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Proceedings of the *Legionella* 2022

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Impact of prolonged hotel closures during the COVID-19 pandemic on *Legionella* infection risks

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In general, it is accepted that water stagnation and lack or poor maintenance in buildings are risk factors for *Legionella* growth. Then, in theory, the prolonged hotel closures due to the COVID-19 pandemic may have increased the risk of *Legionella* infections. However, there are very few field studies comparing the level of *Legionella* colonization in buildings before the pandemic and the new situation created after the lockdown. The objective of this study was to analyze these differences in a group of hotels that experienced prolonged closures in 2020 due to the COVID-19 pandemic. We have studied the *Legionella* spp. results, analyzed by standard culture, from the domestic water distribution systems of 73 hotels that experienced closures (from 1 to >4months) during 2020, immediately after the reopening. The results were compared with those obtained in similar samplings of 2019. For the comparative analysis, we divided the hotels in two groups: Group A that have suffered closures for ≤3months and Group B that remained closed for more than 3months, both in relation to the opening period of 2019. In the Group B (36 sites), the frequency of positive samples in the hot water system increased from 6.7% in 2019 to 14.0% in 2020 ($p<0.05$). In the Group A (37 sites), no significant differences were observed. No statistically significant differences were observed in terms of positive sites (defined as hotels with at least 1 positive sample), *Legionella* spp. concentrations and prevalence of *Legionella pneumophila* sg1 between the samplings of the two periods studied. The results suggest that hotels that suffered the longest prolonged closures (> 3months) could have carried a higher risk of exposure to *Legionella* in the domestic hot water system. These findings highlight the importance of adequate preopening cleaning and disinfection procedures for hotel water systems, and the convenience of considering the most effective disinfection methods especially for hot water systems and after prolonged closure periods.

KEYWORDS

***Legionella*, COVID-19, hotel facility, prolonged closures, domestic water distribution systems**

1. Introduction

It is widely accepted that water stagnation in buildings is a risk factor for microbial growth, including opportunistic pathogens as *Legionella* spp. (World Health Organization, 2007; Nisar et al., 2020; Proctor et al., 2020). Among the causes that can favor this microbial proliferation, the following have been cited: decay or decreased disinfectant residual (Boccelli et al., 2003;

Barbeau et al., 2005; Goyal and Patel, 2015; Branz et al., 2017; Xu et al., 2018; Huang et al., 2020), accumulation of nutrients (iron and other metals) by means of increase of corrosion (Rakić and Štambuk-Giljanović, 2016; Van der Lugt et al., 2017; Cullom et al., 2020; Martin et al., 2020) or due to leaching from the pipes and scaling (Lytle and Schock, 2000; Nescerecka et al., 2014; Cullom et al., 2020; Li et al., 2020) and development of microbial communities that result in biofilm instability and proliferation of opportunistic microorganisms (Guerrieri et al., 2008; Inkinen et al., 2014; Tsagkari and Sloan, 2018; Rahmatika et al., 2022). Following this body of evidence, current *Legionella* guidelines and regulations recognize the risks associated with stagnation emphasizing the need to avoid dead ends and dead legs, the regular flushing of low-used pipes or the cleaning and disinfection of the water systems after periods of non-use, among others (Health and Safety Executive, 2013; ESGLI, 2017; Ministerio de Sanidad, 2022).

However, the undesired effects of stagnation are highly dependent of different factors including water temperature, flow profiles and total stagnation time (Zhang et al., 2021; Rhoads et al., 2022). It has been this last variable that, in the context of the COVID-19 pandemic, has led many public health institutions and other relevant authorities to promote preventive guidelines for the safe reopening of buildings after months of prolonged mandatory closures. Hotels and other commercial accommodation sites have been severely affected by "stay-at-home" orders and travel restrictions, in particular in touristic destinations where hotels are used mainly for recreational purposes and were not considered essential business. This has been the case in Spain, where the "state-of-alarm" decree (Ministerio de la Presidencia, Relaciones con las Cortes y Memoria Democrática, 2020) forced many hotels to close from March 14, 2020, until the end of June 2020. In addition, many hotels continued to be closed for several more months, due to a lack of customers. This extended closure, even for seasonal hotels that usually close for several months each year during the winter season, is unprecedented, adding an additional period of total closure, during the summer season. In addition, it should be added that the prolonged closure of nonessential buildings also significantly altered the patterns of water demand in the municipal distribution networks of many tourist areas, contributing to further increase the period of stagnation and thus the water age.

In addition, hotels and commercial accommodation sites have been historically associated with a significant number of *Legionella* infections. In Europe, around 15–20% of all reported *Legionella* infections are travel associated (Beauté, 2017). Moreover, it is known that the prevalence of *Legionella* spp. in the water systems of hotels is usually higher than 50%, at least in the Mediterranean area (Borella et al., 2005; Erdogan and Arslan, 2007; Kyritsi et al., 2018; Doménech-Sánchez et al., 2022). Therefore, it is of interest to study the impact that this prolonged closure could have had on the presence of *Legionella* in the domestic water systems of hotels, in particular immediately after the reopening, when, in theory, the consequences of the stagnation could have been more noticeable.

The purpose of this study is to analyze the quality of potable water in reopened hotel establishments, after the prolonged closure due to the COVID-19 pandemic, in terms of the presence of *Legionella* spp. in a group of hotels in the Balearic Islands (Spain), comparing the results with those obtained the previous year in similar samplings. In Spain, hotels are legally obliged to carry out a cleaning and disinfection procedure of the entire domestic water system before reopening the establishment after a period of 1 month of closure, in accordance with an established protocol (Ministerio de Sanidad y Consumo, 2003) and to carry out a sampling for *Legionella* spp. testing, following a standardized procedure (UNE 100030:2017. *Guidelines for prevention and control of proliferation and spread of Legionella in facilities*). To our knowledge, this is the first study of its kind that includes a relatively large number of hotels whose water systems had previously been disinfected through protocolized procedures and compares the results of similar samplings carried out before and after the extended closures due to the COVID-19 pandemic.

2. Materials and methods

2.1. Experimental design

Water samples collected from a group of hotel facilities in Spain, that had experienced prolonged closures throughout 2020 due to COVID-19 pandemic, were tested for *Legionella* spp. immediately after opening (first sampling). The frequency of positive samples, the prevalence of positive sites, the concentrations of *Legionella* spp. and the presence of *Legionella pneumophila* sg1 were analyzed. The results were compared with those obtained for the same hotels in similar samplings of 2019. All the hotels included in the study operated seasonally. All hotels had their domestic water systems disinfected prior to reopening according to local regulations. All the samplings were conducted at least 2 weeks after the disinfection procedure. For the comparative analysis, the hotels were divided in two groups: Group A that have suffered closures for ≤3 months and Group B that have remained closed for more than 3 months, both in relation to the opening period of 2019.

2.2. Definitions

(a) Positive sample: any sample with a positive result of ≥10 CFU/L.
(b) Positive site: hotel with at least 1 positive sample for *Legionella* spp.
(c) Prolonged closure: any closure in a determined hotel of at least 1 month (30 days) in 2020 due to the COVID-19 pandemic, in relation to the normal opening period in 2019; period of prolonged closure calculated as: closing months in 2020 minus closing months in 2019.

2.3. Sampling

The samplings were conducted in accordance with the Spanish standard UNE 100030:2017 (*Guidelines for prevention and control of proliferation and spread of Legionella in facilities*). The number of samples collected from each hotel depended on the number of rooms being approximately 0.5 $\sqrt{n}$ for the hot water systems and 0.25 $\sqrt{n}$ for the cold water systems [n = number of rooms; Asociación Española de Normalización (AENOR), 2017]. The sampling points included the cold and hot water tanks and several terminal points, taps and showers, from different areas of the building. All the samples were taken after 1 min of flushing, followed by measuring the temperature and the biocide level (in cold water) before collecting 1 liter of water

in a sterile container with sodium thiosulphate pentahydrate (0.01%, w/v) and transported immediately at ambient temperature into an isothermal bag to the laboratory. *Legionella* samples were taken and tested by the same accredited laboratory (Biolinea Int. S.L.). Water temperature was tested using a calibrated digital thermometer at the time of the sample collection after 1 min of flushing.

2.4. Detection and enumeration of *Legionella* spp. by culture

The samples were processed within 24 h of collection. The detection and enumeration of *Legionella* spp. in the water samples was carried out according to the UNE-EN ISO 11731:2017 standard. The isolates were further identified and serotyped (*L. pneumophila* serogroup 1, *L. pneumophila* serogroup 2–14 and *Legionella* non-pneumophila species) using latex agglutination reagents (Oxoid, Spain).

Water samples where *Legionella* spp. was not detected or when containing <10 CFU/L were considered negative, and the result equated to 0 CFU/L for the statistical analysis.

2.5. Statistical analysis

For the statistical analysis, the 850 data were tested for normality using the Kolmogorov–Smirnov test. The nonparametric chi-squared test was used to compare the proportion of positive results in two groups. The Mann–Whitney U test was used to compare the concentration of *Legionella* spp. IBM SPSS Statistics V22.0 was used for statistical analysis. Results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of the hotel groups

Seventy three hotels were included in the study, 37 in the Group A and 36 in the Group B. The average number of floors and rooms were, respectively, 4.1 ± 2.3 and 177 ± 138 in group A and 5.3 ± 2.9 and 194 ± 125 in Group B. The prolonged closure periods in 2020 for both groups, the number of hotels for each prolonged closure period and the average water temperatures of the samples from the cold and hot water systems from both years are shown in Table 1.

3.2. Microbiological results

Four hundred fourteen water samples were collected in 2019 and 436 in 2020. The proportion of positive samples in the cold water system and the hot water system per year and per group, and the pertinent *p* values is shown in Table 2. Overall, there were 35 positive samples (8.5%) in 2019 and 46 positive samples (10.6%) in 2020 ($p > 0.05$). Only the proportion of positive samples in the hot water system of Group B experienced a statistically significant increase (6.7% in 2019 and 14% in 2020, $p < 0.05$).

The number of positive sites was 27 (37.0%) in 2019 and 22 (30.1%) in 2020 ($p > 0.05$).

The number of positive samples to *Legionella pneumophila* sg1 was 10 (28.5%) in 2019, involving 9 hotels, and 17 (36.9%) in 2020, from 10 hotels ($p > 0.05$). No statistically significant differences were observed either by groups or by water system.

The results of *Legionella* concentrations in the periods 2019 and 2020 for the different samplings are summarized in Table 3. There were no significant differences between the concentrations of *Legionella* spp. found in 2019 and 2020 (Figure 1). No statistically significant differences were observed either by groups or by water system.

4. Discussion

Although water stagnation in large buildings, like hotels, is considered a risk factor for microbial proliferation, its real impact in terms of both *Legionella* colonization and *Legionella* infections is not well understood. This is due, in part, to the intrinsic difficulties of studying the effects of building stagnation under real conditions, in numerous buildings, under relatively well controlled conditions and over long periods of time. Thus, there are studies that have analyzed these effects along short periods, e.g., days or weeks (Lautenschlager et al., 2010; Montagnino et al., 2022), studies that have evaluated some buildings (De Giglio et al., 2020; Liang et al., 2021), other studies mixing different types of buildings and water systems (Turetgen, 2021) and, finally, laboratory investigations using lab-scale models (Wang et al., 2012; Rhoads et al., 2017; Tsagkari and Sloan, 2018; Martin et al., 2020). However, field studies carried out with many buildings that have suffered stagnation for relatively long periods and that have been sampled under similar conditions before and after the period of stagnation are rare or non-existent. The COVID-19 pandemic has provided an unprecedented favorable situation to carry out this type of research, by causing prolonged closures of numerous buildings and situations of low demand for water in large areas of the distribution network.

Hotels are ideal buildings for this type of studies. First, because stays in hotels and other commercial tourist accommodation establishments have been associated historically with a very significant number of cases of legionellosis (Beauté et al., 2012) and there is, in Europe, a well-established epidemiological surveillance system, the European Legionnaires' disease Surveillance Network (ELDSNet), for cases of legionellosis associated with travel (European Centre for Disease Prevention and Control, 2017). Second, because the relatively high rates of *Legionella* colonization in hotels makes it relatively easy to obtain meaningful statistical data when conducting pre-and post-intervention studies. And finally, because it is a sector that, at least in Spain, is regulated in terms of *Legionella* prevention thus following standardized guidelines for certain practices, such as annual disinfection procedures and routine samplings. We have taken advantage of this situation by analyzing the results of a group of hotels for which we had microbiological data for the year 2019, corresponding to a first sampling carried out after the annual disinfection, which, in Spain, is mandatory and must be carried out within 1 month before the reopening in seasonal hotels. This, together with the fact that the sampling must be done in a standardized way in terms of the number of samples and the sampling points, has allowed us to make a comparative analysis of the pre-and post-pandemic results, ensuring a reasonable degree of homogeneity in both initial yearly samplings.

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TABLE 1 Prolonged closure periods in 2020 of Groups A and B and average water temperatures of the samples taken in 2019 and 2020 (CWS=cold water system; HWS=hot water system).

	Months of prolonged closure	Number of hotels	Average water temperatures (°C)			
			2019		2020	
			CWS	HWS	CWS	HWS
Group A	≥1–2	8	22.8 ± 1.3	52.7 ± 4.6	25.7 ± 1.8	52.6 ± 4.4
	>2–3	29	21.4 ± 1.3	53.4 ± 6.2	24.7 ± 1.9	51.2 ± 8.4
Group B	>3–4	30	20.7 ± 2.0	56.0 ± 4.7	25.6 ± 2.6	55.9 ± 5.4
	>4	6	19.4 ± 1.5	56.0 ± 5.3	25.9 ± 1.9	56.4 ± 5.5

TABLE 2 Number of samples (N), number of positive samples (*n*), proportion of positive samples (% pos) per Group (A, B) and year (2019, 2020), and *p* values (CWS=Cold Water System; HWS=Hot Water System).

	Group A			Group B		
	CWS	HWS	TOTAL	CWS	HWS	TOTAL
	N/n (% pos)	*N/n* (% pos)	*N/n* (% pos)	*N/n* (% pos)	*N/n* (% pos)	*N/n* (% pos)
2019	55/2 (3.6)	135/21 (15.6)	190/23 (19.2)	75/2 (2.7)	149/10 (6.7)	224/12 (9.4)
2020	59/5 (8.5)	42/19 (13.4)	201/24 (21.9)	85/1 (1.2)	150/21 (14.0)	235/22 (15.2)
p	0.102	0.172	0.30	0.110	0.038	0.302

TABLE 3 Range of *Legionella* spp. concentrations by water system (cold and hot water systems), year (2019, 2020), and Group (A, B; *n*=number of samples, %=proportion of positive or negative samples, CFU/L=Colony Forming Unit per Liter).

Legionella spp. (CFU/L)	Group A			Group B		
	n	%	Mean (CFU/L)	*n*	%	Mean (CFU/L)
Cold water system						
2019						
≤1 × 10^2	1	1.8	8.0 × 10^1	1	1.3	3.6 × 10^1
1 × 10^2–1 × 10^3	0	0	-	1	1.3	3.6 × 10^2
≥1 × 10^3	1	1.8	8.0 × 10^3	0	0	-
Positive	2	3.6	-	2	2.6	-
Negative	53	96.4	-	73	97.3	-
2020						
≤1 × 10^2	2	3.4	6.0 × 10^1	1	1.2	6.0 × 10^1
1 × 10^2–1 × 10^3	2	3.4	1.2 × 10^2	0	0	-
≥1 × 10^3	1	1.7	1.0 × 10^4	0	0	-
Positive	5	8.5	-	1	1.2	-
Negative	54	91.5	-	84	98.8	-
Hot water system						
2019						
≤1 × 10^2	10	7.4	2.1 × 10^1	6	4.0	1.5 × 10^1
1 × 10^2–1 × 10^3	6	4.4	1.9 × 10^2	0	0	-
≥1 × 10^3	5	3.7	2.4 × 10^4	4	2.7	1.6 × 10^4
Positive	21	15.6	-	10	6.7	-
Negative	114	84.4	-	139	93.3	-
2020						
≤1 × 10^2	8	5.6	2.2 × 10^1	10	6.7	3.1 × 10^1
1 × 10^2–1 × 10^3	5	3.5	2.4 × 10^2	4	2.7	4.2 × 10^2
≥1 × 10^3	6	4.2	4.5 × 10^3	7	4.7	9.9 × 10^3
Positive	19	13.4	-	21	14.0	-
Negative	123	86.6	-	129	86.0	-

The results of this study suggest that only hotels that suffered the longest prolonged closures (>3 months) could have carried a higher risk of exposure to *Legionella* in the domestic hot water system. Indeed, only the Group B showed a significant increase in the proportion of positive samples for *Legionella* spp. in 2020, after their reopening. In contrast, the hotels in Group A, with closures ≤3 months, although they tended to have higher frequencies of positives, these were not statistically significant. However, these data must be balanced against the absence of significant changes in the number of positive hotels, *Legionella* concentrations and presence of *Legionella pneumophila* sg1. It is very likely that these factors, especially the last two, play an important causal role in the chain of infection, given the preponderance of the *Legionella pneumophila* sg1 in the set of *Legionella* infections (Yu et al., 2002) and the general relevance of the concentrations of the pathogenic germ at source in quantitative microbial risk assessments (Hamilton et al., 2019; Sharaby et al., 2019). Then, this is a relatively modest impact, being limited only to the frequency of positive samples in the hot water system, which, in the absence of relevant changes in the bacterial loads and prevalence of *Legionella pneumophila* sg1, might have little relevance in terms of actual infections, although we do not have data on this last aspect. In fact, the data on the incidence of legionellosis cases associated with travel for 2020 are hardly relevant given the significant travel restrictions that still existed this year, being difficult to compare with the previous year (the number of reported cases to the travel-associated surveillance scheme of ELDSNet decreased by 67% in 2020 compared with 2019; European Centre for Disease Prevention and Control, 2020).

In general, the results of our study are in line with other studies that have also observed increased risk of exposure to *Legionella* after prolonged facility closures due to the COVID-19 pandemic (Turetgen, 2021), although in our study only the hotels that were closed the longest showed significant increases. This seems logical, considering that the total stagnation time and water age in buildings are relevant factors in the persistence of microbial colonization, resulting in higher

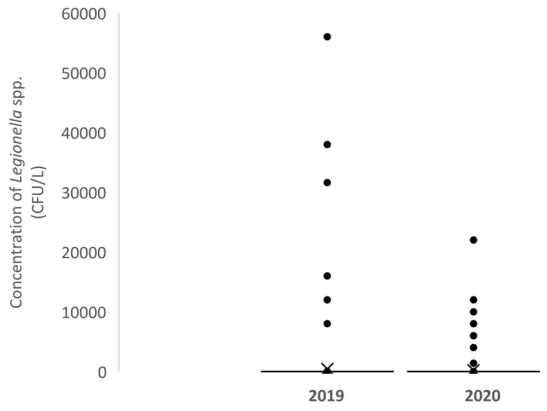

FIGURE 1
Box plot of *Legionella* spp. concentrations (CFU/L) in 2019 and 2020.

bacterial loads (Rhoads et al., 2016) and the formation of more dense and complex biofilms (Tsagkari and Sloan, 2018). We must highlight that our study evaluated the presence of *Legionella* following the reopening of the hotels, after having carried out a mandatory cleaning and disinfection of the water systems, that is, under normal operating conditions, in contrast to other studies that were carried out before returning to normal activities (De Giglio et al., 2020; Liang et al., 2021). In this sense, our results suggest that these cleaning and disinfection procedures, in preparation for the reopening after the closure induced by COVID-19 pandemic, could have been insufficient to eliminate the highest bacterial loads and dense biofilms in the hotels that suffered the longest shutdowns.

The fact that significant increases in the frequency of positive samples have been observed only in the hot water system, and not in the cold water, may be due to several factors. Firstly, different studies suggest that *Legionella* preferentially colonize hot water systems of buildings (Nisar et al., 2020; Doménech-Sánchez et al., 2022; Gamage et al., 2022). Second, because the Spanish regulations allow choosing between chemical or thermal disinfection, it is possible that, at least in a significant number of installations, the hot water system had been thermally disinfected (raising the water temperature up to 70°C for 2 h), a method that is generally considered less effective and has fewer lasting effects than chemical disinfection (Molina et al., 2022). Since we have not investigated the precise methodology used in pre-opening disinfections, we cannot rule out that a preferential use of one or the other may have affected the results. Finally, the presence of biocide in the cold water system (in Spain, 0.2–1.0 mg/L of residual free chlorine is required in cold drinking water) may have helped to recover the baseline conditions in this system earlier after reopening.

