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OXFORD FEMS

FEMS Microbiology Ecology, 2025, **101**, fiae164

DOI: 10.1093/femsec/fiae164

Advance access publication date: 17 December 2024

Research Article

Extensive environmental survey of free-living amoebae and their elusive association with *Mycobacterium bovis* or *Mycobacterium avium* subsp. *paratuberculosis*

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Editor: [Martin W. Hahn]

Abstract

Free-living amoebae (FLA) are described as environmental reservoirs for some bacteria able to resist their phagocytosis. In the environment, the fate of *Mycobacterium bovis* (*Mbo*) and *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) responsible for bovine tuberculosis and paratuberculosis, respectively, remains poorly understood and is considered potentially problematic in the eradication and control of these diseases. We hypothesize that FLA may play a role in the persistence of *Mbo* and *Map* in the environment. In this study, 90 samples were collected from herds affected by one or both diseases to investigate the diversity of amoeba and their associated bacteria. Metabarcoding analyses revealed that *Acanthamoeba*, *Copromyxa*, *Naegleria*, and *Vermamoeba* were the most represented genera of FLA, with Pseudomonadota being the bacteria most commonly found associated with FLA. Although no *Mbo* and *Map* DNA were identified by sequencing, traces were detected by ddPCR (digital droplet PCR), specifically targeting these bacteria. In conclusion, we described a wide diversity of FLA and associated bacteria in this environment. It also suggests that *Map* and *Mbo* could be associated, even weakly, with FLA in the environment. However, this needs to be confirmed by detecting a highest amount of DNA and, if possible, cultivable *Map* and/or *Mbo* associated with these environmental FLA.

Keywords: amoeba; *Mycobacterium bovis*; *Mycobacterium avium* subsp. *paratuberculosis*; tuberculosis; bovine; environment

Introduction

Free-living amoebae (FLA) are ubiquitous protozoa found in the environment. They primarily feed on bacteria through phagocytosis (Rodríguez-Zaragoza 1994, Samba-Louaka et al. 2019). However, several significant pathogenic bacteria, such as *Legionella pneumophila* and *Mycobacterium* spp., can resist phagocytosis and even multiply within FLA (Greub and Raoult 2004). Furthermore, the growth of *L. pneumophila* in FLA enhances its virulence toward human cells (Cirillo et al. 1994) and its resistance to antimicrobial treatments (Barker et al. 1992). Mechanisms of resistance to FLA are similar to those involved in macrophage resistance, explaining FLA's role in increasing virulence (Escoll et al. 2014). It is believed that FLA serve as the primary reservoir for *L. pneumophila* development in the environment (Molmeret et al. 2005). However, the role of FLA as reservoirs of *Mycobacterium* spp. in the environment remains to be clarified.

The two primary mycobacterioses of veterinary significance, particularly in livestock, are bovine tuberculosis (bTB), caused by *Mycobacterium bovis* (*Mbo*), and paratuberculosis (paraTB), caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) (Biet et al. 2005, Pérez-Lago et al. 2014). bTB ranks among the most common zoonoses worldwide, associated with significant health impacts and substantial socioeconomic costs (Marsot et al. 2016). *Mbo* circulates within a multihost system, involving cattle and wild fauna such as badgers (*Meles meles*), wild boars (*Sus scrofa*), and red deer (*Cervus elaphus*) as potential wildlife reservoirs (Rivière et al. 2014). Recently, Allen et al. reviewed the potential role of the environment in bTB control and eradication. They suggest that infection from environmental sources of *Mbo* could pose a disease transmission risk, but the environmental fate of *Mbo* remains poorly understood (Allen et al. 2021). ParaTB, also known as Johne's disease, is widespread in domestic and wild ruminant populations in nearly all countries worldwide, resulting in significant economic losses not only due to decreased productivity but also from income loss due to premature culling (Whittington et al. 2019). It has been demonstrated that *Map* shedding into the environment can infect local livestock or other animals and can persist for many months in agricultural slurry and the wider environment (Pickup et al. 2006). The challenge in addressing these diseases stems from the presence of these mycobacteria in a multihost system, where environmental contamination poses a proven epidemiological risk for both livestock and wildlife, as reviewed by

Received 4 October 2024; **revised** 6 November 2024; **accepted** 16 December 2024

our group and others (Biet and Boschiroli 2014, Pérez-Lago et al. 2014).

Regarding mycobacteria-FLA interactions, several *in vitro* studies have shown that depending on the species, mycobacteria may be digested, survive, or multiply within FLA (Drancourt 2014, Claeys and Robinson 2018). FLA may even increase the virulence of mycobacteria (Cirillo et al. 1997, Bakala N'Goma et al. 2015). It has even been hypothesized that protozoa have played a central role in the evolution of mycobacterial pathogenesis (Primm et al. 2004). Consequently, FLA could be considered a component of the reservoir and training ground for mycobacteria in the environment (Salah et al. 2009). Since *Mbo*/*Map* DNA have also been found in water and soil (Rhodes et al. 2013, Barbier et al. 2016), we hypothesize that, among other factors, FLA might serve as a reservoir and vector for *Mbo*/*Map* in the environment.

Several *in vitro* interaction studies between FLA and *Mbo*/*Map* have been published, demonstrating FLA's permissiveness to infection by these bacteria (Taylor et al. 2003, Mura et al. 2006, Sanchez-Hidalgo et al. 2017). A recent study used *A. castellanii* as a tool to study *Map* virulence factors and concluded that this FLA is a good model to simulate macrophage infection by *Map* (Phillips et al. 2020).

Besides, the interactions between environmental FLA and *Mbo*/*Map* in the environment have been significantly underexplored (Drancourt 2014). To our knowledge, only few studies have addressed these interactions in the environmental context. The initial study reported environmental FLA with *Map*-positive PCR detection (White et al. 2010). Subsequently, a study described acid-fast bacteria found within FLA cultivated from badger latrines (Mardare et al. 2013). A third study, conducted by our team, isolated FLA from water on infected farms, revealing a *Map*-positive qPCR signal. Additionally, this *Map* DNA underwent genotyping, demonstrating similarity to a local pathogenic strain associated with cattle, suggesting FLA's role as a reservoir of *Map* in the environment (Samba-Louaka et al. 2018). Recently, we described the co-occurrence of *Map* and FLA in the environment of bovine herds (Rochard et al. 2023). There is a need for further environmental studies to thoroughly assess the potential role of FLA in the persistence and virulence of *Mbo* and/or *Map* in the environment.

In this study, we conducted an extensive 1-year sampling campaign on farms known to be positive for bTB. We collected water, soil, and faeces samples from watercourses, ponds, drinking troughs, streams, badger's burrow, and badger's faeces within farm. FLA were cultured from all these samples, and their total DNA was extracted. This total DNA was utilized for detecting DNA from *Mbo* or *Map* using digital droplet PCR. Additionally, the total DNA underwent high-throughput sequencing of 18S and 16S rRNA amplicons to identify the FLA and their associated bacteria (i.e present in the same samples).

