et al., 2016). Most bacteria that were transiently identified or persisted through multiple replication cycles in the 4 studied FLA were in the Alpha (such as *Bosea*), Beta (namely *Ideonella, Kinneretia, Massilia*, *Paucibacter*), and Gamma (*Acinetobacter*, *Escherichia/Shigella*, *Pseudomonas*). A similar abundance of Proteobacteria classes was found amongst digestion-resistant bacteria in surveys of marine and freshwater ciliates, another group of ubiquitous aquatic bacterivorous protists (Gong et al., 2016). These authors suggested that bacteria with type IV and VI secretion systems (T4SS and T6SS) could have a possible role in promoting protist-bacteria associations (Gong et al., 2016). Other examples that secretion systems can play an important role in amoebae-bacteria interactions include *A. castellani* and *P. aeruginosa* type III secretion system (T3SS) (Matz et al., 2008) and *D. discoideum* and *Vibrio cholera* type VI secretion system (T6SS) (Pukatzki et al., 2006).

Besides bacteria–host interactions, competition and facilitation between bacteria also likely play a role in amoeba microbiome composition. For instance, the bacteria *Paraburkholderia agricolaris*, *P. bonniea*, and *P. hayleyella* (which are internal symbionts of wild-collected *D. discoideum*) facilitate the carriage of other bacteria by the social amoeba (Brock et al., 2020). The presence of the bacterium *Protochlamydia amoebophila* protects its amoebal host *A. castellanii* from pathogenic infection with L. *pneumophila*, likely by outcompeting *L. pneumophila* for limited intracellular resources (König et al., 2019). It is fair to assume that more cases of facilitation and competition between bacteria could exist in other microbiotes of amoebae. Still, these bacteria–bacteria interactions may depend on where the bacteria are located within the amoeba host, as some inhabit host-derived vacuoles, while others persist in the cytoplasm (Haselkorn et al., 2021). Two intra-amoeba bacteria may not interact if one of them is contained within a vacuole. Herein, we could observe bacteria living freely in the cytoplasm of *P. ustiana* trophozoites, whilst in the other amoebae (namely *N. australiensis*), the bacteria were mainly located within vacuoles. Bacteria-virus interactions can also impact amoebae microbiome diversity. Indeed, Arthofer and co-authors (2022) recently showed that another chlamydia-like bacteria such as *Parachlamydia acanthamoebae* can protect their amoebal hosts (*Acanthamoebae* sp.) against different giant virus infection (Arthofer et al., 2022). Future studies are necessary to further elucidate the localization of the bacteria in amoeba and how location affects interactions within the bacterial community or with intra-amoebal viruses.

5. Conclusion

- Cysts of wild amoebae isolated from recreational baths in Guadeloupe carry diverse bacterial genera (including some being pathogenic to humans), protecting their bacterial microbiota from water disinfection treatment.
- Different amoebae genera such as *Naegleria*, *Paravahlkampfia* and *Vahlkampfia* have ecological relationships with different bacteria. Permanent or transient relations can be observed depending on the food source available and the number of passages.
- As amoebae can harbor different bacterial genera that those detected in water, water quality monitoring tools should be updated to include FLA and amoebae-associated bacteria. The current approach for ensuring public health safety by monitoring bacteria using culture-based methods is unable to determine the true concentration of these infectious bacteria, as it is not considering FLA as a critical condition promoting bacteria's growth.
- Exploring the amoebae-bacteria interaction may also help us to understand the evolution of symbiosis and the microbiome formation in basal eukaryotic organisms. For this, complementary analysis of the transcriptome and metabolome of FLA exposed to these various bacteria may help us to understand the interaction between amoeba and bacteria, particularly the defense against ingestion of the ARBs.

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CRediT authorship contribution statement

Sample preparation: AD, FH, AS, OG and IM. Data acquisition and analysis: AD, IQ, FH, AS, OG, IM. Conceptualization: AD and IM. Methodology: AD, IQ, AS, OG, IM. Formal analysis: AD, IQ, AS, OG, IM. Investigation: AD, IQ, FH, AS, OG, AT and IM. Data curation: IQ and IM. Writing/original draft preparation: IM. Writing—review and editing: all authors. Supervision: IM. Project administration: IM, AT. Funding acquisition: AT. All authors have read and agreed to the published version of the manuscript. All authors reviewed the manuscript. The authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availabilityThe 16S rRNA gene metabarcoding data presented in the study were deposited in NCBI under the BioProject accession number PRJNA996907. Free-living amoebae ITS sequences have been deposited on GenBank and are available under accession numbers: OR335806 (*Paravahlkampfia ustiana*), OR335807 (*Vahlkampfia* sp. AK-2007), OR335808 (*Naegleria australiensis*) and OR335809 (*Naegleria* sp. WTP3) (Supplementary Table 2). Bacteria 16S sequences have been deposited on GenBank and are available under accession numbers: OR326959 (*Acinetobacter baumanii*), OR326960 (*Escherichia coli*), OR326961 (*Pseudomonas otitidis*), OR326962 (*Enterobacter cloacae*), OR326963 (*Microbacterium paraoxydans*) and OR326964 (*Ideonella dechloratans*) (Supplementary Table 4).

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