Our study has several strengths and some limitations. First, because of the regulations in Spain, we had the opportunity to compare results from the same group of hotels in 2019 and 2020 in quite similar conditions (first sampling after the annual disinfection procedure), thus avoiding possible bias due to longer periods of normal operation after reopening and subsequent recovery of baseline conditions. The average hot water temperatures at which the samples were taken in both years did not differ significantly, thus allowing a fair comparative analysis between the 2 years. Our study also benefits from the fact that all samplings and analysis were done by the same laboratory, which has accredited both procedures, sampling and testing, thus avoiding the possible biases that could occur with different samplings and/or analysis systems in other studies. Finally, the methodology used for the domestic water system disinfection was essentially the same for all the sites, following the same regulation, so, in theory, this procedure should not have affected from 1 year to another.

The study also presents some limitations. First, both the number of hotels and the number of samples is too low for deriving strong conclusions. We also acknowledge that the proportion of positive samples in 2019 for the Group B (6.7%) was quite low in comparison of the proportion of positive samples for Group A (15.6%). The causes of this difference are not clear, given that the structural characteristics of both groups of hotels (number of floors, number of rooms) were not much different, although we cannot completely rule out some significant structural and operational differences (piping materials, flushing regimes, chlorine levels, etc.) that were not considered and could have conditioned the results in one way or another. Indeed, hot water temperatures in Group B were a bit higher than in Group A. However, we know that Group B presented a similar percentage of positives in 2021 in relation to 2019 (7.5%, data not shown in the results section), which suggests that the increase observed in 2020 was genuine and not the result of an exceptionally low result from the previous year. We also acknowledge the limitations of culture methodology for estimating the number of viable bacterial cells in water samples. It is known that stagnation and other environmental stresses can promote generation of viable but non-culturable (VBNC) *Legionella* cells (Li et al., 2014). So, it is likely that our study underestimated the actual bacterial load, especially in the 2020 sampling. Finally, it should be noted that the 2020 samplings were generally carried out in the middle of summer, while in 2019 they were carried out much earlier, mostly in winter. This may have at least affected the results in the cold water system (the average temperature of the cold water samples in 2020 was higher than in 2019). However, this fact does not seem to have had relevant effects since we have not observed significant increases in this system. Likewise, it is possible that this fact affected the pattern of water use in the hotels but in absence of precise data, e.g., occupancy rate, water consumption per person, it is difficult to assess this possible effect.

The results of this study are only applicable to hotels that operate seasonally, which abound in the tourist areas of the Mediterranean basin. It has been suggested that seasonal hotel operation, as opposed to hotels that operate all year around, favors the colonization by legionellae in hotel water systems (Mouchtouri et al., 2007). In this sense, seasonal hotels also could offer an interesting field model to investigate the effect of prolonged stagnation. However, in these cases the stagnation is not usually total since the facilities are subject to a certain degree of both water use and maintenance. On the other hand, the closing periods tend to occur in typically regular periods and generally shorter than those forced by the pandemic. In this case, all the hotels included in the study operated seasonally, but they suffered longer closures than the merely seasonal ones, which also included part of summer. Situations of prolonged closures in hotels can also occur, to a greater or lesser extent, in other cases, for example in periods of low occupancy or during closures for renovations or modifications, although again, in these cases, the stagnation is usually only partial or temporary.

In summary, this study suggests that prolonged closures of hotels longer than 3 months due to the COVID-19 pandemic caused an increased risk of exposure to *Legionella* in the hot water systems. However, the practical consequences of this increase in terms of actual *Legionella* infections, in the absence of other risk factors (higher *Legionella* concentrations, more positive establishments, or more prevalence of *Legionella pneumophila* sg1) may have been relatively modest. In any case, these findings highlight the importance of adequate preopening cleaning and disinfection procedures for hotel water systems after periods of closure and the convenience of considering the most effective methods after closures that are longer than usual, especially in hot water systems.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SC: conceptualization. JM and MB: data curation. JM, SC, and MB: investigation. JM and EP: data analysis. SC and EP: supervision. SC and JM: writing—original draft. SC, JM, and EP: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Protein sociology of ProA, Mip and other secreted virulence factors at the *Legionella pneumophila* surface

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The pathogenicity of *L. pneumophila*, the causative agent of Legionnaires' disease, depends on an arsenal of interacting proteins. Here we describe how surface-associated and secreted virulence factors of this pathogen interact with each other or target extra- and intracellular host proteins resulting in host cell manipulation and tissue colonization. Since progress of computational methods like AlphaFold, molecular dynamics simulation, and docking allows to predict, analyze and evaluate experimental proteomic and interactomic data, we describe how the combination of these approaches generated new insights into the multifaceted "protein sociology" of the zinc metalloprotease ProA and the peptidyl-prolyl *cis/trans* isomerase Mip (macrophage infectivity potentiator). Both virulence factors of *L. pneumophila* interact with numerous proteins including bacterial flagellin (FlaA) and host collagen, and play important roles in virulence regulation, host tissue degradation and immune evasion. The recent progress in protein-ligand analyses of virulence factors suggests that machine learning will also have a beneficial impact in early stages of drug discovery.

KEYWORDS

Legionella pneumophila, surface-associated proteins, secreted effectors, zinc metalloprotease ProA, macrophage infectivity potentiator, interactomics, computational biology

1 Introduction

Legionellosis emerged in the second half of the 20th century as a consequence of engineered warm-water habitats, which enable increased reproduction of *Legionella pneumophila* and efficient transmission of pathogen-containing aerosols to humans (McDade et al., 1979; Breiman, 1996; Albert-Weissenberger et al., 2007; Campbell and Cianciotto, 2022). Hot water facilities such as cooling towers, whirlpools, hot tubs, or showers are sources and technical vectors of infection, since natural freshwater reservoirs

usually contain lower bacterial densities (Jernigan et al., 1996; Keller et al., 1996; Atlas, 1999; Fields et al., 2002; Inoue et al., 2015; Molina et al., 2022).

L. pneumophila is a Gram-negative, obligate aerobic and rod-shaped bacterium from the ɣ-Proteobacteria class. In its natural habitat, replication occurs intracellularly within protozoa, especially amoebae of the genus *Acanthamoeba, Naegleria* and *Vermamoeba* (Rowbotham, 1980; Watanabe et al., 2016; Nisar et al., 2020; Price and Abu Kwaik, 2021). Due to improved diagnostics, as well as demographic and climatic changes, the number of cases of legionellosis continues to rise (Fischer et al., 2020; de Jong and Hallström, 2021; Portal et al., 2021; Barskey et al., 2022). Thus, *Legionella* became the most significant waterborne pathogen in terms of its spread and the severity of infection (Neil and Berkelman, 2008; Walker, 2018). Evoked respiratory diseases are differentiated between the self-limiting Pontiac fever with flu-like symptoms and the life-threatening Legionnaires' disease (Fraser et al., 1977; Glick et al., 1978).

Legionnaires' disease is a multisystemic form of legionellosis associated with atypical pneumonia. It is caused by bacterial proliferation predominantly in alveolar macrophages but also in lung epithelial cells, which results in necrotic damage to the pulmonary tissue (Horwitz and Silverstein, 1980; Blackmon et al., 1981; Mody et al., 1993; Cianciotto et al., 1995). Risk groups of Legionnaires' disease especially include elderly, males, smokers and patients with immunosuppressive diseases or treatments (Hoge and Breiman, 1991; Marston, 1994; Neil and Berkelman, 2008). The mortality rate varies considerably depending on risk factors, the source of infection and antibiotic therapy. About one out of ten patients dies from respiratory failure (Buchholz et al., 2010).

The pathogenicity of *L. pneumophila* for humans is considered to be a result of its co-evolution with protozoa pre-adapting the bacterium to phagocytes and likewise enabling its replication within human alveolar macrophages. The infection of these evolutionarily distant hosts shows strong similarities and is based on the pathogen's ability to inhibit phagolysosomal degradation (Horwitz, 1983; Bozue and Johnson, 1996; Gao et al., 1997; Segal and Shuman, 1999; Albert-Weissenberger et al., 2007). While other bacterial species undergo enzymatic digestion after acidification, the *Legionella*-containing vacuole (LCV) matures into an ER-like (endoplasmic reticulum) compartment with rather neutral pH values (Swanson and Isberg, 1995; Wieland et al., 2004). Although conservation of intracellular, eukaryotic signaling pathways enables the bacterium to multiply in our lungs, it is not adapted to humans as hosts, since further transmission from person to person does not take place (Abu Kwaik, 1996; Molofsky and Swanson, 2004).

L. pneumophila shows a biphasic life cycle switching between a replicative, intracellular and a transmissive, infectious state. In this context, expression of important virulence traits and flagellation are closely linked to the transmissive, stationary growth phase, when *L. pneumophila* requires access to new host cells (Swanson and Hammer, 2000; Molofsky and Swanson, 2004; Chauhan and Shames, 2021). For the infection of many different host species, *L. pneumophila* possesses an exceptionally versatile spectrum of virulence factors. Remarkably, the bacterium acquired numerous eukaryotic-like genes or domains *via* horizontal gene transfer from its protozoan hosts (Brenner et al., 1979; Cazalet et al., 2004; Mondino et al., 2020a; Hilbi and Buchrieser, 2022). Specific interactions in mammals, such as the manipulation of the NF-κB signaling pathway, further suggest that simple environmental metazoans also played a role in the evolution of *L. pneumophila*. These virulence factors are key elements for influencing host cell trafficking and immune evasion, and thus pre-adapt and enable the pathogen to evoke severe pneumonia in humans (Cazalet et al., 2004; Steinert et al., 2007; Lurie-Weinberger et al., 2010; Best and Abu Kwaik, 2018). In contrast to amoebal infections, the life cycle in higher organisms additionally requires extracellular pathogenicity for tissue dissemination and protection from the host response. This is primarily mediated by surface proteins and secreted effectors, which are of high importance during Legionnaires' disease.

In this review, we focus on secretory and surface-associated virulence proteins of *L. pneumophila* and their respective interactions with each other as well as with host structures. We describe how bacterial effector secretions reprogram host cells and determine the pathogenicity of Legionnaires' disease. The major secreted protease ProA and the membrane-associated peptidyl-prolyl *cis/trans* isomerase (PPIase) Mip of *L. pneumophila* will be especially highlighted, since they exhibit a multifaceted "protein sociology" with bacterial and host interaction partners. We discuss overlapping target structures of ProA and Mip like bacterial flagellin and the host ECM protein collagen and describe how the combination of X-ray structures, interactomic approaches and modern computational biology methods were used for simulation and exploration of biochemical processes at the pathogen-host interface. Moreover, we will briefly outline potentials related to the use of computational biology in drug discovery.

2 Surface-associated bacterial proteins target extra- and intracellular host proteins and contribute to different phases of infection

Proteins on the cell surface of *L. pneumophila* play a central role for pathogen-host interactions (Table 1). In the case of macrophages, recognition, adherence and complement-mediated phagocytosis of *L. pneumophila* takes place *via* the host cell receptors CR1 and CR3. The major outer membrane protein MOMP serves as the interacting acceptor molecule on the bacterial site (Horwitz and Silverstein, 1981; Payne and Horwitz, 1987; Bellinger-Kawahara and Horwitz, 1990). However, complement-independent attachment is likewise possible *via* type IV pili. Those are expressed by the pathogen in different lengths from 0.1 to 1.5 µm. Adherence to different host cells is only mediated by longer forms with at least 0.8 µm, for which a functional *L. pneumophila* pilin $pilE_L$ is necessary. They are also involved in the colonization of biofilms or human lung tissue, as well as the invasion of free-living amoebae (Stone and Abu Kwaik, 1998; Lucas et al., 2006). Gene expression and pili biogenesis are increased at 30°C compared to 37°C and correlate with the natural

TABLE 1 Surface-associated and secreted proteins of *L. pneumophila* and their extra- and intracellular host targets addressed in this review.

Factor	Protein type	Host target	Activity and function	Reference
Surface proteins				
FlaA	Flagellin	Targeted by TLR5, Naip5/Nlrc4	Immunogenic properties, bacterial motility and invasion	Dietrich et al., 2001
MOMP	Major outer membrane protein	CR1 and CR3	Cell entry, acceptor molecule for complement-mediated phagocytosis	Bellinger-Kawahara and Horwitz, 1990
Mip	Peptidyl-prolyl *cis/trans* isomerase	Collagen I-VI	Moonlighting activities, contributes to infection, transmigration, and colonization of nematodes	Karagöz et al., 2022
PilD	prepilin peptidase		Promotes pilus biogenesis, protein secretion and intracellular growth	Rossier and Cianciotto, 2001
$pilE_L$	Pilin		Associated with bacterial adherence, colonization, invasion	Stone and Abu Kwaik, 1998
PilY1	Type IV pili biogenesis protein		Promotes twitching motility, adherence, invasion and replication	Hoppe et al., 2017
TolC	Efflux pump		Stress resistance, important during early phase of host cell infection	Ferhat et al., 2009
Secreted proteins				
AnkB	F-box protein	E3 ubiquitin ligase, Parvin B	Promotes proteasomal degradation, essential for intracellular life cycle	Richards et al., 2013
ChiA	Endochitinase	MUC5AC, C1-INH	Mucinase activity, survival and transmigration in human lungs	Rehman et al., 2020
DotA	T4SS component		Effector secretion, promotes intracellular replication	Nagai and Kubori, 2011
LapA	M28 peptidase	Aminopeptides	Nutrient acquisition, promotes infection of *A. castellanii*	White et al., 2018
LipA	Lipase	Mono- and triacylglycerols	Lipolytic activity	Aragon et al., 2002
LpnE	SLR protein	OBSL1	Cell entry, trafficking of the LCV	Newton et al., 2007
NttA	T2SS substrate	Phosphoinositides like PtdIns $(3,5)P_2$	Association with the LCV, replication in *A. castellanii*	Portlock et al., 2020
PlaA	GDSL lipase/ acyltransferase	Cholesterol, ergosterol, lysophospholipids	Major LPLA activity, transfers propionic acid, promotes LCV destabilization for bacterial exit	Lang et al., 2017
PlaC	GDSL lipase/ acyltransferase	Cholesterol, ergosterol, diacylphospholipids	Major PLA and GCAT activity, transfers palmitic acid, promotes growth within amoebae	Lang et al., 2012
PlcA	Phospholipase C		Catalyses pNPPC hydrolysis	Aragon et al., 2002
ProA	M4 zinc metalloprotease	Various, e.g., TNF-α, collagen IV, IL-2, IL-6, CD4	Hemolytic and cytotoxic, tissue degradation, intracellular growth, transmigration and immune evasion	Scheithauer et al., 2021
SdhA	T4SS substrate	OCRL protein	Promotes LCV integrity, evasion of death pathways and intracellular growth in macrophages	Choi et al., 2021
SrnA	RNase		Replication in *V. vermiformis* and *N. lovaniensis*	Tyson et al., 2013

competence of *L. pneumophila*, hence promoting genomic recombination with transformed DNA. Therefore, these pili are referred to as CAP for competence and adherence associated pili (Liles et al., 1998; Stone and Kwaik, 1999). Studies have shown that the membrane-localized protein PilY1, which is involved in type IV pili biogenesis, accordingly represents a virulence factor affecting twitching motility, adherence and invasion but also replication of the pathogen (Hoppe et al., 2017).

Even though alveolar macrophages feature the main reservoir of *L. pneumophila* in human lungs, the pathogen is also able to infect pulmonary epithelial cells, which makes invasion strategies more important. The surface protein and chaperone Hsp60 of the GroEL family is able to promote the entry into those non-phagocytic host cells and, moreover, induces the establishment of the LCV. It is especially synthesized in the early phase of infection and secreted into the phagosome, leading to the recruitment of mitochondria and increased cytokine expression in macrophages (Fernandez et al., 1996; Garduño et al., 1998; Chong et al., 2009; Zhan et al., 2015). Factors which play a role in cell entry and manipulation of the intracellular transport often contain the predominantly eukaryotic SLR motif (Sel1 repeats) for protein-protein interactions (Newton et al., 2007). Examples are LpnE and the periplasmic EnhC, which additionally influences survival under H_2O_2 stress, cell integrity and immune evasion (Liu et al., 2008; Liu et al., 2012).

As a Gram-negative bacterium, *L. pneumophila* has the ability to release effectors in so-called extracellular vesicles (EVs), which contain a variety of immunomodulatory proteins and display surface-associated proteins and lipopolysaccharides (LPS) on the outside. They are used for communication between bacteria, but can also fuse with host cells and transmit virulence factors including RNA (Sahr et al., 2022). In this way, EVs cause lung tissue damage and provide replication advantages for the pathogen (Jäger et al., 2015). Various effectors such as the chitinase ChiA, the macrophage infectivity potentiator Mip, the zinc metalloprotease ProA and LPS can be components of EVs (Galka et al., 2008). The LPS of *L. pneumophila* differs significantly from that of other bacteria due to its composition and low endotoxic potential. The membrane-bound lipid A exhibits particularly long-chain and branched fatty acids. Therefore, LPS can act as an adhesion factor that anchors the bacterium in the host cell membrane (Zähringer et al., 1995; Neumeister et al., 1998). In addition, it allows evasion of the pathogen from lysosomal digestion (Seeger et al., 2010). Depending on the phase of infection, *L. pneumophila* is able to express two different forms of LPS. A more hydrophilic variant facilitates the close spatial proximity of *Legionella* in the phagosome, while a hydrophobic form facilitates adhesion to host cell membranes, as well as survival and transmission of the bacterium in aerosols (Reichardt et al., 2010; Palusinska-Szysz et al., 2019). Although Mip is also an EV-associated surface protein, it does not play a role in bacterial adherence to host cells, but rather confers resistance of the pathogen to intracellular digestion by the host (Cianciotto et al., 1990; Cianciotto and Fields, 1992). The FK506-binding protein is of decisive importance for the proliferation rate of *L. pneumophila* in protozoa and macrophages. Its particular role for Legionnaires' disease will be discussed in a later chapter of this review.

For motility in the transmissive phase, *L. pneumophila* possesses a single monopolar flagellum on its cell surface, which also plays a crucial role in the establishment of infection. Over 50 different genes are involved in its functional expression assembling a membrane-spanning basal body, a hook and an extracellular filament (Chilcott and Hughes, 2000). On the cytoplasmic side, the motor complex MotAB generates the required rotational energy *via* proton motive force. Extracellular components are secreted by a type III-like export apparatus. The filament itself is formed by polymerization of the 48 kDa subunit FlaA, which is, assisted by FliD, gradually incorporated at the tip (Altegoer et al., 2014; Appelt and Heuner, 2017).