Materials and methods

Sample collection

Twenty cattle farms situated in Nouvelle-Aquitaine and Normandie participated in this study, with sampling conducted between July 2021 and October 2023, under the supervision of LDAR24 (Fig. 1). Samples were chosen based on their location, focusing on specific sites: (i) unsecured watering points, such as drinking troughs, ponds, and streams, were targeted as they are deemed high-risk locations for bovine transmission due to their accessibility to wildlife and (ii) soil samples from badger burrows and badger faeces were also sampled when present in the livestock areas. Further details regarding the sampling locations and methods are provided in a supplementary table (Supplementary Data 1). All samples were divided into two groups: one set was designated for DNA detection of *Mbo* and *Map* association with amoebae and the other set was allocated for the direct DNA detection of *Mbo* and *Map* in the samples.

Amoebae culture and isolation

Water samples were vigorously homogenized prior to allowing them to settle for 10 min. Soil and faeces samples (1–3 g) were resuspended in 30 ml of PAS buffer (Page's Amoebae Saline; containing 1 g/l sodium citrate, 4 mM $MgSO_4$, 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, 0.4 mM $CaCl_2$, pH 6.5), vigorously homogenized, and allowed to settle for 10 min. Prefiltration was performed using sieves with pore sizes of 500 and 200 µm. Subsequently, the samples were filtered through a 3-µm-pore-sized nitrocellulose membrane. The filter was then cut and placed on non-nutrient agar plates seeded with live *Escherichia coli* Δ*tolC* (Lagkouvardos et al. 2014). Two plates from each filter were incubated at 20°C and two plates at 37°C for up to 7 days to cultivate amoebae with different optimal growth temperatures until a characteristic migration front was observed. The amoeba migration fronts were recovered using a sterile swab and preserved in 5 mM Tris buffer pH 8.5 for total DNA extraction, mycobacterium culture, and storage in a −20°C freezer.

For isolation, a square of agar from the migration front was placed in liquid PAS supplemented with *E. coli* at OD_{600nm} 0.5 at 20 or 37°C to subculture amoebae. After observing amoeba growth, critical dilutions were performed until only one morphology was observed. The purity of the culture was confirmed by sequencing the 18S rRNA gene using Sanger sequencing. All isolates were then cryopreserved at −80°C in 7.5% dimethyl sulfoxide (DMSO). The sequences generated have been submitted at NCBI under SUB14758417.

Mycobacteria culture and isolation

To isolate mycobacteria, the environmental samples were decontaminated according to two methods in parallel, as previously described (Rochard et al. 2023). Briefly, the first decontamination used 0.9% hexadecylpyridinium chloride (HPC) (weight/volume) and the second decontamination used 3% sodium dodecyl sulphate (SDS). For both methods, Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) with 0.2% glycerol and supplemented with 10% albumin dextrose catalase (ADC, Becton Dikinson, Le Pont de Claix, France) to inoculate two Herrold's tubes with mycobactin J and ANV (Becton Dickinson, Le Pont de Claix, France) was used. The tubes were incubated horizontally for 1 week and then vertically until cultures appeared (in a maximum of 16 weeks).

The amoebic lysates are inoculated on 7H11 agar supplemented with OADC and 50 µg/ml of nalidixic acid, incubated for 18 weeks at 37°C.

Mycobacterial identification at the species level was performed by *hsp65* sequencing. The method for the rapid identification of mycobacterial species was adapted from Telenti et al. (1993). For the PCR, 100 µl of culture in a stationary phase was frozen at −80°C for 1 h then bacteria were heat-killed for 15 min at 95°C. PCRs were performed with 5 µl of DNA from the thermolysate supernatant added to a final volume of 50 µl containing 0.2 µl of GoTaq Flexi DNA polymerase (5 U/µl), 2 µl dimethyl sulfoxide (DMSO), 2 mM of dATP, dCTP, dGTP, and dTTP (Promega); 10 µl of 5 × PCR buffer supplied by the manufacturer; 1 µM of

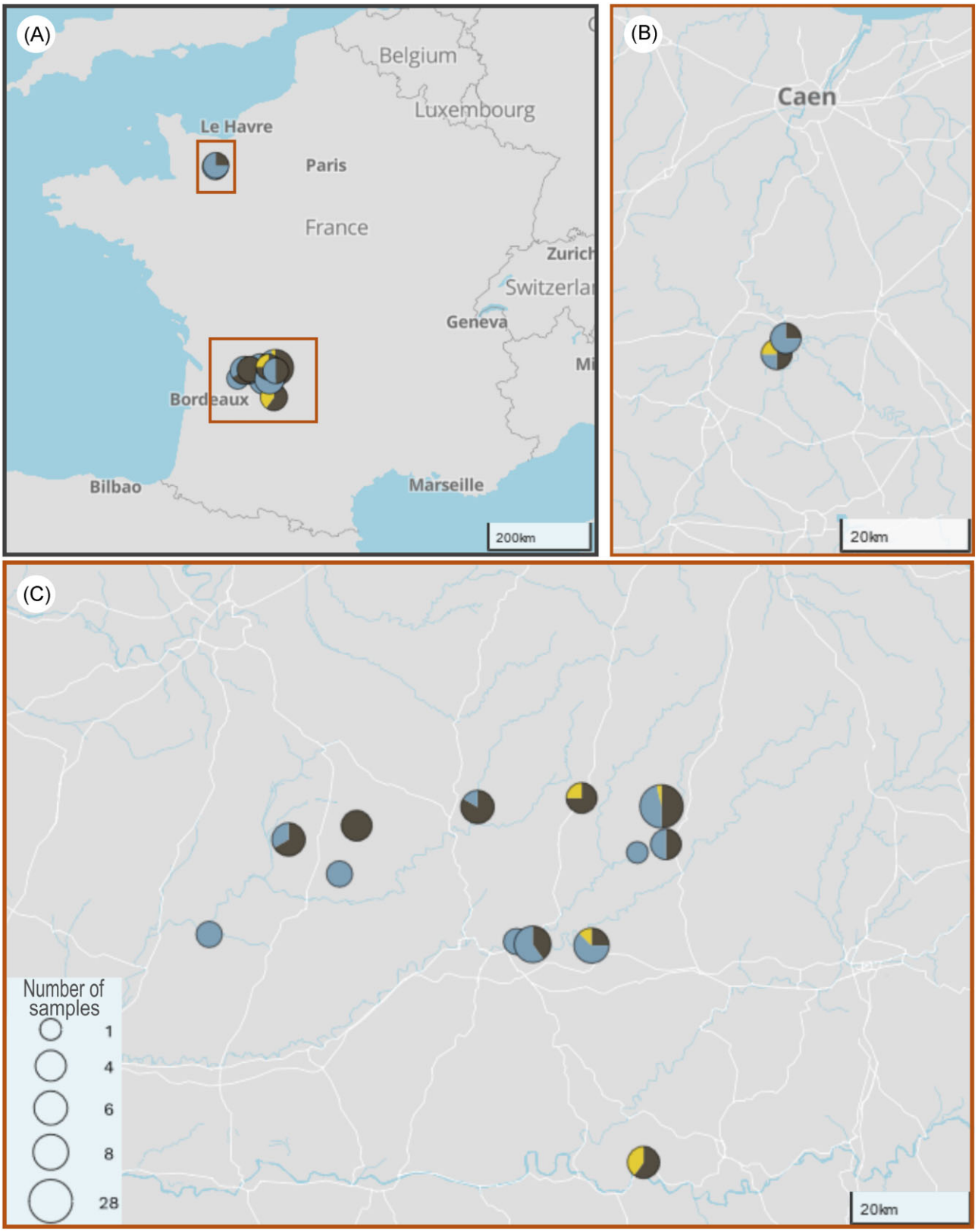


Figure 1. Location of the sampled bovine herds. A total of 90 samples were collected on 20 farms. Water samples are represented with blue colour, soil samples with brown colour, and faeces samples with yellow colour. A size marker indicates the approximate number of samples collected at each point. Some point represents several herds because of their closed location. A) Global location in France B) Location in Normandie region C) Location in Nouvelle-Aquitaine region.