Transcription of the flagellin gene *flaA* is temperature-dependently regulated by the alternative sigma factor σ^{28} FliA (Heuner et al., 1995). Thus, FlaA expression and motility are primarily determined by the growth phase, but also by environmental factors such as the availability of amino acids and the viscosity or osmolarity of the medium (Heuner et al., 1999). FliA itself is part of the post-exponential regulatory cascade triggered by the alarmon ppGpp in response to nutrient limitation. At this point of the *L. pneumophila* life cycle, the flagellum facilitates access to new host cells and increases the rate of invasion. Therefore, proliferation of a *flaA* deletion mutant is attenuated in amoebae and human cell lines (Dietrich et al., 2001; Teruya et al., 2007). However, free, unassembled flagellin induces the innate immunity of alveolar macrophages as it is recognized by various host receptors such as Toll-like receptor 5 (TLR5) on the cell surface (Palusinska-Szysz and Janczarek, 2010). TLR5 is of great importance regarding the bacterial clearance or susceptibility towards Legionnaires' disease and regulates, for example, the recruitment of polymorphonuclear neutrophils (PMNs) into the alveolar space (Hawn et al., 2007). After stimulation of the receptor TLR5, the adapter molecule Myd88 (myeloid differentiation primary response 88) is induced first. The signal is further transmitted *via* the IκB kinase IKK and the mitogen-activated protein kinase p38 MAPK (Yu et al., 2003). As a result of phosphorylation, the inhibitor IκB is degraded and its target NF-κB is released. The activation of the transcription factor NF-κB finally leads to the expression or secretion of immunoregulatory factors such as tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8) and IL-6, as well as other chemokines. A nonsense mutation in the TLR5 gene, therefore, significantly increases susceptibility to *L. pneumophila* infections (Hawn et al., 2003; Hawn et al., 2007). Furthermore, it was shown that flagellin monomers are occasionally translocated into the host cell *via* the bacterial Dot/Icm type IV secretion system (T4SS) (Fontana and Vance, 2011). On the cytosol side, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) such as neuronal apoptosis inhibitory protein 5 (Naip5) and the NLR family CARD domain-containing protein 4 (Nlrc4, formerly: Ipaf) are stimulated in a flagellin-dependent manner. These in turn can prevent the proliferation of *Legionella* in murine macrophages by activating caspase-1 and, moreover, contribute to the maturation of inflammatory cytokines such as IL-1β and IL-18. Naip5 induces early apoptosis, whereas binding of Nlrc4 leads to lysosomal digestion of the bacteria (Amer et al., 2006; Ren et al., 2006; Lamkanfi et al., 2007). Akhter et al. showed that the activation of caspase-7 plays a crucial role in these processes as well (Akhter et al., 2009). Both NLRs together form the Naip5/Nlrc4 inflammasome, which ultimately leads to pyroptosis, a rare form of cell death associated with the initiation of a severe inflammatory response (Kay et al., 2020; Chauhan and Shames, 2021). The proliferation of *L. pneumophila* is effectively limited by intact NLRs in mice making them resistant against Legionnaires' disease. The human orthologues also mediate immune defense against flagellin-expressing bacteria, however, the host cells can still be infected and severe courses of the disease occur (Vinzing et al., 2008).

3 Type I, II and IV effector secretion orchestrates host cell manipulation and increases the outreach of protein interactions in tissue

Selection for survival in the environment and intracellular replication in diverse protists has resulted in the accumulation of a broad arsenal of effectors by *Legionella* (Table 1). Thus, besides an interaction of the pathogen with host cells *via* membrane-bound surface proteins, *L. pneumophila* is most notably able to secrete a

versatile repertoire of virulence factors to the extracellular space or directly into the host cell cytosol (White et al., 2019; Costa et al., 2021). Accordingly, the bacterium features different secretion systems that are fundamental to cellular and tissue pathogenesis.

The type I secretion system (T1SS), encoded by the lssXYZABD locus, plays also an important role regarding the host cell entry and is involved in sliding motility of the pathogen (Jacobi and Heuner, 2003; Fuche et al., 2015). One of its central components represents the membrane protein TolC, which acts as an efflux pump contributing to the early phase of invasion and intracellular replication (Ferhat et al., 2009). Even though it is not able to directly inject effectors into the host cell cytoplasm, the T1SS plays a major role for the intracellular life cycle in various amoeba species, alveolar macrophages as well as lung epithelial cells. It contributes to the persistence of the pathogen in lung tissue and attenuates the host's chemokine and cytokine response (McCoy-Simandle et al., 2011; Cianciotto et al., 2013; Mallama et al., 2017). Additionally, it regulates growth of *L. pneumophila* at low temperatures as well as biofilm formation (Söderberg et al., 2004; White and Cianciotto, 2019).

Like the T1SS, the type II secretion system (T2SS) lsp (*Legionella* secretion pathway) is involved in the secretion of surfactants for sliding motility. These surfactants additionally exhibit antibacterial properties and create a selective advantage for *L. pneumophila* over other *Legionella* species (Stewart et al., 2011). The T2SS is built up from the membrane proteins LspD in the inner membrane, LspF in the outer membrane, and the ATPase LspE. It is also dependent on the protein PilD, which is essential not only for the biogenesis of type IV pili but for the processing of the pseudopilins LspG-K (Liles et al., 1998; Rossier and Cianciotto, 2001). Studies have shown that some T2SS substrates first utilize the twin-arginine translocation (Tat) system of the inner membrane for export to the periplasm and additionally require the lsp system for final secretion (Rossier and Cianciotto, 2005). The T2SS translocates over 25 effector proteins such as the M4 zinc metalloprotease ProA, phospholipases A and C, RNAses, and NttA-E, which are essential for optimal infection of different amoeba species (Rossier et al., 2009; Tyson et al., 2013; Portlock et al., 2020). In this context, the protease ProA was the first discovered substrate of a T2SS which is involved in the intracellular infection of a bacterial pathogen (Hales and Shuman, 1999; Rossier et al., 2004). The tissue destructive protease not only promotes replication in several amoebal hosts but also determines extracellular pathogenicity during Legionnaires' disease (Moffat et al., 1994a; Edelstein et al., 1999; Tyson et al., 2013).

The most important secretion system, which is essential for the whole life cycle of *L. pneumophila*, is the Dot/Icm type IVB secretion system (T4SS) (Lockwood et al., 2022). Translocated effector proteins control the bacterial uptake and egress, disrupt the phagolysosome fusion, and manipulate many critical processes in the eukaryotic cell, such as vesicle transport, signal transduction, gene expression and protein translation, ubiquitination, cytoskeleton dynamics, autophagy, apoptosis pathways, and host defense (Berger and Isberg, 1993; Segal et al., 2005; Michard and Doublet, 2015; Hilbi et al., 2017; Allgood and Neunuebel, 2018; Schuhmacher et al., 2018; Yu et al., 2018; Gomez-Valero et al., 2019; Kitao et al., 2020). Interestingly, T4SS effectors have recently been shown to reverse the activity of mitochondrial FoF_1-ATPase to ATP-hydrolysis, which even enables the pathogen to precisely time host cell death (Escoll et al., 2021; Kubori et al., 2022). Moreover, bacterial effectors not only mediate post-translational modifications of eukaryotic proteins but also are able to enter the host cell nucleus for a direct upregulation of transcription (Schuelein et al., 2018).

With more than 300 substrates the Dot/Icm type IVB secretion system secretes the largest repertoire of virulence-associated effectors of any pathogen species known to date (Al-Quadan et al., 2012; Lockwood et al., 2022). They enable the bacterium to infect many hosts from different phyla and with evolutionary distance (Boamah et al., 2017; Graells et al., 2018; Moreno et al., 2019). In fact, over 18 000 putative Dot/Icm substrates have been identified across the genus *Legionella*, which is known for the additional expression of so-called metaeffectors. This type of effectors represents a rather unique level of regulation since it is able to target other bacterial effectors and control their activity *via* different modes of action such as degradation or activation (Kubori et al., 2010; Gomez-Valero et al., 2019; Mondino et al., 2020b; Joseph and Shames, 2021). A large number of T4SS substrates also exhibits functional redundancy, which effectively protects the pathogen from a defect in intracellular replication (Ninio and Roy, 2007; Isberg et al., 2009). Substrate recognition and secretion occurs *via* the membrane-bound T4 coupling complex (T4CC). This is assembled from a DotLMNYZ core with DotL as the ATPase and the chaperones IcmSW (Meir et al., 2020). DotA of the inner membrane is essential for effector secretion and in turn for the intracellular proliferation of *L. pneumophila*. Its own secretion *via* the T4SS and the high recombination rate suggest direct interaction with the host. However, the exact function of DotA has not yet been clarified (Nagai and Kubori, 2011; Gomez-Valero and Buchrieser, 2019).

4 Major secretory protein ProA

4.1 ProA promotes infection of certain cell types and contributes to lung tissue destruction

ProA was first described in 1981 by Thompson et al. analyzing the *L. pneumophila* serogroups 1-6 (Thompson et al., 1981). Continuative studies regarding the distribution of different T2SS effectors in the genus *Legionella* revealed that ProA occurs as one of only two core substrates in all 57 species tested (White and Cianciotto, 2019). On the whole, *L. pneumophila* codes for various proteases, some of them annotated as homologues of other pathogens suggesting important virulence-associated functions. According to the MEROPS peptidase database, a total of 188 confirmed or putative proteases are listed for the bacterium, 57 of them are classified as zinc metalloproteases (www.ebi.ac.uk/merops/). In this context, ProA is the most important and best-characterized representative in *L. pneumophila*. The M4 enzyme was originally referred to as the major secretory protein (Msp), since it is the most abundant protein in the supernatant of *Legionella* cultures (Blander et al., 1990).

ProA does not appear to hold a universal function during intracellular proliferation of *L. pneumophila*, comparing different environmental hosts and infected cell types. Infection studies with amoeba strains of natural aquatic habitats revealed importance of ProA for optimal proliferation in *Vermamoeba vermiformis* and *Naegleria lovaniensis*, with a variety of tasks in *N. lovaniensis* and activation of bacterial PlaC as the key feature in *V. vermiformis*. In terms of *Acanthamoeba castellanii*, experiments from 2013 did not reveal any biological relevance for infection. Regarding the initiation phase, this was also attested in recent experiments from 2019 (Rossier et al., 2008; Tyson et al., 2013; White et al., 2019). Nevertheless, as replication progresses, a *proA*-deficient mutant showed reduced growth rates with lower cell counts compared to the *Legionella* wild type. Due to its extracellular enzymatic activity, ProA might promote, together with other T2SS effectors, the accessibility of amino acids, especially important in later stages of infection (Rossier et al., 2008; White et al., 2019).

In addition to the life cycle in natural environments, early experiments in guinea pigs also pointed towards importance of ProA as a virulence factor for Legionnaires' disease. Thus, intranasal inoculation with purified protease caused inflammation and lung lesions with alveolar hemorrhage, edema, tissue necrosis, and infiltration of PMNs and macrophages. These histomorphological changes closely resembled findings of a real *L. pneumophila* infection and were associated with the localization of ProA (Baskerville et al., 1986; Conlan et al., 1986; Williams et al., 1987). Moreover, increased humoral and cellular immunity of guinea pigs could be demonstrated after subcutaneous administration of the protease (Blander and Horwitz, 1989; Blander et al., 1990). Infections with a *proA*-negative mutant, however, led to contradictory results in animal models. Early studies in this context have not been able to display virulence-associated functions of the protease (Blander et al., 1990). Later, influence of ProA on bacterial replication, the course of disease, and necrosis of pulmonary tissue was described in guinea pigs (Moffat et al., 1994a; Edelstein et al., 1999). Forty years after discovery, the role of ProA was specified recently in human lung tissue explants (HLTEs) using purified protease as well as *proA* mutant strains. This detailed elucidation evidenced protease-dependent tissue destruction with swelling and disintegration of alveolar septa as well as increased bacterial proliferation, transmigration, and immune evasion of ProA-expressing *Legionella* strains in the physiological human background (Figure 1) (Scheithauer et al., 2021; Scheithauer et al., 2022).

Regarding monocellular systems, ProA could be detected intracellularly in amoebae and alveolar macrophages of guinea pigs or humans 24 h post infection (Rechnitzer and Kharazmi, 1992; Moffat et al., 1994a). Interestingly, the zinc metalloprotease was shown to be translocated into the host cell cytoplasm and, after 6 h, accumulates on the surface of the LCV. In this context, the first evidence for a T4SS-independent cytosol transport was found indicating semi-permeability of the LCV membrane (Truchan et al., 2017). Although ProA is produced during intracellular replication in multicellular hosts, no relevance to bacterial growth or host cell death could initially be demonstrated in macrophages (Szeto and Shuman, 1990; Moffat et al., 1994a; Rossier et al., 2008). However, subsequent analyzes by Edelstein et al. indicated diminished proliferation of the *proA*-negative *L. pneumophila* mutant in macrophages as well as lungs of guinea pigs (Edelstein et al., 1999). Analogously, slightly reduced bacterial replication was revealed in human cell lines, especially in A549 lung epithelial cells (Scheithauer et al., 2021). Attenuation after *proA* deletion can therefore be partly attributed to a restricted intracellular proliferation. However, epithelial cells, though susceptible, do not represent the preferred reservoir of *Legionella* in human lungs (Jäger et al., 2014). Phenotypes and histological observations regarding to ProA are therefore assumed to mainly result from extracellular activity of the secreted protease, for example, against structural host tissue factors.

The zinc metalloprotease ProA possesses an extraordinarily broad and diverse spectrum of substrates, and displays hemolytic as well as cytotoxic activity (Figure 1) (Conlan et al., 1986; Keen and Hoffman, 1989; Quinn and Tompkins, 1989). Despite highest sequence homology to the elastase pseudolysin from *Pseudomonas aeruginosa* ProA is not able to cleave human elastin fibers. Nevertheless, structural proteins such as casein, gelatin and collagen are among the known substrates (Thompson et al., 1981). Many M4 proteases function as key factors for pathogenesis, since they degrade targets with important roles during infection. The versatile spectrum of ProA substrates comprises human host proteins as well as bacterial effectors (Figure 1). By proteolysis of structural and immunomodulatory compounds, ProA provokes tissue degradation and bacterial evasion, especially important in infection of higher organisms. Furthermore, it can influence various processes of the cellular life cycle by regulating other virulence factors of *L. pneumophila*. Truchan et al. revealed that ProA associates in a ring-like structure along the outer leaflet of the LCV membrane enabling direct interaction with proteins of the host cell cytoplasm (Truchan et al., 2017). Presumably, ProA anchors itself *via* a putative farnesylation domain at the C-terminus and cleaves host factors to influence downstream signaling pathways. It is also conceivable that ProA contributes to the acquisition of nutrients for the proliferation of *L. pneumophila*, thus, supporting known mechanisms of the virulence factor AnkB, which exploits the ubiquitination machinery of the host cell (Richards et al., 2013; Truchan et al., 2017; White et al., 2018).

4.2 ProA targets host immunity and degrades human collagen for tissue transmigration

Substrates of the zinc metalloprotease ProA comprise various host proteins, which enable the enzyme to intervene in different areas of pathogenesis (Figure 1). Outside its protective intracellular replication niche, *L. pneumophila* needs to fight the host immune system. Accordingly, extracellular proteases like ProA can act as a first line of defense against humoral or cellular immune factors to inhibit both innate and acquired defense mechanisms. Early studies in 1980 demonstrated a direct cleavage of serum proteins by the bacterial metalloprotease (Müller, 1980). ProA might represent, together with LPS and ChiA, one of the pivotal factors to promote

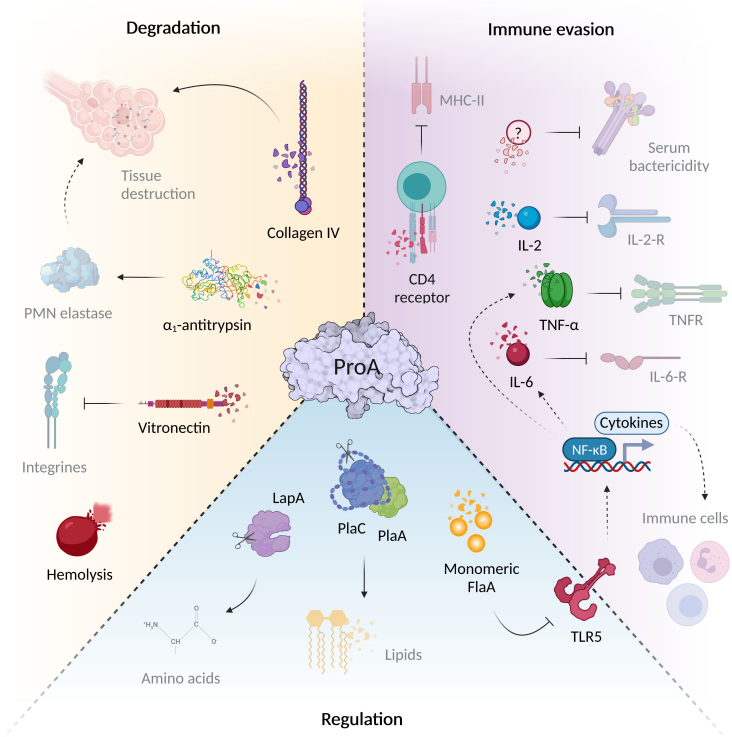

FIGURE 1
ProA mediates virulence regulation, host tissue degradation and immune evasion. The virulence factor ProA can interfere with a wide variety of infection-associated processes *via* the diverse range of substrates. During Legionnaires' disease, it significantly mediates tissue degradation due to structural host targets such as collagen IV of the basal lamina and the cell adhesion protein vitronectin. ProA also cleaves α_1-antitrypsin, important for controlling of host proteases. In addition, ProA exhibits hemolytic properties and contributes significantly to immune evasion. The zinc metalloprotease not only cleaves humoral serum components and chemokines (IL-2, IL-6, TNF-α), but also influences the recruitment and proliferation of various immune cells *via* these signaling molecules of specific surface receptors. However, ProA also attacks cellular structures directly, for example, by degrading the CD4 receptor. Aside from host targets, the protease also affects other bacterial proteins that play an important role during the intracellular life cycle of *L. pneumophila*. *Via* cleavage of exogenous flagellin, the TLR5-mediated immune response and thus the cytokine expression are reduced. Through direct processing, ProA can additionally activate virulence factors such as PlaA/PlaC and LapA, which contribute to lipid degradation at the LCV and amino acid synthesis (created with BioRender.com).

serum resistance of the pathogen and its protection from complement-mediated lysis (Khan et al., 2013; Rehman et al., 2020; Scheithauer et al., 2021). With regard to the bactericidal effect, factors of the complement system function as important serum components. They not only mediate direct lysis, but also contribute to the removal of the pathogen by phagocytic immune cells *via* opsonization. Among different *L. pneumophila* strains, high resistance to complement factors is a characteristic trait of virulent in contrast to non-virulent strains (Plouffe et al., 1985; Caparon and Johnson, 1988). Purified ProA is able to degrade, for example, the human serum protein and important inhibitor α_1-antitrypsin, which controls activity of host proteases in pulmonary tissue (Conlan et al., 1988). It is therefore conceivable that *L. pneumophila* pursues a strategy similar to *P. aeruginosa* causing substantial destruction of lung tissue by endogenous serine proteases *via* M4 enzyme activity (Morihara et al., 1979). Another humoral *in vitro* substrate of ProA and well-known pro-inflammatory cytokine is TNF-α, which limits intrapulmonary replication of the pathogen in the early phase of infection (Hell et al., 1993; McCoy-Simandle et al., 2011). It is mainly released by macrophages and important for the PMN-mediated controlling of legionellosis (Blanchard et al., 1988). *L. pneumophila* mutant strains lacking the ProA-secreting T2SS showed significantly enhanced levels of TNF-α in infected U937 cells or mice lungs (McCoy-Simandle et al., 2011). Regarding the cellular immune response, ProA was shown to be able to impair the chemotaxis of PMNs and monocytes in a concentration-dependent manner, to impede the production of reactive oxygen species, and thus bacterial elimination *via* an oxidative burst (Rechnitzer and Kharazmi, 1992; Sahney et al., 2001). Additionally, activation and proliferation of T-cells are both inhibited by direct cleavage of the CD4 receptor and interleukin-2 during assays with purified protease (Mintz et al., 1993). Moreover, a ProA-dependent reduction of the B-cell differentiation factor IL-6 was also observed in cell culture infections (McCoy-Simandle et al., 2011). Even though *in vivo* studies on this topic are rare, ProA was found active against multiple important immune regulators.

Considering these effects on humoral and cellular defense mechanisms, the *L. pneumophila* zinc metalloprotease seems to be especially important during the pathogenesis of Legionnaires' disease and may represent one of the virulence factors that enable the pathogen to infect multicellular organisms. This also holds true

for the interaction of *L. pneumophila* with structural components of the tissue at the site of infection, since ProA contributes to bacterial proliferation and dissemination in human lungs as well as formation and progression of pulmonary damage causing the severe pneumonia-related symptoms in patients. Evaluation of ProA effects in HLTEs as well as guinea pig models revealed significant tissue destruction with inflammation and immune cell infiltration comparable to the histopathology of Legionnaires' disease (Baskerville et al., 1986; Scheithauer et al., 2021). Occurring lesions demonstrated a correlation between the extent of tissue damage and the protease concentration used (Williams et al., 1987). A prominent histomorphological feature was the significant swelling of alveolar septa due to ProA treatment. These observations can be attributed to structural changes and decomposition of connective tissue fibers at the basal lamina resulting in edema formation (Baskerville et al., 1986; Scheithauer et al., 2021). Early histopathological studies of deceased patients already described an edema-related widening of alveolar septa as a characteristic feature associated with Legionnaires' disease (Hernandez et al., 1980). Thus, quantification of the alveolar septal thickness represents a suitable indicator for inflammatory processes and the extent of tissue damage in the HLTE model system. As a barrier between individual alveoli during infection, destruction of these septa represents a central step in the development of fibrosis and progression of the disease (Ohta et al., 2012). Accordingly, transmigration and growth studies in treated HLTEs showed reduced spread and proliferation of a *proA* deletion mutant with less invasive bacteria in deeper tissue layers compared to the *L. pneumophila* wild type (Scheithauer et al., 2021). These effects are also in line with earlier observations in guinea pig models (Moffat et al., 1994a).

Altered and finally dissolved alveolar septa are predominantly made up of collagen and elastin fibers. In contrast to the M4 homologue pseudolysin, ProA exhibits no elastase activity (Thompson et al., 1981; Conlan et al., 1986). However, early studies indicated possible collagen degradation by the zinc metalloprotease in azocollagen assays and histological examinations of guinea pig lungs following intranasal administration of ProA (Baskerville et al., 1986; Conlan et al., 1986). Recently, direct proteolysis of human collagen IV, the predominant collagen type of interalveolar septa, was verified *in vitro*. In these assays, highly assembled complexes were rapidly degraded. Likewise, collagen IV antibody staining and quantification of the septal width revealed modification and disintegration of the collagen backbone in HLTEs (Scheithauer et al., 2021). Moreover, activity against human vitronectin, which mediates primarily cell adhesion in pulmonary tissue, was also demonstrated *via in vitro* degradation assays (Hayman et al., 1985; Schvartz et al., 1999; Scheithauer, 2022). Thus, ProA promotes dissemination of *L. pneumophila* by cleavage of different structural host targets, especially disrupting the collagen IV-assembled interalveolar barrier in human lungs. The protease is therefore required in later stages of the intracellular life cycle and the infection.

4.3 Sequence homology and X-ray structure analysis classify ProA into the family of M4 zinc metalloproteases with broad substrate specificity

During the stationary phase, expression of ProA is induced *via* the alternative sigma factor RpoS and the response regulator LqsR (Tiaden et al., 2007). The open reading frame (ORF) of ProA comprises a 1629 bp gene sequence, which codes for a translational product with 534 amino acids and 60.8 kDa (Black et al., 1990). The strikingly conserved spatial proximity to the lipA gene of a monoacylglycerol lipase might indicate a common regulation or functional coordination of both gene products (White and Cianciotto, 2019). The inactive ProA precursor is first transported across the inner membrane in a Sec-dependent manner and then further secreted by the T2SS (Hales and Shuman, 1999). In addition to the peptidase unit, the preproenzyme is composed of a 24 amino acids long N-terminal signal peptide and the propeptide with 183 amino acids, making up about a third of the total length (Figure 2A). After export, both are cleaved off autocatalytically. The resulting 37.8 kDa mature protease encompasses the C-terminal region of the translational product beginning with a glutamate at position 208 (Moffat et al., 1994b). Its isoelectric point (pI) ranges between 4.20 and 4.42 and the optimum of enzyme activity is dependent on neutral pH values between 5.5 and 7.5. At temperatures of at least 40°C a significant loss of activity occurs, whereas 60°C or more lead to a complete denaturation of the enzyme. Since ProA possesses a central catalytic zinc cofactor, the proteolytic activity is effectively reduced by complexing agents such as EDTA (ethylenediaminetetraacetic acid) or EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid). However, this effect can be reconstituted by the addition of free zinc ions. Interestingly, enzyme activity can also be partly restored using other divalent cations such as Fe^{2+}, Cu^{2+} or Mn^{2+} (Thompson et al., 1981; Dreyfus and Iglewski, 1986). In this context, it is noticeable that ProA is expressed less when cultivated in chemically defined minimal medium with limited zinc source compared to complex media (Szeto and Shuman, 1990).

In many infectious bacteria, M4 proteases such as ProA are of particular relevance during pathogenesis. The best-known representative of this enzyme family is thermolysin from *Bacillus thermoproteolyticus*, which is the reason why these endopeptidases are also known as thermolysin-like proteases (TLPs). Characteristically, translation of TLPs results in much longer preproenzymes, which also comprise a signal and a propeptide in addition to the actual peptidase unit. As a chaperone, the latter supports correct folding and, due to its inhibitory function, prevents destructive activity in the bacterial cytoplasm. Autocatalytic processing to the active form only occurs after export to the periplasm. However, the cleaved fragment remains associated with the mature protease until secretion is completed (Yeats et al., 2004; Gao et al., 2010). Due to their extracellular site of action, TLPs are usually involved in the breakdown of foreign proteins and thus contribute to the generation of important nutrients, the bacterial

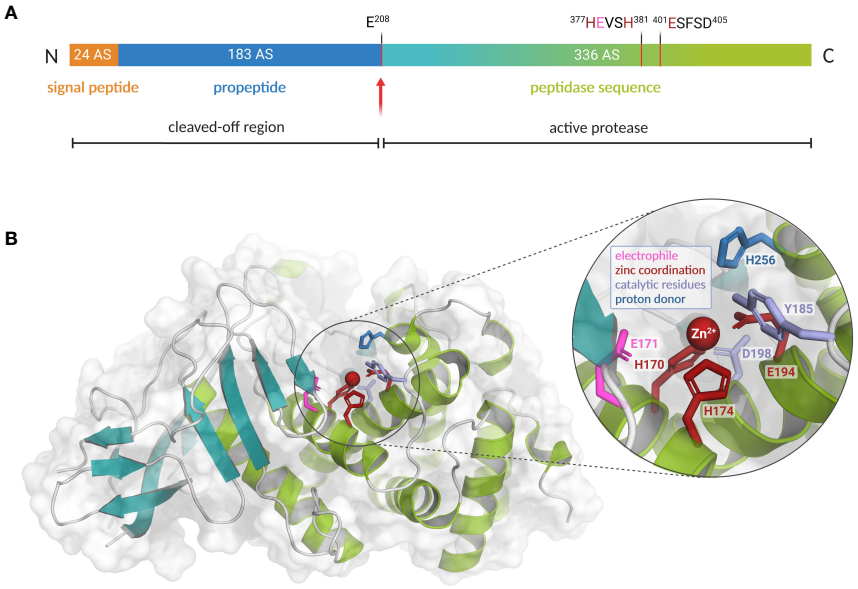

FIGURE 2
Preproenzyme composition, structure and catalytic center of *L. pneumophila* M4 zinc metalloprotease ProA. **(A)** Schematic overview of the ProA preproenzyme containing a signal- and a propeptide (60 kDa, 543 amino acids). Secretion and autoprocessing at residue Glu 208 results in a 38 kDa mature peptidase unit (teal and green). Important residues for cofactor coordination (red) and proteolytic activity (pink) are highlighted within the zinc-binding motifs. Especially the HExxH motif is highly conserved among the M4 family of TLPs. **(B)** Two domain structure of ProA (PDB code: 6YA1) with N-terminal β-sheets (teal) and C-terminal α-helices (green). A close-up of the active center displays the zinc cofactor as well as amino acid residues for coordination and catalysis (created with PyMOL 2.0 and BioRender.com).

spread in multicellular organisms and the defense against host immune factors (Miyoshi and Shinoda, 2000). All M4 proteases exhibit a rather broad range of substrates and hydrolyze peptide bonds zinc-dependently. Even though sequence identity varies significantly between different members, there is a high level of structural identity, particularly regarding the conserved amino acid residues for hydrolysis and zinc coordination. As part of the active site, the cofactor is located in the interdomain region between N-terminal β-sheets and C-terminal α-helices. It is coordinated by two histidines of the HExxH motif, a glutamate residue of a second zinc-binding motif, and a water molecule (Jongeneel et al., 1989; Jiang and Bond, 1992; Hooper, 1994; Adekoya and Sylte, 2009). These considerable structural homologies imply functional comparability of ProA and other important virulence factors of the TLPs, which also occupy key positions in pathogenesis. Within the M4 enzyme family, ProA shares highest sequence homology of 62.9% with the lasB-encoded elastase pseudolysin from *P. aeruginosa*. Both enzymes show distinct similarities on structural as well as functional level (McIntyre et al., 1991; Poras et al., 2012; Scheithauer et al., 2021). Moreover, efficacy of several competitive inhibitors, such as phosphoramidon, suggests strong conservation regarding substrate binding and conversion (Black et al., 1990). Based on a co-crystallization of pseudolysin and the inhibitor HPI (N-(1-carboxy-3-phenylpropyl)-phenylalanyl-α-asparagine), the structure of ProA was modeled for the first time in 2012. In this context, a highly specific fluorescent substrate was generated for ProA to facilitate the detection of *Legionella* (Poras et al., 2012). Its genomic distribution and immunogenic potential makes the extracellular protease constantly interesting for detection and quantification of the pathogen, and the development of vaccines (Blander and Horwitz, 1989; Poras et al., 2012).

To overcome deficiencies of the elastase-based model, the ProA crystal structure was finally resolved at 1.48 Å by X-ray crystallography in 2020 (Figure 2B). Proteolytic and cytotoxic activity was found to be mediated by the glutamate residue Glu 171 of the mature enzyme; experimental studies have already shown that it is essential for substrate conversion and autoprocessing of ProA (Moffat et al., 1994b; Scheithauer et al., 2021). Due to a highly conserved active centre among M4 proteases, with superimposable amino acid residues for zinc binding and catalysis, similar targets are plausible. Nevertheless, significant discrepancies, such as lacking elastase activity of ProA in contrast to pseudolysin, must arise from important differences in peripheral structural elements (Thompson et al., 1981). These variable regions might be involved in substrate recognition or binding. In this context, three flexible, unique loops were identified by comparing ProA to other TLPs. Furthermore, other representatives often possess different numbers or types of cofactors in addition to the common central zinc ion. Calcium is widely distributed in various TLPs and occurs, for example, in pseudolysin and vibriolysin. In these enzymes, it is important for production and processing as well as thermostability (Olson and Ohman, 1992; Eijsink et al., 2011). However, in terms of ProA no additional cofactor could be identified in the native crystal structure apart from the catalytic zinc. According to this, only one zinc ion per protease molecule was detected in atomic absorption spectroscopy (AAS) by Dreyfus and Iglewski in 1986 (Dreyfus and Iglewski, 1986; Scheithauer et al., 2021). Interestingly, de Kreij et al. assumed that differences in the substrate spectrum of

M4 proteases are not determined by general sequence or structural differences but are due to a small number of specific amino acids in substrate-binding motifs (de Kreij et al., 2000). The hydrophobic substrate binding pocket S_1' is the most important recognition site concerning substrate determination. Site-directed mutagenesis in this region can therefore lead to fundamental changes in the catalytic properties of the proteases. Characteristics of the TLP from *Bacillus stearothermophilus* were already demonstrated to be successfully converted into those of thermolysin by exchanging a single amino acid residue (de Kreij et al., 2000; de Kreij et al., 2001). In pseudolysin, five amino acids from this substrate binding site S_1' are also postulated to determine the recognition of the structural human protein elastin (Yang et al., 2015). Four of them can be found at equivalent positions in ProA. Only Phe 129 from pseudolysin is replaced by Met 159 in ProA. If this specific sequence difference is a crucial reason for the lacking elastase activity of ProA or if other factors appear to be more importantly involved in the binding and proteolysis of elastin, remains to be investigated. Nevertheless, structural analyzes showed that Met 159, together with three additional amino acid residues, also narrows the substrate binding pocket of ProA characteristically. Presumably, the zinc metalloprotease is therefore able to expand its binding pocket in the flexible interdomain region through a conformational change upon substrate recognition (Scheithauer et al., 2021). The spatial restriction, however, might hinder binding of stiff and bulky substrates like connective fibers. In contrast to the solid triple helices known from most collagen types, collagen IV forms highly flexible, thin and sheet-like structures at the basal lamina of interalveolar septa. Interestingly, its subunit furthermore exhibits a characteristic curvature 30 nm from the N-terminus, which corresponds perfectly to the obstructive narrowing of ProA's substrate binding pocket. While the catalytic centre of the protease is less accessible for straight and rigid proteins, this might reflect an adaptation to collagen type IV as a physiologically occurring target (Hofmann et al., 1984; Martin et al., 1985; Ricard-Blum, 2011; Scheithauer et al., 2021).

4.4 ProA cleaves and activates bacterial substrates and camouflages *L. pneumophila* by degradation of monomeric flagellin which can be modeled by computational methods

Besides a diverse range of host substrates, ProA shows activity against several *Legionella*-own proteins and thereby influences the intracellular life cycle indirectly (Figure 1). The zinc metalloprotease not only regulates these factors by degradation but some T2SS effectors also by specific post-translational activation. The two phospholipases A (PLA) PlaA and PlaC represent virulence-associated factors in the extracellular space, which are activated by ProA *via* processing of a sterically inhibiting disulfide loop (Banerji et al., 2006; Lang et al., 2012; Lang et al., 2017). In this way, *L. pneumophila* most likely prevents itself from enzymatic damage within the cytoplasm. PlaC in particular, but also PlaA, acts as a glycerophospholipid:cholesterol acyltransferase (GCAT), which is expected to hydrolyze various host cell lipids. As a lysophospholipase A (LPLA) and antagonist of the T4SS effector SdhA, which promotes phagosome integrity, PlaA destabilizes the LCV membrane to facilitate the bacterial exit after completed replication (Hiller et al., 2018). Interestingly, ProA expression increases this LPLA activity from PlaA, but abolishes GCAT activity at the same time. In PlaC, however, ProA cleavage induces both PLA and GCAT activity, while the LPLA function appears to be generally independent of the protease (Banerji et al., 2005; Lang et al., 2017). ProA, moreover, processes the bacterial aminopeptidases LapA and LapB, which contribute to the generation of amino acids. LapA exhibits activity against aminopeptides containing leucine, isoleucine, methionine, phenylalanine, valine, aspartate and tyrosine, and is the first representative of secreted aminopeptidases promoting intracellular infection (White et al., 2018). Thus, ProA at least indirectly affects amino acid acquisition and LCV biogenesis *via* its activated bacterial substrates. Nevertheless, ProA can also regulate excess of certain proteins by simple degradation. For example, it reduces free immunogenic flagellin monomers in the extracellular space, which are otherwise recognized by the host immune system (Mascarenhas and Zamboni, 2017; Scheithauer et al., 2022).

The finding that the flagella subunit FlaA from *L. pneumophila* is a physiological substrate of the zinc metalloprotease ProA might be of high relevance during the infection of human lung tissue. Interestingly, only monomeric flagellin was shown to be digested, while polymerized FlaA assembling the filament remains stable (Scheithauer et al., 2022). This is important to preserve its crucial functions for the intracellular life cycle. Originally, flagellar expression was associated with the general ability of *L. pneumophila* to initiate infections. In this context, co-regulation with other essential virulence factors represents a central aspect (Pruckler et al., 1995; Bosshardt et al., 1997). FlaA is not expressed while bacterial replication but during the stationary phase to gain access to new host cells (Byrne and Swanson, 1998). Nevertheless, infection of human A549 lung epithelial cells as well as THP-1 macrophages demonstrated significant attenuation of a *flaA* deletion mutant after 24 h and 48 h compared to the *L. pneumophila* wild type (Teruya et al., 2007; Scheithauer, 2022). Correspondingly, a reduced invasion rate was reported, particularly in HL-60 cells (Dietrich et al., 2001; Pereira et al., 2011). Studies of the lung pathogen *P. aeruginosa* revealed that the flagellum stimulates bacterial internalization into the host cell *via* opsonin-independent phagocytosis (Mahenthiralingam and Speert, 1995). Nevertheless, extracellular and unassembled FlaA triggers the immune response upon receptor recognition by the host. Interestingly, both TLR5 stimulation and proteolytic degradation exclusively occur with monomeric flagellin. Thus, recognition by receptor and protease is assumed to be mediated *via* a non-exposed part of FlaA located within the polymerized flagellum. It was already published that TLR5 binding especially involves specific amino acid residues of the highly conserved D_1 domain of flagellin but also of the polymerization domain D_0 (Forstnerič et al., 2017; Song et al., 2017). The *L. pneumophila* zinc metalloprotease most

likely exhibits a similarly located recognition site. By using AlphaFold v2.2 multimer modeling, we were able to generate a structure of the ProA-FlaA complex with a high score of prediction quality referring to the interface between both proteins (Figure 3A). Indeed, this binding interface is localized within the D_0 polymerization domain of FlaA strengthening our assumption that ProA is only able to degrade flagellin monomers since an assembly of the subunits will cover the protease cleavage sites. By aligning the *L. pneumophila* FlaA structure with the known filament Cryo-EM structure of *P. aeruginosa*, we also modeled polymeric flagellin (Wang et al., 2017; Karagöz et al., 2022). The integration of ProA (magenta) into the multimer prediction resulted in a deranged flagellar structure (marine blue) with steric clashes indicating an impossible protein-protein interaction (Figure 3B). This becomes particularly clear when compared with the typically assembled filament, where ProA has no access to the flagellin cleavage site (Figure 3C). This lead to the conclusion that ProA is not able to attack the intact surface of a polymerized flagellum but has immunomodulatory potential by degradation of free FlaA.