each primer (hsp65-F: ACCAACGATGGTGTGTCCAT and hsp65-R: CTTGTCGAACCGCATACCCT); and 1.5 mM of $MgCl_2$. The reactions were carried out using a TC-512 thermal cycler (Techne). PCR conditions were as follows: 1 cycle of 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C; and 1 cycle of 7 min at 72°C.

PCR products (441 bp) were visualized by electrophoresis using 1.5% agarose gels (agarose electrophoresis grade; Invitrogen) and sequenced by GENEWIZ of Azenta Life Sciences (Bahnhofstrasse 86, 04158 Leipzig, Germany). Sequences were analysed using the leBIBI-QBPP website https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi.

DNA extraction and Oxford Nanopore sequencing (18S and 16S)

To assess the global diversity of FLA and their associated bacteria, total DNA was extracted from the migration fronts for sequencing of partial 18S and 16S rRNA genes by Oxford Nanopore Technologies. Total DNA extraction was conducted using the DNeasy Blood & Tissue Kit (Qiagen) on the QIAcube apparatus, following the manufacturer's instructions, with an additional initial step for cell lysis. Briefly, cells from the migration front, preserved in Tris buffer, were transferred into a screw cap tube containing 300 mg of 0.1 and 2 mm glass beads and filled with a 1:3 ratio of ATL lysis buffer (Qiagen) to sample volume and lysed for two cycles of 30 s at a speed of 6 m/s on a FastPrep homogenizer (Fisher Scientific). Lysed cells were then maintained on ice until DNA extraction. Primers targeting both the 18S and 16S rRNA genes, known to amplify a wide diversity within both amoeba and bacteria phyla, were selected (Needham and Fuhrman 2016). Subsequently, all samples were amplified with Q5® High-fidelity DNA polymerase (New England Biolabs) in final volume of 50 µl containing Q5® reaction buffer 5×, 0.2 mM dNTPs, 0.5 µM of each primer (forward 5′-GTGYCAGCMGCCGCGGTAA-3′; reverse 5′-CCGYCAATTYMTTTRAGTTT-3′), Milli-Q water, and 2 µl DNA. The DNA was amplified as follows: 98°C for 30 s, 30 cycles at 98°C for 10 s, 51°C for 20 s, 72°C for 45 s, and a final step at 72°C for 2 min. All amplified DNA was purified with the QIAquick PCR Purification Kit (Qiagen) on the QIAcube instrument. DNA was quantified using a Qubit fluorometer (Thermo Fisher Scientific) and equimolarly pooled per farm location. A second quantification of pooled DNA was performed using a Qubit, and ∼200 fmol of amplicon DNA was used for library preparation employing the Oxford Nanopore Technologies Native Barcoding Kit (SQK-NBD114.96), following the manufacturer's protocol. The library was then loaded on a flow cell (R.10.4.1) and sequenced with MinION Mk1C using the high-accuracy settings for 24 h. Fast5 raw files were exported and processed for demultiplexing, barcodes trimming, and basecalling using Guppy v6.4.6. Basecalled positions with a minimum quality score of 9 were kept for subsequent analyses. Taxonomic inferences were achieved using emu v3.4.5 (Curry et al. 2022). The obtained taxonomic assignments were then manually inspected, parsed, and further explored in R and graphically formatted using the package ggplot2. The sequences generated in this study have been deposited at NCBI under the BioProject ID PRJNA1167211.

Droplet digital PCR

The ddPCR reactions were carried out following the Bio-Rad ddPCR system guidelines, using the droplet generator QX200 (Bio-Rad, USA) and the QX200 Droplet Reader (Bio-Rad, USA). The reaction mixture was prepared in a 21 µl final volume as follows: ddPCR™ EvaGreen® Supermix 2× (Bio-Rad, USA), forward and reverse primers (2 µM each) targeting the RD4 flanking region and IS900 sequence to detect *Mbo* and *Map* DNA, respectively, nuclease-free water to a total volume of 20 µl and DNA extracted from migration fronts. The total reactional volume and the droplet generation oil (70 µl) were carefully dispensed into wells of a DG8™ cartridge for droplet generation and transferred into a 96-well plate (Bio-Rad, USA) and heat-sealed with an aluminium foil and the PX1™ PCR Plate Sealer at 180°C for 5 s. The PCR reaction was performed in a T100™ Thermal Cycler (Bio-Rad, USA), including an initial denaturation cycle at 95°C for 5 min, followed by 45 cycles of 30 s at 95°C and 1 min at 60°C. A final signal stabilization cycle was performed at 4°C for 5 min, followed by 90°C for 5 min. A 2°C/s ramp was utilized to ensure proper temperature transition for each stage of the cycle. QuantaSoft™ and QuantaSoft™ Analysis Pro (Bio-Rad, USA) software were employed for manual thresholding and absolute quantification (in copies/µl).

Results

FLA are present in almost all the 90 samples collected

The objective was to conduct an extensive sampling campaign on farms known to be affected by bTB or paraTB, to recover environmental FLA. A total of 90 samples were collected in the regions of Nouvelle-Aquitaine and Normandie, comprising 44 water samples, 40 soil samples, and 6 faeces samples (Fig. 1). Each sample was filtered and incubated at two different temperatures: 20 and 37°C. From the 90 cultivated samples, a total of 235 migration fronts were obtained and subsequently analysed. In total, almost all samples (97%) exhibited a characteristic migration front, indicating the presence of cultivable amoebae. The majority of FLA migration fronts (70%) were observed at 37°C, with only 30% detected at 20°C. This discrepancy can be attributed, in part, to the presence of contamination by fungi on the plates incubated at 20°C, which exhibited faster growth and hindered the recovery of the amoebae.