Immune evasion by the protease was already elucidated *via* a reporter-based TLR5 stimulation assay. Purified and active ProA demonstrably abolished the receptor activation caused by monomeric flagellin. Moreover, continuative experiments displayed diminished recognition of a *flaA* deletion mutant but increased values with a *proA* deletion strain compared to the *L. pneumophila* wild type using bacterial suspensions as well as HLTE infection supernatants (Scheithauer et al., 2022). Conclusively, ProA leads to a significant reduction of flagellin-dependent TLR5 stimulation in a physiological human background. Similar activity and effects were described in detail regarding the homologous pseudolysin and the alkaline protease ArpA, both from *P. aeruginosa*. In an *arpA*-deficient mutant, TLR5 activation is increased 100-fold compared to the wild type, indicating responsibility for a complete degradation of exogenous flagellin (Bardoel et al., 2011; Casilag et al., 2016). In pulmonary tissue, TLR5 is expressed particularly by alveolar macrophages, but also by respiratory epithelial cells, type II pneumocytes, neutrophils, plasma and dendritic cells (Honko and Mizel, 2005; Shikhagaie et al., 2014). Activation of this Toll-like receptor plays a crucial role in fighting legionellosis, which is why people with a $TLR5^{392STOP}$ polymorphism suffer from a significantly enhanced susceptibility to Legionnaires' disease (Hawn et al., 2003). In this case, flagellin-mediated signaling is disrupted by an alteration in the ligand-binding domain, resulting in a decreased innate immune response of the lung epithelium. The cleavage of free FlaA by ProA hence may represent a major advantage for immune evasion and thus for pathogen proliferation (Figure 1). One of the most important pro-inflammatory factors that are induced in a TLR5-dependent and therefore FlaA-dependent manner is IL-8 (Yu et al., 2003; Schmeck et al., 2007; Im et al., 2009). Studies have already shown that IL-8 is

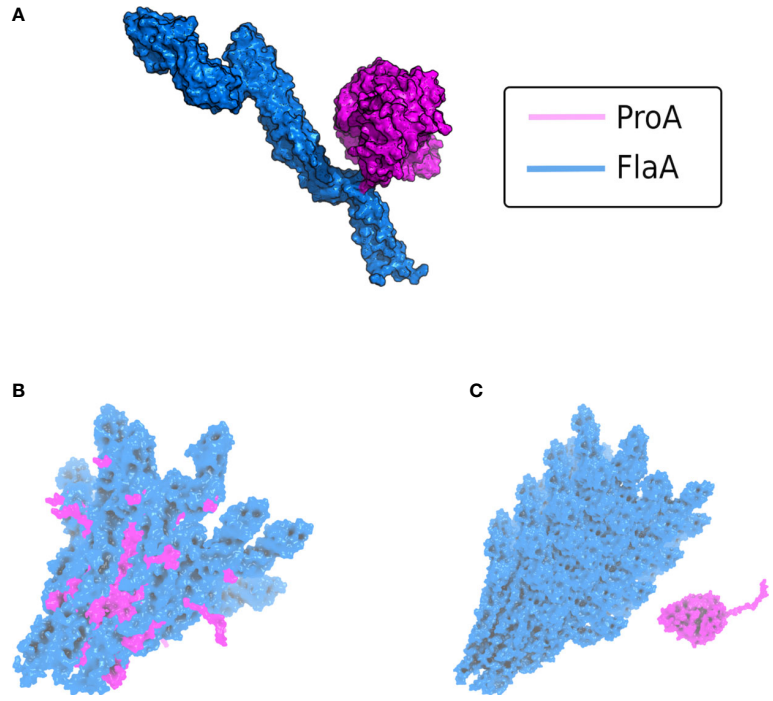

FIGURE 3
AlphaFold v2.2 multimer modeling of the interaction between *L. pneumophila* ProA and FlaA. **(A)** Complex of the protease ProA in magenta and monomeric FlaA in marine blue. The binding interface with the most contacts is localized within the D_0 polymerization domain of flagellin. **(B)** Modeling of ProA (magenta) and generic polymerized FlaA (marine blue). The flagellar filament was generated by aligning the predicted *L. pneumophila* FlaA structure on the known Cryo-EM structure of the *P. aeruginosa* filament (PDB ID: 5WK6) (Karagöz et al., 2022). ProA was directly integrated into a deranged flagellar structure, due to steric clashes in the FlaA polymerization region. These clashes were quantified using the repulsive Lennard-Jones (LJ) Energy score from the Rosetta scoring modeling suite according to Alford et al., 2017 and corroborated that this is not a possible protein-protein interaction (Alford et al., 2017). **(C)** Typically assembled filament without access for ProA (ProA placed randomly in vicinity). The conserved cleavage sites of FlaA are hidden within the polymerized flagellar filament.

also the most common messenger in the supernatant of *Legionella*-infected macrophages (McCoy-Simandle et al., 2011). The chemokine contributes to the recruitment of neutrophils and other immune cells that are crucial for elimination of the pathogen (Tateda et al., 2001; Hawn et al., 2007; Mascarenhas et al., 2015). Additionally, TLR5-mediated recognition of flagellin *via* NF-κB induces TNF-α production in alveolar macrophages. TNF-α is a multifunctional signaling factor, which provokes apoptosis of infected host cells and thus reduces proliferation of *L. pneumophila* in the lungs (Blanchard et al., 1988; Kawamoto et al., 2017). *Pseudomonas* flagellin also triggers the expression of cytokines like MIP-2, IL-17 and IL-22 and antimicrobial peptides such as β-defensin 2 or CRAMP (cathelin-dependent antimicrobial peptide) in a mouse model (Yu et al., 2010). Eventually, FlaA is not only recognized extracellularly by TLR5 on the host cell surface but also detected in the cytoplasm by the Naip5/Nlrc4 inflammasome. Since ProA is likewise translocated from the LCV, it is conceivable that the protease cleaves cytosolic flagellin in order to reduce the inflammasome stimulation. For future works, alteration of pro-inflammatory mediators in downstream pathways should be examined, since already identified targets of ProA include a wide range of host factors.

5 Macrophage infectivity potentiator (Mip)

5.1 Peptidyl-prolyl-*cis/trans*-isomerase Mip binds collagen and enables bacterial transmigration across tissue barriers

The *L. pneumophila* membrane-associated surface protein Mip was the first genetically identified virulence factor of *L. pneumophila* (Cianciotto et al., 1989; Engleberg et al., 1989). Deletion of the respective gene results in reduced intracellular replication in human alveolar macrophages and protozoa (Cianciotto et al., 1989; Cianciotto and Fields, 1992; Fischer et al., 1992). Mip (24 kDa) possesses an N-terminal signal sequence which is cleaved off while the protein is transported through the cytoplasmic membrane (Wintermeyer et al., 1995). The basic protein (pI 9.8) forms a stable homodimer, and the 2.4 Å crystal structure revealed that each monomer consists of a N-terminal dimerization module, a long connecting α-helix (α3) and a C-terminal peptidyl-prolyl-*cis/trans*-isomerase (PPIase) domain (Riboldi-Tunnicliffe et al., 2001; Helbig et al., 2001; Köhler et al., 2003; Galka et al., 2008). The fold of the C-terminal domain (residues 100-213) shows high homology to the human FK506-binding protein 12 (FKBP12) (Schmidt et al., 1996; Fischer et al., 1998), and the macrolides FK506 or rapamycin efficiently inhibit the PPIase activity of the respective proteins (Schiene-Fischer et al., 2013). Further Mip targeting inhibitors such as cycloheximide, pipecolic acid, as well as non-immunosuppressive FK506 derivatives independently corroborated the observation of moonlighting activities of Mip in fundamental processes of infection (Scheuplein et al., 2020; Rasch et al., 2015; Rasch et al., 2014; Juli et al., 2011; Pomplun et al., 2018; Ünal and Steinert, 2014).

Nuclear magnetic resonance (NMR) solved the solution structure of free Mip^{77-213} and the Mip^{77-213}-rapamycin complex, and comparisons with the structures of free FKBP12 and the FKBP12-rapamycin complex suggested an identical binding mode for both proteins (Ceymann et al., 2008). Molecular dynamics simulations of the Mip dimer yielded two different correlation times for the two domains and thus confirmed the independence of the domain motions. Thus, mediated by a hinge in the long α-helix, both FKBP domains of the dimerized Mip appear highly flexible for cooperative binding of potential target structures (Horstmann et al., 2006).

PPIases of pathogens such as *Burkholderia*, *Chlamydia*, *Clostridium*, *Neisseria*, *Klebsiella* and others are generally involved in a broad spectrum of phenotypes including virulence, metabolism, and multiple stress responses (Lundemose et al., 1991; Norville et al., 2011; Ünal and Steinert, 2014; Ünal et al., 2018; Ünal et al., 2019; Christodoulides, 2022; Iwasaki et al., 2022). Likewise, the PPIase Mip of *L. pneumophila* contributes to infection, collagen binding, phospholipase C-like activity, transmigration across tissue barriers, nematode colonization, surface translocation, and growth at suboptimal temperature (Figure 4) (DebRoy et al., 2006; Ünal et al., 2011; Rasch et al., 2019; Rasch et al., 2016). Although previous studies employing genetic and biochemical methods together with different infection models revealed that Mip impacts the course and outcome of infection on multiple levels (Wintermeyer et al., 1995; Köhler et al., 2003; Wagner et al., 2007; Söderberg and Cianciotto, 2008), our knowledge about binding partners or substrates of Mip remained very limited. In contrast, the structural understanding of Mip improved steadily. Site-specific mutagenesis of highly conserved amino acid residues within the FK506-binding pocket, in which Asp 142 was replaced by leucine and Tyr 185 by alanine, revealed pronounced loss of PPIase activity of the purified recombinant protein *in vitro* (residual activity 6.2% for the D142L mutant and 2.0% for the Y185A mutant). Surprisingly, wild type phenotypes in infection studies with *A. castellanii* or human macrophage-like cell lines were observed, when the same site specifically mutated variants of *mip* were used to complement *L. pneumophila mip*-negative mutants (Wintermeyer et al., 1995). This suggests that either the residual enzymatic activity of the mutated Mip was still sufficient for PPIase-dependent phenotypes, or additional properties other than the PPIase activity are important during intracellular infection. Strikingly, guinea pig infections with *L. pneumophila* strains expressing Mip variants, that were unable to dimerize or had a low PPIase activity, were significantly attenuated (Köhler et al., 2003). This was in good agreement with the observation that Mip-deficient bacteria were found to be attenuated and unable to disseminate systemically in guinea pigs (Wagner et al., 2007).

The different consequences of a reduced PPIase activity in monocellular infection models and guinea pig infections indicated additional activities of Mip in the course of the more complex infection of mammalian tissues. This interpretation was further corroborated when transwell assays with *L. pneumophila* and

recombinant *Escherichia coli* HB101 strains revealed that Mip enables these bacteria to transmigrate across a barrier of NCI-H292 lung epithelial cells and extracellular matrix (NCI-H292/ECM barrier). Further evidence for an extracellular function of Mip resulted from the observation that Mip binds to collagen I-VI (Figure 4) (Wagner et al., 2007). Especially interesting is the fact that the best binding of Mip was determined for collagen IV, which is known to be the prevalent collagen type in the human lung (Gelse et al., 2003). Mip binds to a surface-exposed sequence in the NC1 domain of the collagen IV α1 chain, and a corresponding collagen IV-derived peptide (IPPCPSGWSSLWI; P290) co-precipitated with Mip and competitively inhibited the Mip-collagen IV binding (Ünal et al., 2011). Moreover, P290 efficiently inhibited transmigration of *L. pneumophila* across a barrier of NCI-H292 lung epithelial cells and extracellular matrix. This significantly reduced transmigration was comparable to the inefficient transmigration of PPIase-negative Mip mutant or rapamycin-treated *L. pneumophila*. Based on NMR data and docking studies, a model for the mode of interaction of P290 and Mip was developed. The amino acid residues of the hydrophobic cavity of Mip, D142 and to a lesser extent Y185, were identified to be part of the interaction surface (Wintermeyer et al., 1995).

5.2 Computational methods allow instructive interpretations of Mip interactions with stringent starvation protein SspB, hypothetical protein Lpc2061, and flagellin FlaA

Although being the first identified virulence factor of *L. pneumophila*, it remained largely unknown for a long time, how Mip exerts its diverse functions. A recent interactomic approach, however, paved the way for more straight forward functional studies, since the stringent starvation protein B (SspB, LPC_0434), hypothetical protein Lpc2061 (LPC_2061) and flagellin (FlaA, LPC_0756) were identified as *in vivo* bacterial interaction partners of Mip (Figure 4) (Karagöz et al., 2022). Addition of the macrolide FK506 in co-immunoprecipitation assays revealed that only Lpc2061, but not SspB or FlaA, requires the C-terminal PPIase binding pocket of Mip for interaction. Recent machine learning models suggest the docking sites of FK506 to the Mip homodimer and indicate why additional binding of Lpc2061 is inhibited (Figure 5) (Corso et al., 2022). The macrolide FK506 occupies the basis of the α-helix at the dimerization region and the cavities of both PPIase domains (Figure 5A). Since Lpc2061 requires

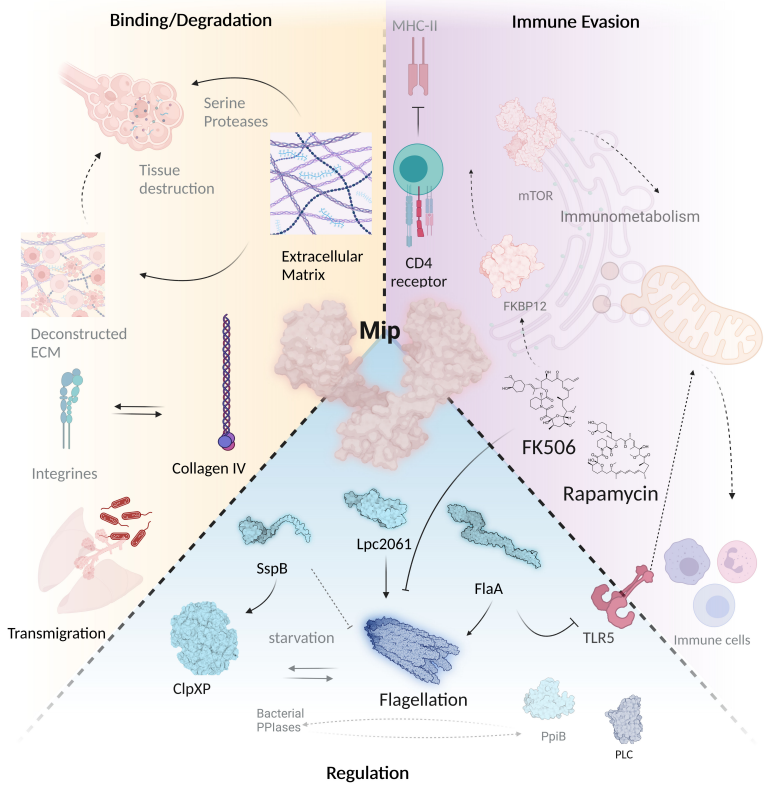

FIGURE 4
Mip mediates virulence regulation, host tissue degradation and immune evasion. The virulence factor Mip interferes with a wide variety of infection-associated processes *via* different interaction partners. During Legionnaires' disease, it is important that bacteria migrate through different tissue barriers. This is facilitated by binding of Mip to collagen IV, which is subsequently degraded by a serine protease. Mip binds to SspB, Lpc2061 and FlaA, and promotes flagellation by its PPIase domain that can be inhibited by FK506 and rapamycin. Binding of FK506 and rapamycin to PPIases modulates immunometabolism and interferes with bacterial immune evasion. Mip regulates stress response and infectivity in bacteria together with other PPIases and promotes Phospholipase C (PLC) activity (created with BioRender.com).

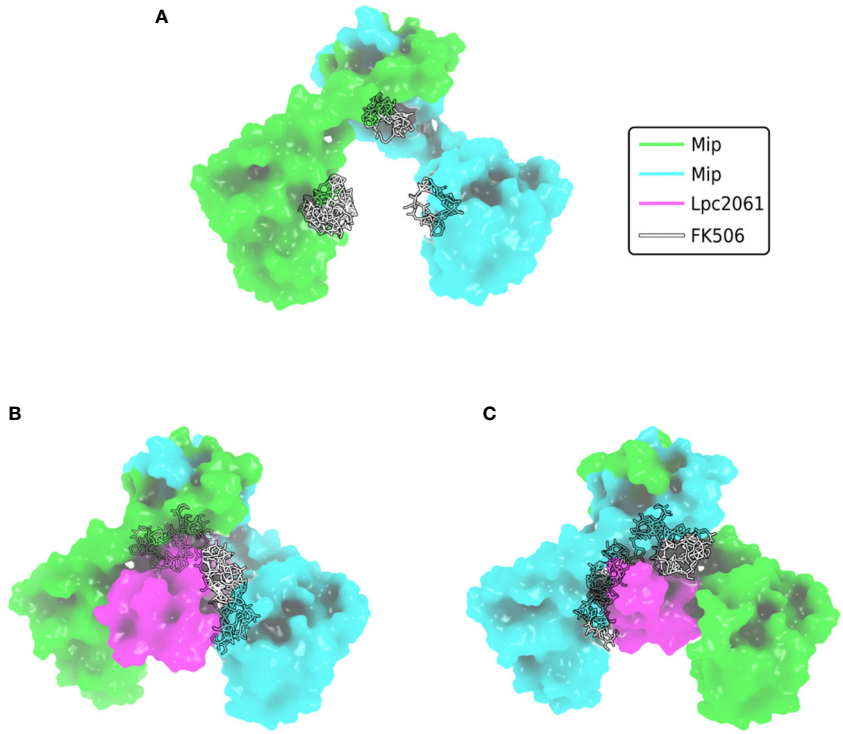

FIGURE 5
Docking of FK506 to Mip and competitive inhibition of Lpc2061 binding. **(A)** Docking of FK506 (grey) to the Mip homodimer (green and cyan) results in binding to the dimerization and the two PPIase domains. **(B)** Lpc2061 (magenta) binding to Mip is inhibited by FK506, since the inhibitor blocks this triangle. **(C)** Overlapping binding regions of Lpc2061 and FK506 after rotation by 180°. Molecular docking was performed using DiffDock (Corso et al., 2022).

this triangle for Mip binding, competitive inhibition by FK506 appears to be the most likely mechanism (Figures 5B, C).

The finding that not all interactions were negatively influenced by FK506 was not surprising since several of the virulence and fitness functions of Mip are not related to enzymatic catalysis, but rather to moonlighting activities in the host (Rasch et al., 2014; Rasch et al., 2015; Ünal et al., 2011). Evaluation of the biochemical data and computational predictions of the respective interactions with Mip consistently suggested that SspB is the strongest binder, followed by Lpc2061 and FlaA. Interestingly, molecular dynamic simulations predicted an increased stability for the tripartite interaction of Mip, Lpc2061 and FlaA compared to the Mip-Lpc2061 binary interaction (Figure 6).

L. pneumophila strains expressing Mip variants with single amino acid substitutions or N-terminally truncated monomers revealed that the dimerization region and the amino acid residue Y185 of Mip are required for the binding of Lpc2061, which is strengthened by FlaA. The binding of SspB occurs independently of the tested Mip variants and is not influenced by the presence of FlaA. Modeling of the interaction partners and global docking with Mip suggested non-overlapping binding interfaces, and molecular dynamic simulation predicted, consistent with the biochemical data, an increased stability for the tripartite interaction of Lpc2061, Mip, and FlaA (Karagöz et al., 2022).

The observed interactions of Mip with the respective bacterial binding partners raise questions regarding their functional implications. SspB homologues of *E. coli* (52% identity) and *Pseudoalteromonas* spp. (62% identity) are well known dimeric adaptor proteins, which increase the rate at which ssrA-tagged substrates are degraded by tethering them to the ClpXP protease (Levchenko et al., 2000; Yin et al., 2021). Respective ClpXP deletion mutants of *Salmonella enterica* Serovar Typhimurium exhibit overproduction of the flagellar protein, a fourfold increase in the rate of transcription of *fliC*, and a hyperflagellated phenotype (Tomoyasu et al., 2002). Whether Mip intercepts SspB and by this means downregulates the ClpXP-dependent repression of the flagellar regulon in *L. pneumophila* remains to be elucidated. The function of the hypothetical protein Lpc2061 with structural homology to glycoside hydrolases also remains to be investigated. The interaction of Mip and FlaA is especially interesting, since the bacterial PPIase was demonstrated to influence flagellation and motility of *L. pneumophila* (Karagöz et al., 2022).

5.3 Mip interactions promote flagellation and bacterial motility which is inhibited by FK506

Recent results demonstrated Mip as a binding partner of FlaA and amplifier of *L. pneumophila* flagellation and motility (Karagöz et al., 2022). Moreover, this phenotype was positively modulated by the Mip interaction partner Lpc2061 (Figure 4). Since FlaA and SspB are both expressed during starvation periods in the post-exponential phase of *L. pneumophila*, it is of future interest to

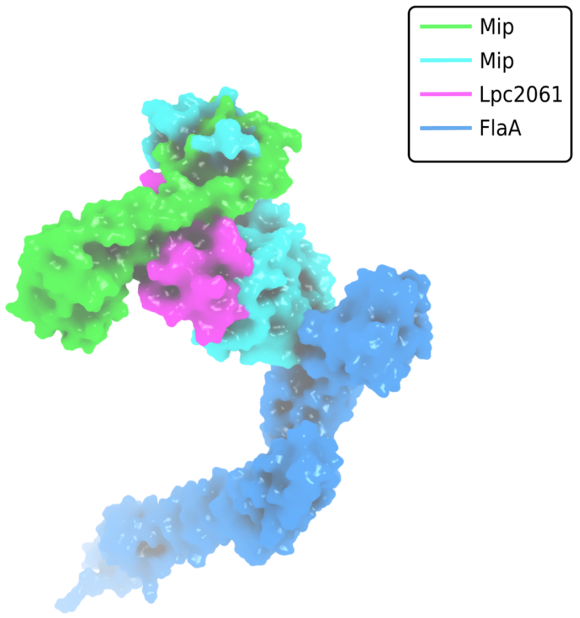

FIGURE 6
Surface plot visualization of the tripartite complex of Mip, FlaA and Lpc2061. Modeling was perfomed by docking of FlaA (marine blue) to the best pose of Lpc2061 (magenta) and the Mip homodimer (green and cyan). While Lpc2061 binds to the Mip dimerization region, monomeric FlaA interacts with the C-terminus of the PPIase domain. This tripartite complex was found to be more stable than the binary Mip-Lpc2061 interaction (Karagöz et al., 2022).

analyze if the Mip-SspB binding also influences this interaction (Appelt and Heuner, 2017).

Biochemical quantifications of FlaA preparations of *L. pneumophila* wild type strains and mutants demonstrated that wild type Mip promotes flagellation of *L. pneumophila* and the yield of FlaA. *L. pneumophila* strains expressing the Mip$^{(Y185A)}$ or the monomeric Mip$^{(77\text{-}213)}$ variant, which bind less Lpc2061, were less flagellated and yielded less FlaA. Also, FK506 treatment resulted in a lower FlaA yield and reduced motility. In accordance with biochemical results showing that FlaA and Lpc2061 mutually reinforce their binding to Mip, which was also suggested by molecular dynamic simulations, it was described that the binding regions of Mip in Mip-Lpc2061 interaction positively influence flagellation (Karagöz et al., 2022).

For a long time it is known that Mip and the major subunit FlaA both positively affect the early phase of infection of eukaryotic host cells such as amoebae and macrophages (Dietrich et al., 2001; Hammer et al., 2002). More recent work suggests that certain effects of Mip or FlaA on *L. pneumophila* pathogenicity are mediated or regulated by their interaction (Karagöz et al., 2022). However, whether or how Mip assists in the flagellar assembly or regulation remains to be elucidated.

6 Conclusion, open questions and future perspectives in drug research

Infection and disease progression largely depend on the outcome of protein-protein interactions between pathogen and host. Since *L. pneumophila* evolved in the aquatic environment and not in humans, ProA and Mip should also have ecological implications. Both virulence factors are important for the replication in certain protist species. Nevertheless, the question remains open, how ProA and Mip became able to recognize target structures like e. g. collagen, which are characteristic for higher eukaryotes. One hypothesis is that an intimate coexistence with native metazoan species as hosts, vectors, or reservoirs lead to the acquisition of eukaryotic protein domains, the development of collagen-binding motifs, and immune evasion strategies (Best and Abu Kwaik, 2018). However, it is also conceivable that most of the human targets represent accidental ProA substrates due to a broad specificity of the protease. Thus, studies clarifying the role of ProA and Mip in multicellular hosts of natural aquatic habitats such as nematodes will be of particular interest. In this context, it appears also promising to apply interactomic approaches to identify further host targets of Mip.

The virulence factors ProA and Mip both target *L. pneumophila* FlaA and the host ECM protein collagen by degradation or binding, respectively (Scheithauer et al., 2021; Scheithauer et al., 2022; Karagöz et al., 2022; Köhler et al., 2003; Wagner et al., 2007). On the phenotypic level, both virulence factors (i) promote bacterial transmigration in tissue, (ii) regulate virulence factors including flagellation, and (iii) influence immune evasion including immunometabolism. This raises the question to what extent the activities of Mip and ProA are cooperative and coordinated in a functional virulence network. Previous work with metabolically labeled ECM revealed that Mip-positive bacteria degrade ECM proteins, whereas Mip-negative bacteria or pure recombinant Mip protein do not cause degradation (Wagner et al., 2007). Moreover, the degradation of ECM could be inhibited by the serine protease inhibitors Pefabloc SC and PMSF, although the Mip PPIase activity was not affected by this treatment (Köhler et al., 2003; Wagner et al., 2007). These observations demonstrated that Mip does not degrade the ECM through an own proteolytic activity and suggested that an additional serine protease activity is required. More recent data revealed that ProA mediates tissue damage in HLTEs by degradation of collagen IV in the basal lamina and the cell adhesion protein vitronectin (Ünal et al., 2011; Scheithauer, 2022). If the effects of Mip and ProA are directly linked, and how a yet not identified serine protease of *L. pneumophila* or the host cells operates in a concerted way, remains to be elucidated. Nevertheless, a pharmacological approach using FK506 or rapamycin in the ECM degradation assay suggested that the PPIase activity is required for the observed proteolysis (Wagner et al., 2007).

Further functional questions arise from the identified tripartite interaction of FlaA, Lpc2061 and Mip and from the degradation of monomeric FlaA by ProA, which reduces the TLR5-mediated immune response. To avoid bacterial clearance by the immune system *L. pneumophila* seems to follow the strategy to either polymerize FlaA into flagella, or to minimize the amount of exogenous monomeric FlaA (Forstnerič et al., 2017; Song et al., 2017). Whether, and if so how, Mip assists FlaA polymerization or ProA degradation is not known. But since Mip-negative *L. pneumophila* strains are less flagellated, yield less FlaA and are non-motile, we hypothesize a coordinated contribution of Mip and ProA to avoid free FlaA monomers.

Since computational approaches have already been successfully applied for the characterization of ProA, Mip and their respective binding partners, it seems possible to also model protein-drug interactions. The fast developing machine learning approaches together with molecular dynamics simulations, docking and X-ray diffraction crystallography or Cryo-EM have the potential to create novel scoring functions to anticipate ligand-binding affinity with high predictive power. By using regression methods and deep learning models trained for ensemble prediction, it is e.g. possible to scan the scoring function space and to gain insight into protein-ligand energetics. Since we currently witness rapid progress due to the application of deep learning methods to predict the 3D structures of proteins, we may soon be able to generate refined protein sociological studies which include options for interference by drugs.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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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.

Publisher's note

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Antibiotic susceptibility pattern of Portuguese environmental Legionella isolates

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Introduction: Legionnaires' Disease is a pneumonia caused by *Legionella* spp., currently treated empirically with fluoroquinolones and macrolides. In this study, we aim to describe the antibiotic susceptibility pattern of environmental *Legionella* recovered in the south of Portugal.

Methods: Minimal inhibitory concentration (MIC) determination of 57 *Legionella* isolates (10 Lp sg 1, 32, Lp sg 2-14 15 L. spp) was achieved by broth microdilution, as described by EUCAST, for azithromycin, clarithromycin, ciprofloxacin, levofloxacin, and doxycycline.

Results: Fluoroquinolones were the most active antibiotic, displaying the lowest MIC values in contrast to doxycycline which had the highest. MIC90 and epidemiological cut-off (ECOFF) values were, respectively, 0.5/1 mg/L for azithromycin, 0.125/0.25 mg/L for clarithromycin, 0.064/0.125 mg/L for ciprofloxacin, 0.125/0.125 mg/L for levofloxacin and 16/32 mg/L for doxycycline.

Discussion: MIC distributions were higher than reported by EUCAST for all antibiotics. Interestingly, two phenotypically resistant isolates with high-level quinolone resistance were identified. This is the first time that MIC distributions, *lpeAB* and tet56 genes have been investigated in Portuguese environmental isolates of *Legionella*.

KEYWORDS

***Legionella*, broth microdilution, MIC, fluoroquinolones, macrolides, environmental, antibiotic susceptibility**

1 Introduction

Legionnaires' Disease (LD) is a pneumonia, often severe, caused by waterborne pathogens of the *Legionella* genera. Despite more than 20 of the 65 known *Legionella* species being able to cause human infection (Chambers et al., 2021), *Legionella pneumophila* (*Lp*) alone is responsible for more than 90% of reported cases (European Centre for Disease Prevention and Control, 2022). Transmission to human hosts occurs primarily through aerosolized water particles, with only one description of person-to-person transmission of LD (Correia et al., 2016). *Legionella* is ubiquitous in water, colonizing natural and artificial environments, such as cooling systems, air conditioners and water supply networks.

The antibiotics used in LD treatment include fluoroquinolones, macrolides, and tetracyclines, given their high cellular penetration, azithromycin, and levofloxacin being considered the first line (Mandell et al., 2007; Woodhead et al., 2011). However, cases of unresolved LD or with slow treatment have already been described. Bruin et al., described one case of susceptibility loss to ciprofloxacin treatment, derived from a mutation in position 83 of the *gyrA* gene which encodes a subunit of the DNA gyrase (Bruin et al.). Additionally, resistance by target mutation has been comproved *in vitro* for macrolides and rifampicin (Nielsen et al., 2000; Descours et al., 2017).

Recently, other resistance mechanisms were reported in this bacterium associated with the LpeAB operon, an analog of the AcrAB efflux pump of *Escherichia coli* (Massip et al., 2017). Mutations upstream of *lpeAB* gene, have been described as responsible for a decreased susceptibility to azithromycin and erythromycin in *Lp* Paris strain. This LpeAB efflux pump has been found in clinical and environmental isolates (Vandewalle-Capo et al., 2017; Nata and Løva, 2019; Cocuzza et al., 2021). Tet56, a tetracycline destructase of the TetX family has been identified in *L. longbeacheae*, *L. nautarum*, and *L. jordanis* isolates (Forsberg et al., 2015; Joseph et al., 2016). Although the presence of Tet56 may not be clinically impactful, as tetracyclines are regarded as an alternative in LD treatment, tigecycline, a synthetic tetracycline, has been successfully used in cases of LD, when treatment with first-line antibiotics has failed (Valve et al., 2010; Slawek et al., 2017).

Antibiotic pollution is a worrying problem. The presence of antibiotics in different aquatic environments, usually in low concentrations (Hughes et al., 2013; Danner et al., 2019; Rodriguez-Mozaz et al., 2020), is known to influence resistance mechanisms (Andersson and Hughes, 2014; Murray et al., 2018; Chow et al., 2021), promote virulence factors (Andersson and Hughes, 2014), and decrease antibiotic susceptibility (Sulyok et al., 2017). However, the full extent of the impact caused by chronic exposure remains unknown (Janecko et al., 2016). As the source of LD infection is from water and soils, special concern should be given to environmental *Legionella* populations.

The high efficacy of LD antibiotic treatment, over reliance on urinary antigen tests and reduction in culture, and the lack of consensus in antimicrobial susceptibility testing (AST) methodology, are contributing factors to the absence of standardization, and clinically defined breakpoint (Portal et al., 2021a). In this study, we aim to characterize Portuguese environmental *Legionella* susceptibility patterns, define ECOFF, and contribute more data on the subject.

2 Methods

2.1 Bacterial isolates

Isolates were collected (n = 57) from November 2021 to June 2022. They were obtained from water samples screened for *Legionella* presence in LAIST (*Laboratório de Análises de Água, Técnico Lisboa, Universidade de Lisboa*), in accordance with the standardized procedures described in the ISO 11731 (International Organization for Standardization, 2017). All isolates were obtained from aerosol producing equipment. Isolates were discriminated into three groups (*Lp* sg 1, *Lp* sg 2-14, *L.* spp) based on serological identification with latex agglutination test (OXOID, UK). Bacterial isolates were then preserved at -80°C in a thioglycolate medium until tested.

2.2 Control strains

In this study, the control strains *Lp* sg1 ATCC 33152 and Paris was used.

2.3 Antibiotic agents

Five antibiotics were selected to be studied: azithromycin (AZT); clarithromycin (CLA); ciprofloxacin (CIP); levofloxacin (LEV) and doxycycline (DOX) [Sigma Aldrich, USA]. Antibiotics were resuspended in the advised solvents, respectively: methanol, DMSO, HCl 1% solution, and water for the last two. The range of concentrations tested were respectively: 0.016 – 4 mg/L; 0.004 – 2 mg/L; 0.016 – 1 mg/L; 0.008 – 0.5 mg/L; and 0.5 – 128 mg/L.

2.4 Susceptibility tests

Tests were performed in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2018; European Committee on Antimicrobial Susceptibility Testing, 2021). Briefly, bacterial suspensions of 0.5 McFarland were prepared using buffered yeast extract medium (BYE). Antibiotics were diluted in ultrapure water in ranges equal to the minimal inhibitory concentration (MIC) distribution of EUCAST (European Committee on Antimicrobial Susceptibility Testing, 2021). Forty microliters of each antibiotic dilution were added to the test wells of the microplate before adding the 160 µL of the bacterial suspension. For positive and negative controls bacterial suspension and BYE medium were used, respectively. All isolates were tested in duplicate.

Microplates were incubated at 37°C for 48 hours in a humid chamber. After incubation, the microplates were read manually according to by EUCAST and CLSI guidelines. Additionally,

absorbances were read in a spectrophotometer at a wavelength of 600 nm and the inhibition rate was calculated using the formula:

$$IR = 100 - \left(\frac{Abs_x - Abs_{C-}}{Abs_{C+} - Abs_{C-}} * 100\right)$$

Where Abs_X represents the absorbance of the sample; Abs_{C-} and Abs_{C+} the absorbance of the negative and positive control, respectively; IR the inhibition rate. To determine MIC a cut-off value of 90% inhibition was used.

2.5 Definition of the wild-type Portuguese ECOFF

ECOFFs were defined through the ECOFFinder program available at the EUCAST website, that follows the methodologies described by Turnidge et al. (Turnidge et al., 2006; European Committee on Antimicrobial Susceptibility Testing, 2023).

2.6 Molecular detection of resistance mechanisms

Molecular amplification of *lpeAB* and *tet56* genes was performed through conventional PCR to determine the presence of possible resistance mechanisms. Briefly, for the gene *lpeAB* the specific primers lpp2879_detec_F2 (5′-GTGATGATTGTCTTATTGG TGCGA-3′) and lpp2879_detec_R3 (5′-ATGGCGTTTAAGATGATGGT GATT-3′) were used (Vandewalle-Capo et al., 2017) and, for the gene *tet56* the primers Fw (5'- ATGTCTAAAAATATCAAAATTCTCGTC-3') and Rv (5'- CTATGATGATTCATATTGAGGTAAGG-3') (Forsberg et al., 2015). The presence of the *lpeAB* gene was investigated in all isolates tested for azithromycin. The presence of the *tet56* gene was investigated in *Lp* sg1 and *Lp* sg 2-14 isolates when MIC for doxycycline was raised to EUCAST tentative highest MICs, 2 mg/L and 32 mg/L respectively, and in all *L.* spp isolates.

2.7 Statistical analysis

To compare the two reading methods, the MIC distributions between serological groups from the present study to the EUCAST values and with the MICs values described in selected studies (Wilson et al., 2018; Assaidi et al., 2020; Cocuzza et al., 2021), non-parametric Mann-Whitney and Kruskal-Wallis test were performed using XLSTAT Software (Addinsoft, France).

3 Results

Thirty eight of the collected isolates were from water distribution systems of hospitals, hotels, and other public spaces, two isolates were from industrial cooling towers, one came from residual waters and, the remaining were of unknown origin. Water samples were collected from the southern region of Portugal, mainly from the Lisbon metropolitan area and Alentejo. In the studied population the three groups are represented: *Lp* sg 1 (n = 10), *Lp* sg 2-14 (n = 32), and *L.* spp (n = 15).

MIC distributions and ECOFFs are shown in Table 1. Overall, both quinolones achieved the lowest ECOFF values: 0.125 mg/L for both CIP and LEV; 0.25 mg/L for CLA; 1 mg/L for AZT; and 32 mg/L for DOX, which is the highest ECOFF.

Comparison of MIC distributions between serological groups (Table 2) showed that *Lp* sg 1 had the highest MIC values. The ranges of the *Lp* sg 2-14 and *L.* spp groups were identical for LEV. For CIP the *Lp* sg 2-14 group achieved the lowest MIC range and for the other antibiotics, the *L.* spp group had the lowest range. Generally, the groups followed the above-described trend in

TABLE 1 Cumulative percentages of MIC distribution of environmental *Legionella* isolates (n = 57) obtained by manual and automated reading (signalized with *).

	MIC distributions (mg/L)													
	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64
AZT				4	28	68	93	98	100					
CLA	7	9	19	74	96	96	98							100
CIP			56	88	93	96							100	
LEV		23	70	89	96				98			100		
DOX								2	14	32	63	95	98	100
AZT*					21	33	86	96	100					
CLA*	2	12	19	44	70	89	96	98+						
CIP*		2	49	77	89	91	96						98+	
LEV*		19	63	75	91	96				98		100		
DOX*								2	7	16	30	72	95	100

+) For two isolates it was not possible to determine the MIC for the automated reading of CLA and CIP, respectively. T, Azithromycin; CLA, Clarithromycin; CIP, Ciprofloxacin; LEV, levofloxacin; DOX, Doxycycline.ECOFFs are represented in grey shading.

TABLE 2 MIC_{50}, MIC_{90}, and range values of five antibiotics for segregated serological groups of 57 environmental isolates obtained by manual and automated reading (within parenthesis).

	Lp sg 1 (n=8)*			*Lp* sg 2-14 (n=32)			*L.* spp (n=15)			Control Strains	
	MIC 50	MIC 90	Range	MIC 50	MIC 90	Range	MIC 50	MIC 90	Range	ATCC *Lp.* 33152	*Lp.* Paris
AZT	0.25 (0.5)	0.5 (1)	0.064 – 0.5 (0.064 - 1)	0.25 (0.5)	0.5 (0.5)	0.125 – 1 (0.064 - 2)	0.25 (0.5)	0.5 (0.5)	0.064 - 0.5 (0.064 - 1)	0.125 (0.125)	0.25 (0.125)
CLA	0.064 (0.064)	0.064 (0.125)	0.032 – 64 (0.032 – 0.125)	0.064 (0.125)	0.125 (0.25)	0.008 - 0.5 (0.016 - 1)	0.064 (0.064)	0.064 (0.5)	0.008 - 0.125 (0.008 – 0.5)	0.032 (0.032)	0.064 (0.064)
CIP	0.032 (0.064)	0.125 (0.125)	0.032 – 32 (0.032 – 0.125)	0.032 (0.032)	0.064 (0.064)	0.032-0.125 (0.032 – 0.125)	0.032 (0.064)	0.25 (0.5)	0.032 - 0.25 (0.016 – 0.5)	0.032 (0.064)	0.032 (0.064)
LEV	0.032 (0.032)	0.032 (0.064)	0.016 – 0.032 (0.032 – 0.064)	0.032 (0.032)	0.064 (0.125)	0.016-0.125 (0.016 – 0.125)	0.032 (0.032)	0.064 (0.25)	0.016 - 0.125 (0.016 – 0.25)	0.016 (0.032)	0.032 (0.125)
DOX	4 (16)	16 (32)	2 – 16 (8 - 32)	8 (16)	16 (32)	1 – 32 (1 - 64)	4 (8)	8 (32)	2 – 16 (2 - 64)	4 (2)	2 (16)

MIC values are presented in mg/L.
*This group excludes the two phenotypical resistant isolates.

MICs, except for CIP and CLA in the *Lp* sg 1 and *L.* spp groups, as in these, CLA presented a lower MIC 90 and range than CIP.

Regarding MIC 50, values were similar in all groups for all antibiotics except for DOX in the *Lp* sg 2-14. Contrarily for MIC 90, values do not follow any trend in the three groups (Table 2).

A closer analysis of discriminatory MIC 50 and MIC 90 values demonstrated that the presence in two isolates showed a disequilibrium in these parameters. These discrepancies were caused by two phenotypically resistant strains (Table 1, supplementary material S1). Both isolates were collected from a hotel water system. The results of these two isolates have been retested in triplicate. These two isolates were also investigated for mutations in the QRDR of gyrA and no mutations were found in positions 83 and 87 (E. coli numbering, Table S3 and Figure S1), suggesting a resistance mechanism different from one starting at GyrA position 83 (Almahmoud et al., 2009).

MIC distributions obtained through manual and absorbance readings were revealed to have statistically significant differences (p-value< 0.05). For all serological groups and antibiotics tested, the absorbance reading method produced higher MICs. Isolates *Lp* sg 1 and *Lp* sg 2-14 showed similar MIC in both methods, except for DOX and CLA. The MICs of *L.* spp were at least twice as high, except for AZT. DOX was the antibiotic that revealed the greatest discrepancy between the reading methods (Table 3).

The evaluation results of the efflux pump gene presence showed that 67% (6/9) of the *Lp* sg 1 isolates and 60% (12/20) of the *Lp* sg 2-14, with MIC values, for AZT, greater than 0.125 mg/L, revealed the presence of the efflux pump gene, as well as 53% (8/15) of the studied *L.* spp. Overall, the *lpeAB* gene was present in 62% (18/29) of *Lp* sg 1 and *Lp* sg 2-14 isolates analyzed. The results showed that the number of isolates without the *lpeAB* gene was more prevalent for MICs equal to or less than 0.250 mg/L. Conversely, for higher MICs, isolates with this gene predominate (Figure 1, supplementary material S2).

Converse to the presence of the *lpeAB* gene, the *tet56* gene was found in only one *L.* spp isolate of those analysed (1/21) and identified by MALDI-ToF as *Legionella anisa*.

In summary, the achieved results did not show significant differences comparing the MIC distributions in the three groups, except the MIC distributions of CLA and DOX in *Lp* sg 2-14 and *L.* spp. MIC distributions obtained manually and automatedly were found to be significantly different (p-value< 0.05). By comparison, our MIC distributions is statistically different from EUCAST and selected studies, Cocuzza et al. (Cocuzza et al., 2021); Assaidi et al. (Assaidi et al., 2020); Wilson et al. (Wilson et al., 2018), except for AZT where similar distributions are found (Table S4).