Acanthamoeba, *Copromyxa*, *Naegleria*, and *Vermamoeba* are the most represented genera after metabarcoding analysis

To assess the diversity of environmental FLA cultivated across all samples, the DNA extracted from the migration fronts, grouped by farm location, was included for metabarcoding analysis. The primers selected for this study were designed to capture the diversity of both FLA and bacterial populations. The sequencing process yielded 2 789 124 sequences corresponding to 185 942 ± 37 544 sequences per sample. Bacterial sequences constituted the majority (representing 73% of the dataset), while eukaryotic sequences comprised 27%. Among the eukaryotic sequences, FLA accounted for 93%. This result underlined that the experimental design is well-suited and selective for FLA studies. FLA were identified at the genus level, and the average relative abundance, along with the occurrence, is illustrated in Fig. 2. The sequencing revealed a broad diversity of genera. Overall, *Naegleria* sp., *Acanthamoeba* sp., *Copromyxa* sp., and *Vermamoeba* sp. were the genera with the highest abundance, comprising 43%, 23%, 16%, and 10% of total eukaryotic sequences (Fig. 2). *Tetramitus* sp., *Vahlkampfia* sp., and *Learamoeba* sp. were present in lower abundance but were detected across all locations. Poorly described FLA genera were detected with this metabarcoding approach such as *Fumarolamoeba*, *Guttulinopsis*, *Willaertia*, and *Rosculus*.

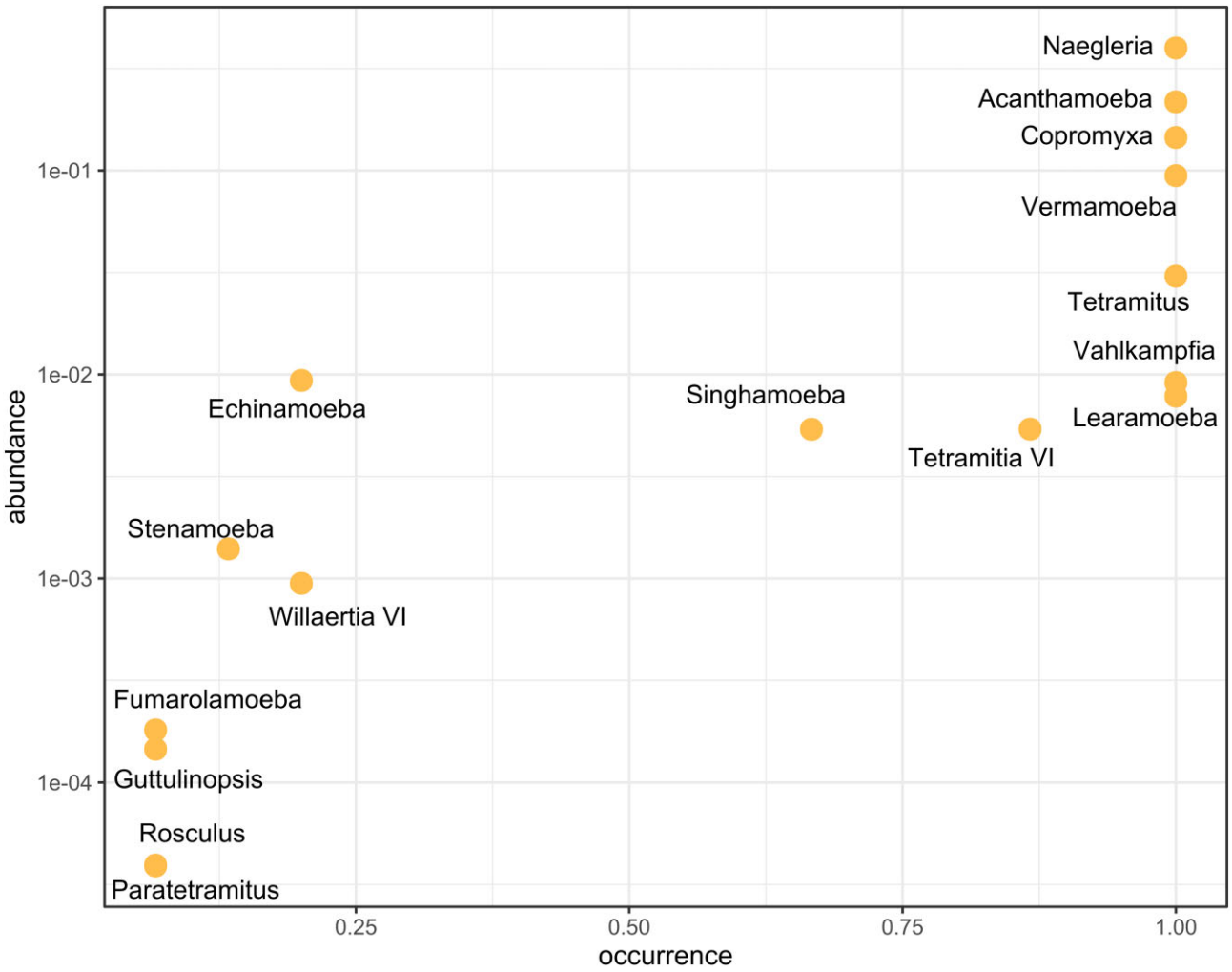


Figure 2. Relative abundance and occurrence of cultivable amoebae from infected cattle environment. Each point represents the average of the relative abundance for one FLA genus found in all farms. Relative abundance threshold fixed at 0.0001%.

A total of 37 FLA is isolated, belonging to 10 different genera

An important aim of this study lies in the establishment of a repository of environmental FLA for subsequent *in vitro* experiments. Substantial effort was devoted to isolating FLA from environmental samples, resulting in the successful isolation and identification of 37 FLA via partial 18S rRNA gene sequencing. Various genera were isolated, predominantly *Acanthamoeba* sp. (20 isolates), *Vermamoeba* sp. (8 isolates), and *Copromyxa* sp. (3 isolates) (Fig. 3A). Other genera were represented by one isolate each. Notably, one isolate presented the highest similarity to a sequence classified as an unidentified heterolobosean FLA; thus, it is not affiliated with any previously described FLA genus. The partial 18S rRNA gene of each isolate was used to assign the phylogenetic positions of our sequences with amoeba reference sequences in phylogenetic trees (Fig. 3B–D). Within the Amoebozoa phylum, all *Vermamoeba* isolates clustered with *Vermamoeba vermiformis*, all *Copromyxa* isolates clustered with *Copromyxa protea*, and the *Echinamoeba* isolate was closely associated with *Echinamoeba exundans* (Fig. 3B). Among the *Acanthamoeba* isolates, phylogenetic analysis revealed intra-genus diversity. For instance, isolate A57 clustered with *Acanthamoeba mauritaniensis*, while isolates A8 and A62 formed a distinct branch from other *Acanthamoeba* isolates. However, other isolates did not exhibit diversity based on the partial 18S rRNA gene used in this study (Fig. 3C). Within the Discoba phylum, the *Tetramitus* isolate was closely related to *Tetramitus entericus*, the *Singhamoeba* isolate was near *Singhamoeba horticola*, and the *Naegleria* isolate was close to *Naegleria gruberi*. The 'unknown' isolate (A41) did not cluster closely with any genus group based on the partial sequence used and may require a complete 18S rRNA gene for phylogenetic analysis (Fig. 3D). Additional details, such as SSU (small subunit)-rDNA sequences, accession numbers of best matches on NCBI, or relative information about isolation, are summarized in Supplementary Data 2.