4 Discussion

In 1999 LD was included for mandatory clinical and laboratory notification as part of the Portuguese infectious disease surveillance scheme. The disease is potentially life-threatening if not treated with the proper antibiotics.

TABLE 3 Ratios comparing automatic to the manual reading of MICs discriminated by serological group of isolates and antibiotic.

Groups	AZT	CLA	CIP	LEV	DOX
Lp sg 1 (n=10)	1,60	1,76	1,66	1,50	5,60
Lp sg 2-14 (n=32)	1,71	2,19	1,29	1,17	1,74
L spp (n=15)	1,67	2,49	2,27	2,26	3,20
All (n=57)	1,68	2,20	1,61	1,50	2,84

The results presented are the averages for each group. Statistical tests (Mann-Whitney and Kruskal-Wallis nonparametric test) were previously used to determine that the results obtained from manual reading and automated reading were statistically different. Statistical differences described elsewhere (Table S4).

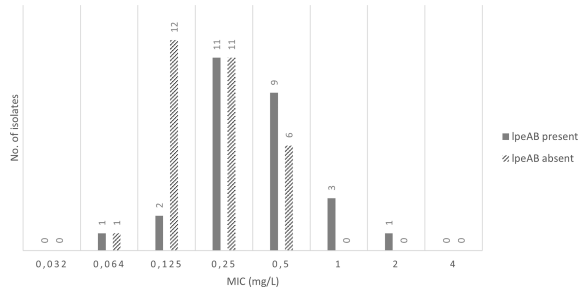

FIGURE 1
Presence of *lpeAB* gene and its distribution according to the AZT MIC of the 57 environmental isolates under study.

Environmental monitoring of *Legionella*, in our country, became mandatory after the publication of Law n. 52/2018, of August 20 (Law n.o 52/2018, of Aug 20, 2023). The Law proposed periodic sampling of water from heat transfer equipment associated with heating, ventilation, air systems, air conditioning units, or air treatment, which produce water aerosols. However, in our study, most of the isolates were recovered from water distribution systems in hospitals and hotels. Growth inhibition, due to a high concentration of disinfectant or another factor, could be a plausible explanation for this lower number of isolates from heat transfer equipment (García et al., 2007; Sanli, 2019).

Regarding the serological distribution, the predominant group was *Lp* sg 2-14, which matches with previous studies (Xiong et al., 2016; Graells et al., 2018; Torre et al., 2018; Assaidi et al., 2020; Cocuzza et al., 2021; Zhan et al., 2022).

Legionella susceptibility analysis is important to predict the evolution of antibiotic resistance and assess its impact on the treatment of LD. This topic has been the subject of several studies, and the results obtained show there is a need for standardization of methodologies, and the establishment of guidelines for all groups of *Legionella* isolates to optimize the detection of resistances. In this study, susceptibility against five antibiotics was evaluated in 57 environmental isolates, and our results corroborate the trend in the effectiveness already reported, with floroquinolones being the most effective antibiotic and tetracyclines the least effective (Xiong et al., 2016; Vandewalle-Capo et al., 2017; Wilson et al., 2018; Assaidi et al., 2020; Cocuzza et al., 2021).

In contrast, MIC 50 and MIC 90 values are not concordant with the literature, being higher for quinolones and macrolides (Vandewalle-Capo et al., 2017; Wilson et al., 2018; Cocuzza et al., 2021). Nonetheless, Xiong et al. (2016) obtained similarly high values for DOX in the *Lp* sg 1 group, and Assaidi et al. (2020), reported elevated MIC 50 and MIC 90 for all antibiotics compared to current guidelines, except for AZT (Xiong et al., 2016; Assaidi et al., 2020). In comparison with EUCAST's values, our MIC 50 and MIC 90 are bigger in *Lp* sg 1 and *Lp* sg 2-14 for AZT and CIP and in LEV and DOX for *Lp* sg 1, CLA for *Lp* sg 1, and LEV and DOX for *Lp* sg 2-14 agreed with the EUCAST guidelines (European Committee on Antimicrobial Susceptibility Testing, 2021).

EUCAST guidelines do not describe any MIC distributions for *L.* spp, although two other studies and these studies were in agreement with our results (Xiong et al., 2016; Assaidi et al., 2020).

The distribution of our MICs proved to be statistically different from those presented by EUCAST and in selected studies for *Lp* isolates (Wilson et al., 2018; Assaidi et al., 2020; Cocuzza et al., 2021; European Committee on Antimicrobial Susceptibility Testing, 2021). The comparison was performed using only studies that employed EUCAST methodologies, but the differences showed how important it is to carry out this type of investigation to provide additional data on *Lp* susceptibility so that an ECOFF can be defined. The global trend of increasing resistance levels implies a reduction of antibacterial treatment effectiveness that could have worrying consequences.

MIC values obtained through automated reading are higher than manual, making a defined cut-off difficult when comparing both methods. Operator variability and subjectiveness can contribute to a lack of standardization and as most results are read manually, so the comparison of MICs values can be controversial in studies.

To the best of our knowledge, this is the first time two phenotypically resistant isolates have been reported with such elevated MIC values (Table S1). These isolates possess an elevated risk, given their diminished susceptibilities to several of the first line antibiotics. While the LD mortality rate typically does not surpass 10% (European Centre for Disease Prevention and Control, 2022), cases have achieved rates of nearly 30%, in hospital-related LD cases (Beauté, 2017). The effect on increased morbidity caused by strains with this high MIC is unknown. Curiously, presence of the efflux pump gene, *lpeAB* was found in both isolates as expected given their MIC values for AZT.

Presence of *lpeAB*, in our isolates, was high. Previous studies reported a 50% prevalence (Portal et al., 2021b), but no studies have reported the *lpeAB* gene outside of *L. pneumophila*. This is the first time resistance has been reported in non pneumophila *Legionella*. Given the similarities between the MIC distributions for *L.* spp, with and without *lpeAB*, it is difficult to determine a clear cut-off value to distinguish the wild-type and *lpAB* positive isolates. This is mainly due to the small population analyzed.

Overall, these results do not diverge from EUCAST. Apart from a single *Lp* sg 2-14 all remaining isolates from this group and *Lp* sg 1 did not change the EUCAST distribution for LpeAB-positive isolates. Previous studies have also reported the presence of the efflux pump gene in isolates whose MIC values for AZT are equal to or exceed 0.125 mg/L (Vandewalle-Capo et al., 2017; Nata and Løva, 2019; Portal et al., 2021b). However, the presence of *lpeAB-negative* isolates with elevated MIC values as high as 0.5 mg/L was also observed in this study, and in previous works (Cocuzza et al., 2021; Yang et al., 2022). In this situation defining an ECOFF is a complex question given that the purpose is to discriminate between wild-type isolates and isolates containing acquired resistance mechanisms, and the efflux pump LpeAB has been described as an acquired resistance mechanism (Turnidge et al., 2006). Therefore, additional studies are required to provide robust ECOFF values.

The *tet56* gene was detected in one isolate with a MIC of 2 mg/L. This gene codes for a tetracycline destructase, and was first described in *L. longbeacheae* (Forsberg et al., 2015).

5 Conclusion

This study reports the susceptibility patterns of environmental Portuguese isolates, whose MIC distributions surpass EUCAST reference values. We also describe two phenotypically resistant isolates with high-level floroquinolone resistance. It would be interesting to pursue further genotypical characterization of these isolates, in particular the *parC, gyrB, parE, rrl, rplD* and *rplV* genes.

The study highlights the presence of the efflux pump gene *lpeAB* in Portuguese isolates, and the presence of the *tet56* gene in a single *L. anisa* isolate which, to the best of our knowledge, is the first reported instance regarding presence of tetracycline destructase in this *Legionella* species.

The current epidemiologic trends of growing incidence of LD cases in several countries, allied with the global antibiotic resistance accentuates the task of establishing robust ECOFF values, clinical breakpoints, and a standardized methodology. The present research points to the current need to increase knowledge about environmental populations of *Legionella* to predict the potential emergence of antibiotic resistance in clinical isolates.

Data availability statement

The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

CC carried out the experiments, analyzed the data, and wrote the original draft manuscript. LR made data interpretations and revised and edited the manuscript. FF isolated *Legionella* and made data interpretations. RS collected the environmental samples and revised the manuscript. PP verified the data and revised the manuscript. MC designed the study, verified the data, and revised and edited the manuscript. 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1141115/full#supplementary-material

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A community outbreak of Legionnaires' disease caused by outdoor hot tubs for private use in a hotel

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During the period October–November 2017, an outbreak of Legionnaires' disease involving 27 cases occurred in the tourist area of Palmanova (Mallorca, Spain). The majority of cases were reported by the European Centre of Disease Prevention and Control (ECDC) as travel associated cases of Legionnaires' disease (TALD). Most cases belonged to different hotel cluster alerts. No cases were reported among the local population residing in the area. All tourist establishments associated with one or more TALD cases were inspected and sampled by public health inspectors. All relevant sources of aerosol emission detected were investigated and sampled. The absence of active cooling towers in the affected area was verified, by documents and on-site. Samples from hot tubs for private use located on the terraces of the penthouse rooms of a hotel in the area were included in the study. Extremely high concentrations (> 10^6CFU/l) of *Legionella pneumophila*, including the outbreak strain, were found in the hot tubs of vacant rooms of this hotel thus identifying the probable source of infection. Meteorological situation may have contributed to the geographical distribution pattern of this outbreak. In conclusion, hot tubs for private use located outdoors should be considered when investigating community outbreaks of Legionnaires' disease of unclear origin.

KEYWORDS

Legionnaires' disease, community outbreak, outdoor hot tubs, hotel, outbreak investigation

1. Introduction

Legionnaires' disease (LD) is a respiratory illness caused mainly by inhalation of aerosolized water contaminated by *Legionella* bacteria. These bacteria are found in both natural and artificial water systems. In addition, *Legionella* can also cause Pontiac fever, an acute febrile, self-limiting, and flu-like illness. Outbreaks of LD have been most frequently linked to cooling towers or evaporative condensWPers, hot water systems and spa-type pool systems although other sources, like humidifiers, ornamental fountains, cooling misting devises, irrigation systems, and others have been identified (World Health Organization, 2007; European Center for Disease Prevention and Control, 2022).

Cases of LD are categorized as being community, travel or hospital acquired based on the type of exposure. For travel-associated cases, experience has shown that clusters initially associated with a certain hotel are actually cases of a more far-reaching community outbreak. Inversely, community-acquired outbreaks can involve international travellers living in other countries. In both cases, exchanging information between the local, national health authorities of the affected countries and ECDC may be of great interest for identifying the origin of the outbreak (Decludt et al., 1999).

Bathing and recreational waters, in their various types, have often been implicated in outbreaks of LD and Pontiac fever. Hot tubs, also called spa pools, whirlpools and whirlpool spas, have been linked to *Legionella* infections, especially when poorly maintained (World Health Organization, 2006). The outbreaks have almost always occurred indoors affecting users or people in the same building area or in close proximity. In these cases, outbreaks can be very large, such as the one that occurred in 1999 in the Netherlands, which affected many visitors to a flower show (Den Boer et al., 2002). When they have occurred outdoors, they have affected a smaller number of people, mostly the users themselves or people that were very close to the source pool (Leoni et al., 2018; Hlavsa et al., 2021).

We describe here a widely dispersed LD community outbreak that almost exclusively affected international visitors to various hotels in a tourist area of Mallorca, Spain, caused by hot tubs for private use located on the terraces of the penthouses of a hotel in the area. The outbreak followed a geographical distribution pattern somewhat reminiscent of those described in different outbreaks caused by cooling towers. The outbreak was investigated to determine the source of infection, to stop further transmission and to prevent further cases. To our knowledge, this is the first community outbreak caused by outdoor hot tubs that has affected a wide geographical area and highlights the importance of considering these systems when investigating community outbreaks of uncertain origin.

2. Methods

2.1. Setting and summary history of the outbreak

Palmanova, a small town, located by the Mediterranean Sea, is one of the most popular tourist destinations on the island of Mallorca. It has a wide variety of tourist accommodation (hotels, aparthotels, and tourist apartments) and leisure areas, all in an area of approximately 1.5 Km^2.

Between 4th October 2017 to 16th November 2017, twenty-seven cases of LD associated with the area of Palmanova were reported to the Spanish Health Authorities. Most cases were reported by ECDC as TALD in different cluster alerts. The ECDC published a rapid risk assessment report about the outbreak on the 23rd October 2017 (European Center for Disease Prevention and Control, 2017).

2.2. Epidemiological investigation

All cases except three were notified to the Spanish Health Authorities by the European Legionnaires' Disease Surveillance Network (ELDSNet) according to standardized procedures (European Center for Disease Prevention and Control, 2012). ELDSNet is an ECDC surveillance scheme. The data reported included, as a minimum, date of onset of symptoms, dates of stay and detail of the accommodation site. In addition, the health authorities of some member states sent directly to the Spanish National Epidemiology Center, additional information on cases, useful for the investigation of the outbreak, e.g., places visited during their stay. Three cases were reported by local Spanish hospitals to the National Epidemiological Surveillance Network following national regulations (Ministerio de Sanidad, Servicios Sociales e Igualdad, 2015). Confirmed and probable case definitions in accordance with the ECDC were used (Official Journal of the European Union, 2008) with an onset date after 1 September 2017 and a history of staying in or visiting the Palmanova area in the 2–10 days before onset of disease. No further epidemiological investigations were carried out at national level.

2.3. Environmental investigation

All tourist establishments associated with one or more TALD cases were inspected and sampled by public health inspectors. The locations of the accommodation sites were georeferenced using visual pinpointing in Google Earth and distances were calculated with the same tool. Possible incidents in the operation of the municipal water distribution network, which was also sampled, were investigated. All relevant sources of aerosol emission detected were investigated and sampled: public and private sprinkler irrigation systems, ornamental fountains, beach showers, street cleaning vehicles, and a car wash station. An active search was carried out, with the help of the local police, of other possible sources of aerosol emission, including misting cooling units that had been in operation during the outbreak period and the previous month. The absence of active cooling towers in the affected area was verified by documents and on-site. Finally, samples of hot tubs for private use located on the terraces of the penthouse rooms of a hotel in the area, Hotel A, were included in the study.

Weather data corresponding to the month of September 2017, were retrospectively retrieved from two meteorological stations, Calvià and Son Ferriol, through the tool SIAR (Spanish Agroclimatic Information System for Irrigation).[1] The data on winds were retrieved through specialized portals from the weather station of a nautical club in the area of Palmanova and from the Port of Palma de Mallorca.[2]

2.4. Microbiological investigation

2.4.1. Clinical specimens

Urine and lower respiratory samples (if available) from cases travelled from United Kingdom were sent to the Respiratory and Vaccine Preventable Bacteria Reference Unit (RVPBRU), UKHSA, for confirmation, detection and typing. Lower respiratory tract samples

1 https://eportal.mapa.gob.es/websiar/SeleccionParametrosMap.aspx?dst=1

2 https://windy.app/forecast2/spot/2648671/Club+N%C3%A1utico+Palmanova/statistics and https://es.windfinder.com/windstatistics/palma_de_mallorca_puerto

were only available for six cases. These samples were tested by a *Legionella pneumophila* serogroup Sg 1 qPCR assay (Mentasti et al., 2015). Sequence Based Typing (SBT) was performed on *L. pneumophila* Sg 1 isolates obtained from two cases (Gaia et al., 2005; Ratzow et al., 2007). Nested SBT was performed on direct clinical samples from the remaining four cases in the absence of isolates (Fry et al., 2006). SBT data were analyzed using the web-based *L. pneumophila* SBT database.[3] This link is undergoing development and is currently unavailable externally but can be accessed internally by the database curators at UKHSA.[4]

Information regarding clinical samples from other cases (non-UK cases) was received from ELDSNet or provided by the local hospitals that reported the cases. SBT data from one clinical *L.pneumophila* Sg1 isolate was received from the National Legionella Reference Laboratory (Spain).

2.4.2. Environmental samples

Environmental samples were collected following standardized protocols (Ministerio de Sanidad y Consumo, 2003) and were analyzed for *Legionella* spp. according to UNE-ISO 11731:2007 – *Water quality-Detection and enumeration of Legionella standard*. The isolates were further identified and serotyped (*L. pneumophila* Sg 1, *L. pneumophila* Sg 2–14 and *Legionella* non-pneumophila species) using latex agglutination reagents (Oxoid, Spain). Further monoclonal antibody (MAb) typing was carried out on the *L. pneumophila* Sg 1 isolates, using an international panel of seven monoclonal antibodies (Dresden panel) (Helbig et al., 1997, 2002).

Genotyping on selected strains (two from each MAb type identified) was performed according to the seven gene protocol from ESCMID Study Group for Legionella Infections (ESGLI) consensus Sequence-Based Typing (SBT) scheme (Gaia et al., 2005; Ratzow et al., 2007) at the National Legionella Reference Laboratory (Czech Republic). The online *L. pneumophila* SBT sequence quality tool was used to determine individual sequence type (ST) for each sample. The standardized protocols including the amplification and sequencing primers for *L. pneumophila* SBT and direct nested SBT, are available at the website https://bioinformatics.phe.org.uk/legionella/legionella_sbt/php/sbt_homepage.php. This website is undergoing development and is currently unavailable externally but can be accessed internally by the database curators at UKHSA (see text footnote 4).

2.5. Control measures

Control measures started on the 6th October 2017, after the outbreak was declared. The following actions were taken in the neighbourhood: the public and private irrigation systems using sprinklers were shut down; the use of street cleaning vehicles was stopped; an urban fountain, a car washing station and the beach showers were closed; one hotel was closed down after deficiencies were detected and several water samples taken at the premises were positive for *Legionella* spp. Hot tubs on the terraces of the vacant rooms were drained and taken out of service. All premises involved in the investigation were ordered to clean and disinfect the closed systems and re-test before reopening, according to local regulations (Ministerio de Sanidad y Consumo, 2003).

3. Results

3.1. Epidemiology

Twenty seven cases of Legionnaires' disease, including a fatal case, were reported during this outbreak (26 confirmed cases and 1 probable case). Twenty four cases were diagnosed outside Spain, upon return of the cases to their country of origin. Cases were reported by the United Kingdom (18), Denmark (2), France (2), Czech Republic (1) and Sweden (1). The cases were aged between 46 and 87 years and include 15 males and 12 females. The illness onset occurred between 11th September 2017 and 17th October 2017. All cases except one were tourists staying in ten different hotels or tourist apartments in the affected area. One case was working at a hotel not previously related to this cluster (Figure 1).

One of the hotels was associated with 10 cases, one with four, one with three, two with two cases, and the rest with one case. All hotels associated with cases were located within a radius of about 500 meters from what was considered initially the epicenter of the outbreak, i.e., the hotel associated with 10 cases (Figure 2).

3.2. Environmental findings

Inspections of potential communal sources (public showers on the beach, ornamental fountains, sprinkler irrigation systems, car wash stations) did not reveal any deficiencies. One of the hotels presented some structural and operational deficiencies in the drinking water system (high corrosion levels in hot water tanks, low hot water temperatures).

Hotel A was located next to the hotel associated with 10 cases and consisted of seven buildings with three or four floors. Each building had penthouses on the top floor with hot tubs on the terraces. These hot tubs are for the exclusive use of the guests staying in these rooms. At the time of the visit, on the 18 and 19 October 2017, there were seventy five hot tubs with a volume of 1.1 m^3 water each, but only fifty nine were in use. The hot tubs were equipped with a recirculation pump, a heater and multiple water jets with passive air mixing. The water was treated manually with bromine tablets put into the filter housing. The average water temperature was 32.7°C. The average total bromine level was 2.1 ppm (required range by Spanish regulations: 2.0–5.0 mg/L).

At the time of the visit, the occupancy rate for the penthouse rooms was about 50%. In the vacant rooms, the hot tubs were left filled with water but without regular checks or maintenance. They were connected to the main electrical switch in the room thus implying that there was no recirculation or treatment when not in use. We found 59% of them with bromine levels <2.0 ppm.