Bacterial identification reveals many Pseudomonadota and FLA-associated bacteria

FLA-associated bacteria were identified at the genus level based on partial 16S rRNA gene sequences. As FLA were cultured on plates seeded with *E. coli*, the sequences from the enrichment were sequenced in high amount and could not be differentiated from sequences originating from environmental FLA-associated *E. coli*. Therefore, for the results presented in this study, all *E. coli* and, consequently, *Shigella* sequences were removed. Bacteria from the Pseudomonadota phylum were the most abundant, comprising 82.15% of the sequences, and occurred at every location (Fig. 4). Bacteroidota and Bacillota accounted for 7.76% and 7.10% of sequences, respectively, and these phyla were also detected in

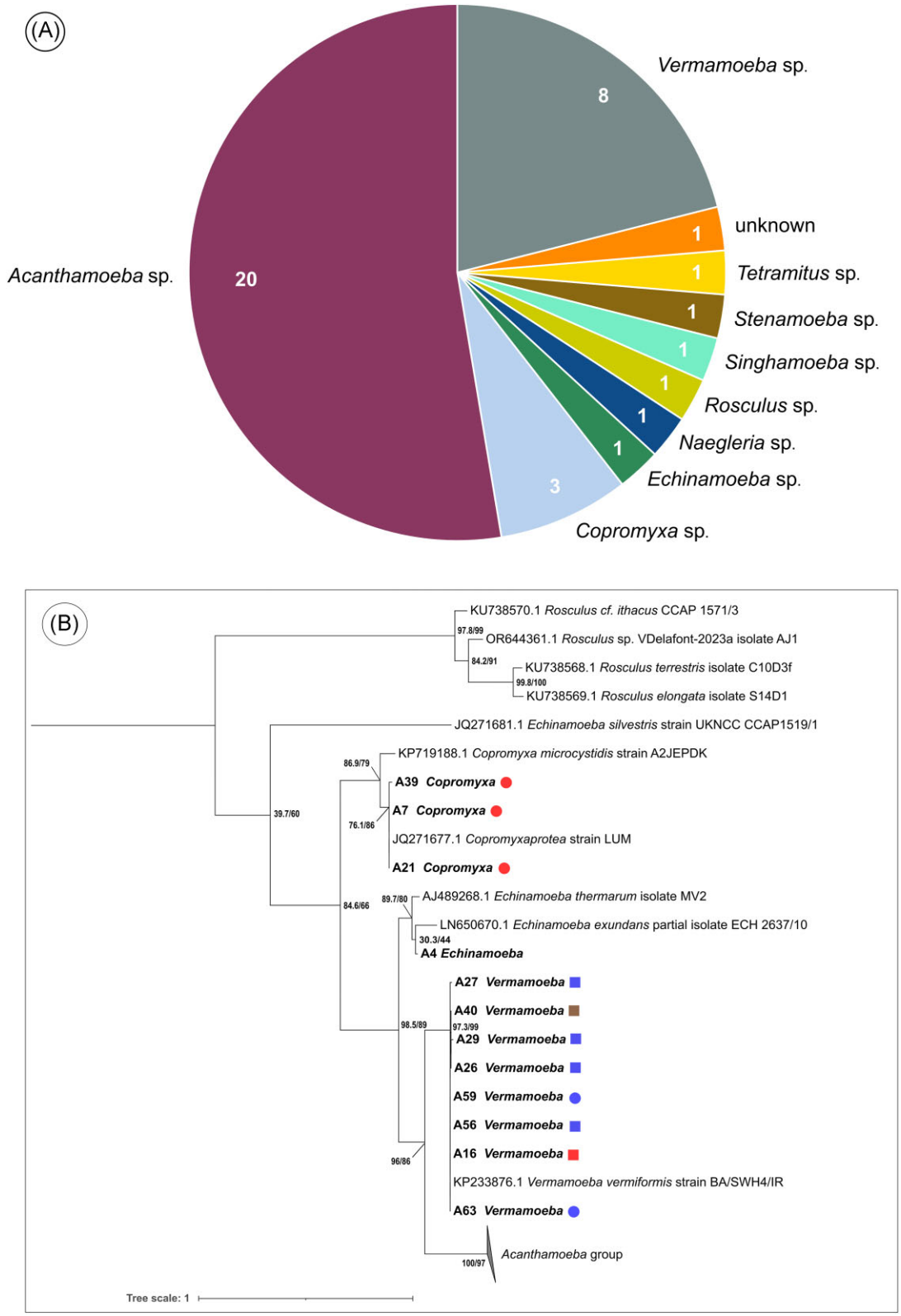
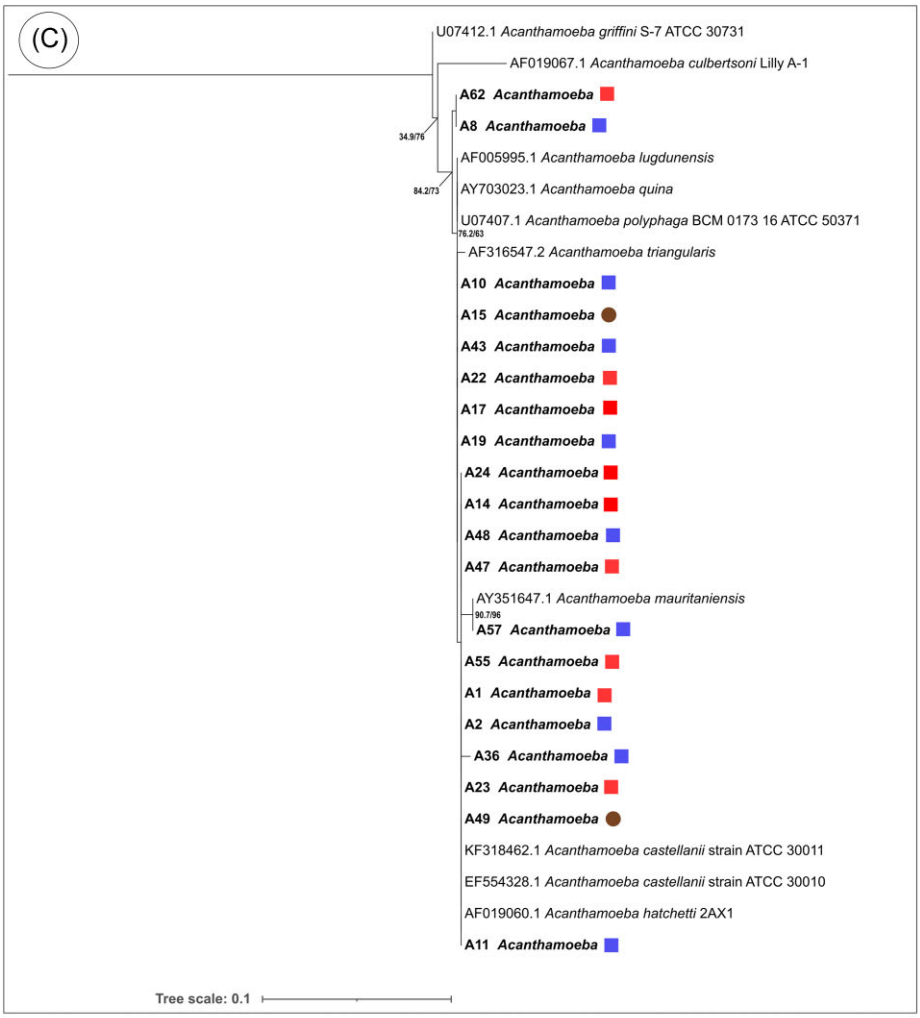


Figure 3. Diversity of environmental amoebae isolates. Identification was obtained via 18S rRNA partial gene with Sanger sequencing. Genera diversity (A) and phylogeny of Amoebozoa (B) with a focus on the *Acanthamoeba* group (C) and Discoba (D) rooted with SAR as outgroup based on 18S rRNA partial gene. FLA were isolated from water (blue), soil (red), and faeces samples (brown) at 20°C (l) or 37°C (n).



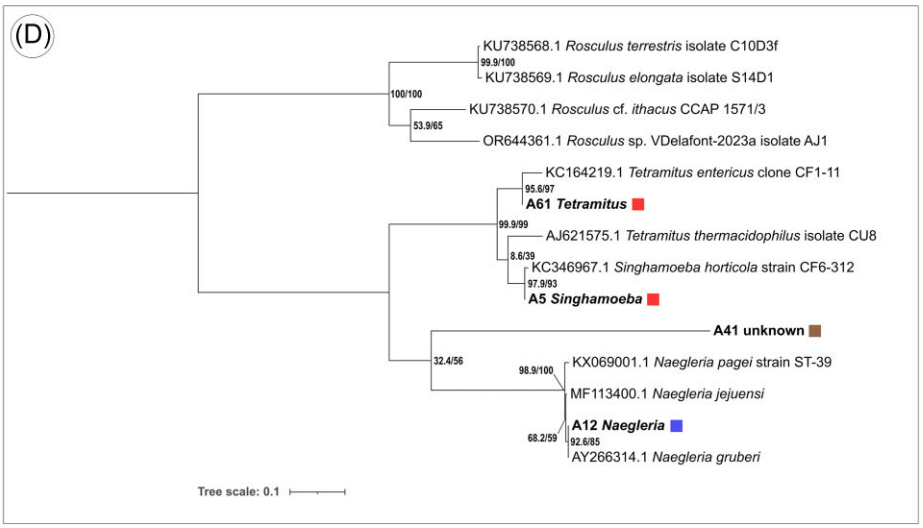


Figure 3. *Continued.*

samples from all locations. Actinomycetota were also identified in all locations, though systematically in low abundances (<1%). Within this phylum, it should be underlined that no mycobacteria sequences were detected. All other phyla also represented <1% of sequences. Among those, several identified phyla are notoriously known for bearing representative that adopt a strict intracellular lifestyle, such as Babelota (synonym Dependentiae), Chlamydiota, and Mycoplasmatota. It should be noted that while lowly abundant, Mycoplasmatota and Chlamydiota were found in 90% and 100% of sequenced samples (Fig. 4).

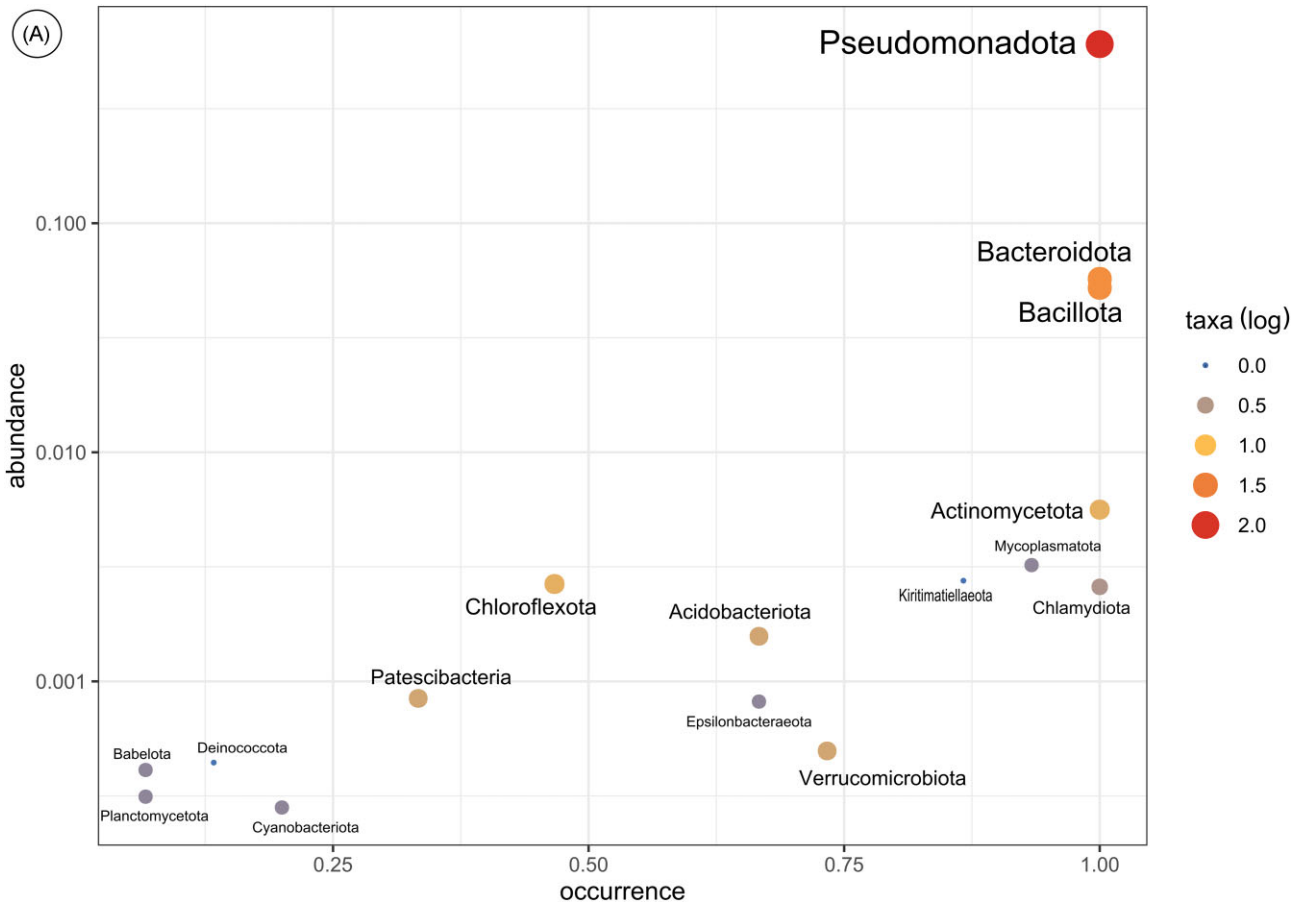


Figure 4. Relative abundance of bacteria associated to cultivable amoebae and occurrence in infected cattle environment. Phyla diversity; each point represents one bacterial phylum and log scale size for each point is in the legend (A). Genera diversity within Pseudomonadota phylum; each point represents one genus. Colours were attributed based on literature when at least one species within the genus was described as amoeba-resistant bacteria (purple), or intracellular in amoeba (green). Grey colour is when no information was found (B). Relative abundance threshold fixed at 0.0001%.

Due to their high relative abundance, occurrence, and richness as FLA-associated bacteria, a specific focus was given on the Pseudomonadota (Fig. 4B). Overall, it represented a total of 108 genus-level identified taxa, among which only 10 were found in all analysed samples. In this core diversity, genera of known amoeba-resistant bacteria such as *Pseudomonas*, *Legionella*, *Coxiella*, and *Stenotrophomonas* were identified, among others (all amoeba-resistant bacteria identified were indicated by purple colour in Fig. 4B). Additionally, genera described as strict intracellular were also identified, such as representative of Diplorickettsiaceae and *Procabacter*.

Traces of *Mbo* and *Map* DNA are found associated to FLA

Our primary objective was to investigate the potential association of *Mbo* and *Map* with environmental FLA. To achieve this, we pursued two approaches: (1) *Mbo* and *Map* DNA detection in DNA extracted from the migration fronts by ddPCR methods, to minimize the effects of inhibitors due to sample partitioning and to produce more accurate results for low levels of nucleic acids than qPCR (Taylor et al. 2017, Nyaruaba et al. 2019) and (2) mycobacteria culture attempts from the migration fronts. *Mbo* and *Map* DNA were detected by ddPCR in several samples (Table 1). This detection was weak, not consistently reproducible, suggesting that DNA from these bacteria was present only in trace amounts. Additionally, *Map* DNA was more frequently detected than *Mbo* DNA, with the latter being detected in only three samples out of the 78 tested. In the positive samples, the main FLA genus isolated was *Vermamoeba*. Besides, FLA lysates from all samples were cultured in an attempt to recover viable *Mbo* or *Map* cells. Unfortunately, no positive cultures were obtained for either bacteria. In conclusion, *Mbo* and *Map*, if present, were not highly associated with the migration fronts, suggesting that they might be poorly associated with FLA recovered in this study in the environment.

Discussion

FLA have been described as reservoirs for several pathogenic bacteria in the environment, such as *L. pneumophila*. Several studies have assessed the interaction of *Mbo* and *Map* and FLA *in vitro*, but very few studies have been focused on how such interaction can occur in the environment. For this reason, our aim was to assess whether FLA can also act as a reservoir of *Mbo* and *Map* by searching for *Mbo* and *Map* in the environment of farm known to be positive to bTB or paraTB. An extensive study over one year allowed to collect 90 samples in 20 farm locations. To our knowledge, no such sampling effort has been described before in Nouvelle-Aquitaine, allowing to harvest 235 migration fronts. The results show that almost all samples were positive for FLA culture, which is not so surprising as FLA is widely known to be omnipresent in a wide array of environments. The metabarcoding analysis of the migration fronts allowed to simultaneously identify both prokaryotic

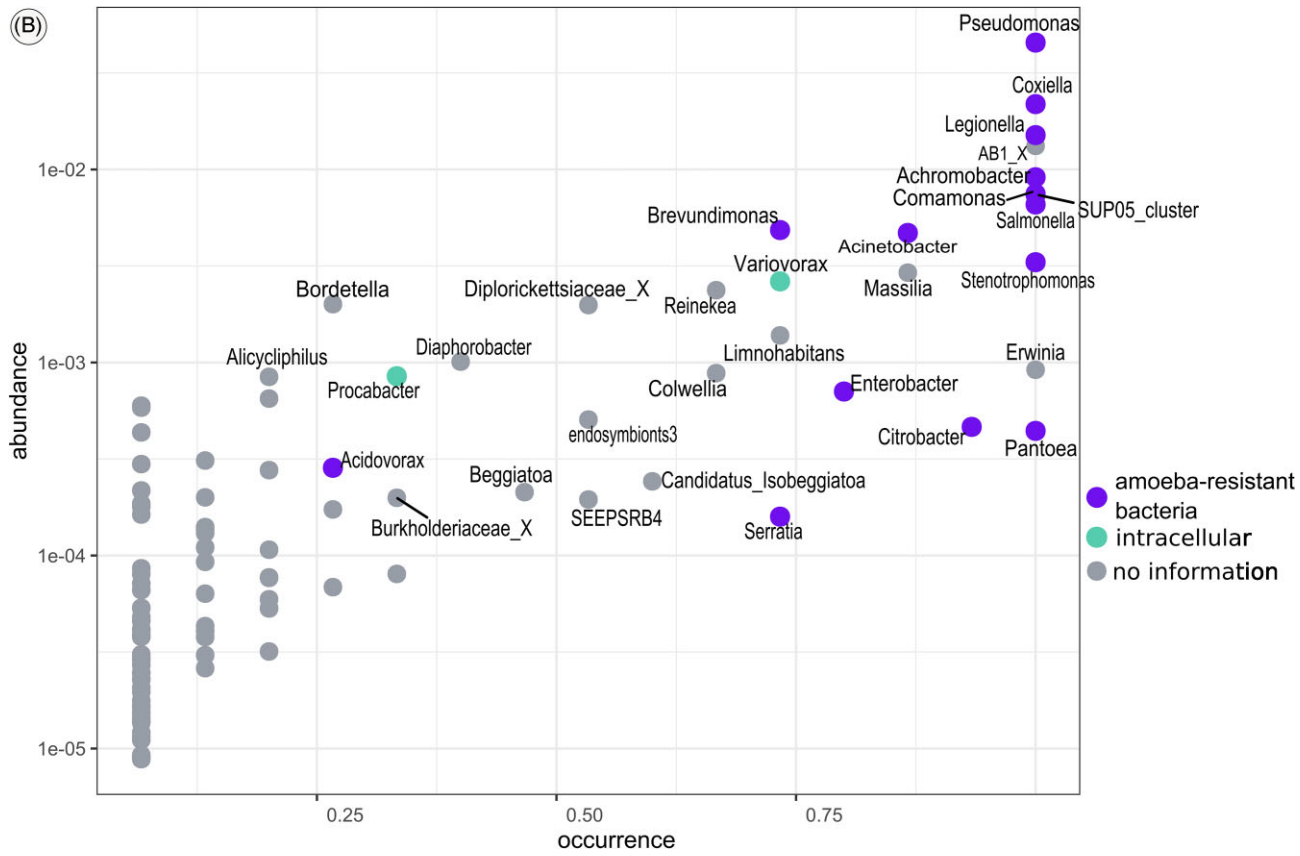


Figure 4. *Continued.*

Table 1. *Mbo* and *Map* DNA associated to amoebae detection by ddPCR.

Mycobacterial DNA targeted	Farm ID	Number of samples	Sample nature	% positive droplet (ddPCR)*	FLA isolated
Mycobacterium bovis	3	2	Water	0.00 279	*Vermamoeba* sp.
	4	12	Soil	0.00 244	
	5	14	Water	0.00 246	
Mycobacterium avium subsp. *paratuberculosis*	1	3	Water	0.00 265	*Vermamoeba* sp.
		3	Water	0.00 267	
		3	Water	0.00 517	
		3	Water	0.00 492	
		6	Water	0.01 104	
		9	Soil	0.00 297	
		10	Soil	0.00 578	
		36	Water	0.00 870	*Vermamoeba* sp.
		38	Soil	0.00 258	
	3	2	Water	0.00 543	*Vermamoeba* sp.
		2	Water	0.00 265	*Vermamoeba* sp.
	4	12	Soil	0.01 016	
	5	15	Water	0.00 820	*Acanthamoeba* sp.
	6	22	Water	0.00 549	
		23	Soil	0.00 246	
	7	25	Soil	0.00 511	
	8	33	Soil	0.00 247	
	9	40	Water	0.00 280	
	11	47	Water	0.00 265	
		51	Soil	0.00 260	
	12	54	Soil	0.00 587	*Acanthamoeba* sp.; *Singhamoeba* sp.; *Copromyxa* sp.; *Acanthamoeba* sp.
	13	58	Water	0.00 560	

*Percentage of positive droplets was calculated on 37 542 ± 2229 droplets.

and eukaryotic diversity in a single PCR assay. According to the PR2 primer database, the primer set we chose for this approach covers 97% of bacteria and 91% of eukaryotes. The sequencing depth reached using Oxford Nanopore sequencing enabled to satisfyingly cover both domains. Noticeably, the overwhelming majority of identified eukaryotic sequences belonged to FLA, indicating the enrichment method used in this study allows to specifically favour this group of protists. Thus, we were able to describe a large diversity of FLA, belonging to >16 genera of FLA, covering most major—yet highly divergent—taxonomic groups bearing FLA, such as Amoebozoa, Heterolobosea, and Rhizaria. Importantly, the primer pair used in this study does not show any particular amplification bias towards Heterolobosea, which is mostly uncovered by many universal eukaryotic primers described in the literature (Delafont et al. 2022). In comparison with the classically implemented approach of FLA identification, consisting in subcloning and Sanger sequencing of isolates, metabarcoding identified twice as much diversity at the genus level. However, all genera classically identified were also recovered by metabarcoding. Indeed, HTS-based identification provides increased sensitivity for diversity analyses, but also points to FLA genera that are more or less likely to be recovered by a classic approach. In line with this, genera such as *Vahlkampfia*, *Rosculus*, *Guttulinopsis*, and *Willaertia* were not recovered in purified cultures, despite being present in the initial migration fronts. Such difficulties in environmental FLA culture were also observed for the *Rosculus* genus, underlying the importance of specific growth conditions (Samba-Louaka et al. 2018, Jessu et al. 2023). Such information should direct future studies in adapting protocols for specific recovery of those FLA genera.

The enrichment method used in this study, consisting in feeding FLA with a lawn of *E. coli*, involves filtering out all *E. coli* sequences from the dataset, including those of potentially FLA-associated ones. Furthermore, it must be kept in mind that the way this enrichment shapes FLA diversity remains mostly unknown; it is likely that part of FLA diversity may not be enriched in such conditions. Culture-independent approaches could bring valuable information regarding this aspect. However, this step remains indispensable to study the association with bacteria and to isolate specific FLA strains. The *Naegleria*, *Acanthamoeba*, and *Vermamoeba* were the most represented in our samples, in line with other environmental study of FLA focused on water and soil samples (Geisen et al. 2014, Denet et al. 2017). Besides, the three *Copromyxa* isolates were isolated from soil. Also, less described genera, but belonging to the same group were also present with high occurrence (i.e. *Tetramitus*, *Vahlkampfia*, and *Learamoeba*).

Regarding the metabarcoding analysis of the bacteria, no *Mycobacterium* sequence were found. It suggests that mycobacteria could not be detected using this method or that these bacteria were not or poorly associated to the FLA migration fronts. Also, *E. coli* were used to feed FLA to isolate them and was sequenced with a high coverage rate, perhaps masking sequences present in lower abundance, such as *Mycobacterium* sp. On the contrary, a high abundance and occurrence of Pseudomonadota were found. The main genera were *Pseudomonas*, *Legionella*, *Stenotrophomonas*, and *Coxiella*, which have been all described before to interact with FLA and to present FLA-resistant bacteria (Rowbotham 1980, La Scola and Raoult 2001, Cateau et al. 2014, Steele et al. 2023). In a previous study of FLA-associated bacteria in drinking water, *Pseudomonas* and *Stenotrophomonas* were the most represented genera found by a similar approach (Delafont et al. 2016).

To go further in the search of *Mbo* and/or *Map* DNA in interaction with FLA, ddPCR experiments were conducted and allowed to detect traces of these DNA in the migration fronts. It should be underlined that these results need to be confirmed by the isolation of the bacteria and not only their DNA to strengthen the hypothesis that FLA might be a reservoir for these bacteria in the environment. Cultivating these slow-growing bacteria from environmental samples is challenging but *Mbo* and *Map* DNA have been already found in water and soil (Rhodes et al. 2013, Barbier et al. 2016). The absence of cultivable *Mbo* and *Map* in the migration fronts suggests that these bacteria were not associated or poorly associated with FLA in the environment or, alternatively, that our samples were not representative of the real situation. Indeed, our sampling may have missed the targeted mycobacterium, which would be really present and/or the difficulty to isolate these bacteria from the environment hinders their presence.

Conclusion

In conclusion, this extensive sampling effort, coupled with HTS sequencing, allowed us to identify a large array of FLA, including some poorly described genera. In addition, the identification of bacteria underlined the presence of many Pseudomonadota and FLA-associated bacteria, but no *Mbo* or *Map* DNA were found by sequencing. However, traces of these DNA were detected by ddPCR, suggesting that *Mbo* and/or *Map* might be present in association with FLA but in very low amounts in our samples. This may be due to a poor association in the environment or a sampling bias, as we are searching for a needle in a haystack. Furthermore, the interactions of FLA isolates with these bacteria should be further characterized *in vitro* to better understand if it could participate to mycobacterium survival and transmission in the environment.

Acknowledgements

We thank Gazal Gokkus for her technical assistance with environmental samples treatment during her internship.

Author contributions

Amélie Jessu (Investigation, Methodology, Writing – original draft), Thierry Cochard (Investigation, Writing – review & editing), Mélanie Burtin (Investigation, Writing – review & editing), Stéphanie Crapart (Investigation, Writing – review & editing), Vincent Delafont (Data curation, Investigation, Methodology, Writing – original draft), Ascel Samba-Louaka (Conceptualization, Funding acquisition, Methodology, Writing – original draft), Franck Biet (Conceptualization, Funding acquisition, Methodology, Writing – review & editing), Jean-Louis Moyen (Conceptualization, Funding acquisition, Methodology, Writing – review & editing), and Yann Héchard (Conceptualization, Funding acquisition, Methodology, Writing – original draft)

Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

Conflict of interest: None declared.

Funding

This work was supported by the "Agence Nationale de la Recherche (ANR-22-CE35-0010, ≪Amitub≫, 01-01-2027). A.J. was

supported by the Laboratoire Départemental d'Analyse et de Recherche de Dordogne (LDAR24) under the Cifre convention.

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Received 4 October 2024; **revised** 6 November 2024; **accepted** 16 December 2024