In September 2017, the average temperature in Son Ferriol was 21.3°C (range: 13.2°C–30.7°C). The relative humidity fluctuated between 21.4 and 98.6% with an average of 73.1% and rainfall was <1 mm. In Port of Palma, the wind speed was generally low, less than

3 https://bioinformatics.phe.org.uk/legionella/legionella_sbt/php/sbt_homepage.php

4 http://legionella-sbt@ukhsa.gov.uk

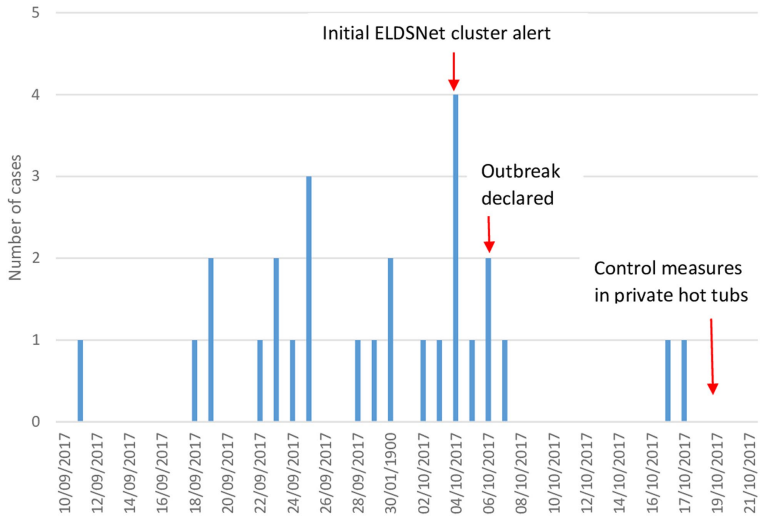

FIGURE 1
Distribution of cases of Legionnaires' disease by date of symptom onset (n=27).

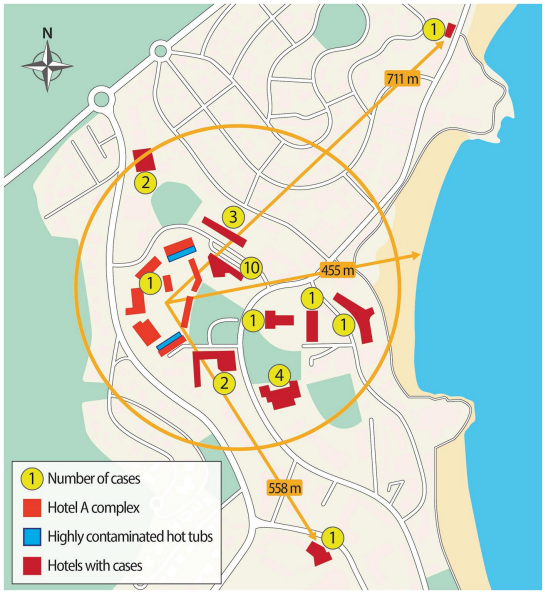

FIGURE 2
Schematic diagram of the affected area, accommodation sites associated with cases, and some selected distances.

6.1 m/s, and the direction of the wind was variable, prevailing N, S, SW, and NE. In the Calvià station, the average temperature in the last 10 days of September was 20.6°C and the average relative humidity was 79.4%. At the Palmanova nautical club station, the prevailing annual winds were S, SW, NE, and NW, being generally light, with a speed of less than 6.1 m/s.

3.3. Microbiological results

Twenty-six cases were laboratory confirmed by urinary antigen and one case by qPCR detection. One clinical isolate (Case 12; Table 1) was identified as *L. pneumophila* Sg 1, MAb type France/Allentown, ST82. *L. pneumophila* serogroup 1, ST 82 isolates were also obtained from two cases travelled from United Kingdom (Case 8 and Case 11; Table 1). Although *L. pneumophila* was not isolated from four additional cases, nested SBT on direct clinical samples yielded partial allelic profile consistent with ST 82 Table 1.

146 water samples from different potential sources initially suspected were collected and tested for *Legionella* spp. (122 samples from domestic water systems of the hotels associated with cases, seven from street cleaning vehicles, six from irrigation water systems, six from misting cooling units, four from aquatic parks, ornamental fountains, and beach showers and one from a car washing station). Nineteen samples yielded positive results to *L. pneumophila* sg1 (range: < 400 CFU/l - > 10^6 CFU/l). These isolates were identified as MAb type OLDA, OLDA/Bellingham, or Benidorm that were distinct from the LD cases (Mab type France/Allentown) Table 1.

Additionally, 48 water samples were taken from the hot tubs at the penthouses of the hotel A. Positive results to *L. pneumophila* Sg 1 were obtained in 38 samples. Twelve samples were from hot tubs in vacant rooms, the concentration of *Legionella* spp. was >10^6 CFU/l. In the rest of the samples, *Legionella* spp. concentrations of 10^2–10^3 CFU/l were obtained (parametric value in Spanish regulations: <10^2 CFU/l). *L. pneumophila* serogroup 1, MAb (France/Allentown), ST82 was identified mixed with OLDA/Bellingham strains Table 1.

No positive results were obtained from samples collected from any of the other sampled systems (drinking water, irrigation, outdoor recreational pool, and indoor hydrotherapy pool) of Hotel A.

4. Discussion

The hot tubs for private use on the upper terraces of penthouse rooms of Hotel A were the most likely source of this community outbreak. Both, the environmental findings (a plausible source, located just beside the epicenter of the outbreak) and the microbiological findings (high proportion of positive systems,

TABLE 1 SBT profiles and MAb types of the relevant clinical specimens and environmental strains.

Clinical specimens				
Origin	SBT (allelic profile)	Isolate	DNA extract	MAb Type
Case 2[a]	SBT: undetermined (5,0,0,10,6,10,6)		Yes	
Case 8[a]	ST 82 (5,1,22,10,6,10,6)	Yes		NT[b]
Case 9[a]	SBT: undetermined (5,0,0,10,6,10,6)		Yes	
Case 11[a]	ST 82 (5,1,22,10,6,10,6)	Yes		NT[b]
Case 12[c]	ST 82 (5,1,22,10,6,10,6)	Yes		France/Allentown
Case 16[a]	SBT: undetermined (5,0,0,10,6,10,6)		Yes	
Case 18[a]	SBT: undetermined (0,0,0,0,0,0,6)		Yes	
Environmental strains				
	SBT (allelic profile)	**Isolate**		**MAb Type**
Hot Tub[d] (Hotel A)	ST 82 (5,1,22,10,6,10,6)	Yes		France/Allentown

[a]Sample tested at RVPBRU, UKHSA.
[b]Not tested.
[c]Sample tested at National Legionella Reference Laboratory, Spain.
[d]Sample tested at National Legionella Reference Laboratory, Czech Republic.

extremely high concentrations of *Legionella* in significant proportion of the hot tubs and identical MAb and sequence type as the outbreak strain) strongly support this conclusion.

This outbreak is extraordinary in different aspects. First, to our knowledge, this could be the first outbreak of LD caused by outdoor hot tubs affecting a wide geographical area. *Legionella* infections in non-users (visitors to the premises where the hot tubs are located, or people exposed nearby) have been well documented in different outbreaks both indoors and outdoors but outbreaks covering a large distance have not been previously reported. This outbreak involved cases staying in hotels located in a radius of about 400 meters from the source, with two exceptions located at 558 m and 711 m, respectively. Almost all the affected hotels were distributed in a circular sector whose radii are oriented to the NE and SE of Hotel A, respectively. There are no cases to the west, as the hotel is located on the western limit of the urbanized area. Not surprisingly, about half of the cases occurred in hotels located adjacent to Hotel A, one with 10 cases, a second with three cases and a third with two cases. However, one of the affected hotels, located just over 300 m from the source hotel, had four affected and another located about 200 m away had 2 cases. It is unlikely that these last six cases would have approached the Hotel A, that was on the outskirts of town and away from the beach. In this sense, the pattern of distribution of cases of this outbreak is very reminiscent of patterns seen in outbreaks caused by cooling towers where bioaerosols can travel hundreds of meters from the source (Sabria et al., 2006; Ferré et al., 2009; Weiss et al., 2017; Dyke et al., 2019) rather than outbreaks caused by hot tubs where typically only users or people staying in their vicinity are affected (Benkel et al., 2000; Götz et al., 2001; Den Boer et al., 2002; Coetzee et al., 2012). This could be due to several reasons. In some hot tubs, the water level dropped below the nozzles and the water jets were directed into the air, causing greater aerosolization. We speculate that this, together with the high number of contaminated hot tubs and the high concentration of *Legionella* in the water combined with the location on the rooftop terraces and dispersal by wind, may explain the dissemination pattern of the aerosols. The case location data are consistent with the pattern of atmospheric distribution that would be expected in such a case: more cases in the vicinity and fewer as we move further from the source.

Second, the high proportion of hot tubs colonized and the very high concentrations of *Legionella* spp. in many of them is surprising. An extensive study of the prevalence of *Legionella* in various water systems of hotels in the Balearic Islands has found that 10.9% of the hot tubs were contaminated with *Legionella* (Doménech-Sànchez et al., 2022). Another study in Quebec found that *Legionella* spp. was detected in 23% of public whirlpool spas (Brousseau et al., 2013). These data are still very far from the 79.1% colonized systems that we found in the Hotel A. The hot tubs were contaminated with the same strain, that was not found anywhere else. A plausible explanation for contamination is by bioaerosols emitted by the contaminated hot tubs seeding adjacent or nearby terraces. The terraces of the penthouses were separated by 1.5 m high walls, although this was reduced to approximately 1 m at the outer edge. As the hot tubs were 0.75 m in height and the distance between each hot tub was only a few meters, it is not difficult to imagine that the emitted aerosols could easily contaminate neighbouring hot tubs. In addition, contaminated hot tubs with high levels of *Legionella* spp. were almost all clustered in two of the seven blocks, suggesting again aerosol contamination. Contamination by other means, for example, through the maintenance staff or their equipment, is less likely since the hot tubs were not regularly maintained in the unoccupied penthouses. In addition, it is likely that stagnation and the reduced maintenance of the hot tubs in the vacant rooms contributed to the growth of the bacteria. In fact, bromine concentrations below required levels and mid-ranged water temperatures could have facilitated microbial growth. It is known that low halogen levels and poor maintenance increase the relative risk of *Legionella* colonization in spa-type pools (Brousseau et al., 2013; Papadakis et al., 2018).

According to the retrieved meteorological data, in September 2017, weather conditions could have favoured transmission and the duration of the outbreak. The average relative humidity was high. It has been reported that increased high humidity is positively associated with increased incidence of LD (Pampaka et al., 2022). Similarly, the winds speeds were low, that could lead to slower dispersion of aerosols.

The variable wind direction and the prevailing winds, especially S and SW, are consistent with the pattern of geographic distribution of the cases. Different studies have suggested that climatic conditions are related to the risk of Legionella infection (Simmering et al., 2017; Walker, 2018) and meteorological studies have usually been included in investigations of legionellosis outbreaks caused by cooling towers or similar equipment (Kirrage et al., 2007; Nygård et al., 2008; Shivaji et al., 2014). To the best of our knowledge, this would be the first time that the analysis of weather conditions has been applied to an outbreak caused by hot tubs, albeit retrospectively.

Another notable aspect of this outbreak was that the hot tubs involved were for the private use of customers in penthouse rooms. This is important since in Spain, swimming pools and similar bathing systems for private use, even if they are in commercial buildings such as hotels and similar, were expressly excluded from the current regulations (Ministerio de Sanidad, Servicios Sociales e Igualdad, 2013), except for the notification of incidents and that their existence, number, and location must be notified before putting them into operation in the regional registry. In this way, these systems for private use were not necessarily subject to the same regulatory obligations as swimming pools and hot tubs for public or collective use, including regular maintenance and sampling. Interestingly, in recent years, the number of hotels that incorporate hot tubs for private use on room terraces has increased in general and the use of these systems has become popular both in the public and private settings. Extensive use of spa pools, combined with inadequate maintenance, has contributed to outbreaks of Legionnaires' disease, which in many cases have originated in a hotel (Centers for Disease Control and Prevention, 2004; Hlavsa et al., 2021). Therefore, the implementation of guidelines and recommendations for good practices for the use and maintenance of hot tubs for private use could help prevent *Legionella* infections. Following this outbreak, the Health Authorities of the Balearic Islands published a guide for the hygienic maintenance of hydro massage vessels that also included those for private use (Govern Balear (GOIB), 2018).

No cases were reported among the local population residing in the area and most cases were British tourists, but this was a very popular with British tourist area (European Center for Disease Prevention and Control, 2017). The people who work in the area are mostly staff from hotels and commercial establishments, so work indoors and unlikely to be exposed. Local doctors are familiar with LD and local hospitals have access to urinary antigen testing. Therefore, it is unlikely that they missed cases among the native population, especially considering that the outbreak was a high profile and reported by the local media. In summary, the pattern of the affected population reflects the pattern of the exposed population well.

This outbreak also highlights the importance of the European surveillance scheme for cases associated with travel ELDSNet. The rapid notification of several clusters associated with hotels in the Palmanova area allowed the early identification of this community outbreak and the subsequent launch of an investigation and implementation of control measures. The exchange of relevant information by ELDSNet relating to the molecular profile of the outbreak strain, was very important for investigating the origin of the outbreak. Only three cases were diagnosed locally, and the investigation would have been very limited in the absence of information on the other cases. Sharing information on community outbreaks that also includes TALD cases allows for active case finding with helpful information on source identification. In this outbreak, the first ELDSNet notification was received on October 4, 2017, when twenty one of the twenty seven cases had already been infected (and had presented with symptoms). Although this frequently happens in outbreaks, it is necessary to emphasize the need to declare legionellosis cases as quickly as possible in order to be able to respond rapidly. Once again, the ELDSNet model, which collects data from all member countries of the network, makes it possible to quickly identify clusters of cases and promote rapid investigations and responses from local health authorities.

In this outbreak, the environmental investigations were key to identify its origin. In the absence of epidemiological data (most reported cases were tourists that had already returned to their country of origin), extensive sampling of possible sources allowed the source to be identified.

This outbreak investigation had several limitations. First, most of the cases were tourists from other countries, so it was not possible to obtain detailed questionnaires about their movements prior to becoming ill as having this information could have facilitated a clearer understanding about the distance travelled by contaminated aerosols. In the absence of this information, it therefore cannot be ruled out that the cases had been close to or even visited Hotel A. Secondly, it was not possible to investigate contaminated hot tubs usage by guests in the Hotel A penthouses. It is probable that some were used given that the occupancy of the penthouse on the sampling dates was 50% and that the proportion of hot tubs found positive for *Legionella* was very high. The fact that only one client of hotel A was affected is surprising but the hot tubs with the highest *Legionella* load were in the unoccupied rooms and their location, on the upper parts of the building, may have facilitated the spread of bioaerosols away from Hotel A. All infections occurred prior to hot tubs sampling, and it cannot be ruled out that the hot tubs in the occupied rooms were in a proper hygienic state. Indeed, it is possible that contamination levels fluctuate significantly over short periods of time (Wéry et al., 2008; Napoli et al., 2009), and even more in low volume systems with manual chemical dosing, thus making it difficult to draw conclusions on the water quality in the days when the infections occurred. In retrospect, we acknowledge that it would have been of interest to study the quality of the hot tub water in more depth, for example, analyzing the presence of *Escherichia coli*, *Pseudomonas aeruginosa*, amoeba and other physicochemical parameters, such as turbidity, which could have shed more light on the hygienic state of the hot tubs in question and perhaps on other aspects such as the pathogenicity or infectivity of the *Legionella* isolates.

No meteorological data was obtained at the time of the events and the data on the weather had to be obtained retrospectively. There was no weather data specifically from the affected area prior to the outbreak, although we believe that the data we have for the meteorological station of Son Ferriol (22 Km from Palmanova) and the Port of Palma de Mallorca (9 km from Palmanova) are quite representative of what the local conditions would have been in Palmanova. Indeed, the partial temperature and wind data that was obtained from the meteorological station located in the nautical club of the affected area was consistent with the above.

Our research shows that private outdoor hot tubs can disseminate aerosols distant from the premises in which they are located and raises the need to register these systems, especially when they are located in highly populated areas. It is also important that manufacturers and

health authorities provide adequate instructions to operators and users for their safe use. In conclusion, our investigation shows that hot tubs for private use located outdoors should be considered as a potential source when investigating community outbreaks of LD of unclear origin and underlines the importance of adequate maintenance, water treatment and controls of hot tubs.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SC, MG, and RC: conceptualization. SC, RC, MG, BS, and AN: data curation. SC, VD, RC, AN, MG, MB, JB, PC, BA, and BS: investigation and writing—review and editing. SC, VD, RC, MG, and AN: data analysis. SC: writing—original draft. 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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Long-read sequencing for reliably calling the *mompS* allele in *Legionella pneumophila* sequence-based typing

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Sequence-based typing (SBT) of *Legionella pneumophila* is a valuable tool in epidemiological studies and outbreak investigations of Legionnaires' disease. In the *L. pneumophila* SBT scheme, *mompS2* is one of seven genes that determine the sequence type (ST). The *Legionella* genome typically contains two copies of *mompS (mompS1* and *mompS2)*. When they are non-identical it can be challenging to determine the *mompS2* allele, and subsequently the ST, from Illumina short-reads. In our collection of 233 *L. pneumophila* genomes, there were 62 STs, 18 of which carried non-identical *mompS* copies. Using short-reads, the *mompS2* allele was misassembled or untypeable in several STs. Genomes belonging to ST154 and ST574, which carried *mompS1* allele 7 and *mompS2* allele 15, were assigned an incorrect *mompS2* allele and/or *mompS* gene copy number when short-read assembled. For other isolates, mainly those carrying non-identical *mompS* copies, short-read assemblers occasionally failed to resolve the structure of the *mompS*-region, also resulting in untypeability from the short-read data. In this study, we wanted to understand the challenges we observed with calling the *mompS2* allele from short-reads, assess if other short-read methods were able to resolve the *mompS*-region, and investigate the possibility of using long-reads to obtain the *mompS* alleles, and thereby perform *L. pneumophila* SBT from long-reads only. We found that the choice of short-read assembler had a major impact on resolving the *mompS*-region and thus SBT from short-reads, but no method consistently solved the *mompS2* allele. By using Oxford Nanopore Technology (ONT) sequencing together with Trycycler and Medaka for long-read assembly and polishing we were able to resolve the *mompS* copies and correctly identify the *mompS2* allele, in accordance with Sanger sequencing/EQA results for all tested isolates (n=35). The remaining six genes of the SBT profile could also be determined from the ONT-only reads. The STs called from ONT-only assemblies were also consistent with hybrid-assemblies of Illumina and ONT reads. We therefore propose ONT

sequencing as an alternative method to perform *L. pneumophila* SBT to overcome the *mompS* challenge observed with short-reads. To facilitate this, we have developed ONTmompS (https://github.com/marithetland/ONTmompS), an *in silico* approach to determine *L. pneumophila* ST from long-read or hybrid assemblies.

KEYWORDS

Legionella pneumophila **SBT, mompS, WGS, Illumina, short-reads, ONT, long-reads**

1 Introduction

The *Legionella*-bacteria can cause a severe and potentially fatal form of pneumonia called Legionnaires' disease (LD). When the bacteria colonize and multiply in man-made systems with favorable conditions for growth, it may pose a threat to human health, through inhalation of bacteria-contaminated aerosols (Bartram et al., 2007; Whiley et al., 2014). There are more than 60 known species of *Legionella* with varying pathogenicity (Parte et al., 2020). *Legionella pneumophila* is the species implicated in at least 90% of the reported LD cases worldwide (reviewed in Herwaldt and Marra, 2018; Chauhan and Shames, 2021). *L. pneumophila* can be subtyped into at least 15 serogroups based on surface molecules and also into sequence types (STs) determined by the seven genes *flaA, pilE, asd, mip, mompS, proA* and *neuA* (Gaia et al., 2005; Ratzow et al., 2007). The current number of defined STs is over 3100 (Legionella-SBT, 2023).

Sequence-based typing (SBT) is a valuable tool for source investigations and epidemiological studies of *L. pneumophila* that allows for rapid molecular typing and inter-laboratorial comparison (Gaia et al., 2005; Ratzow et al., 2007). Although *L. pneumophila* SBT performed by Sanger sequencing is still considered the gold standard, more recently, whole-genome sequencing (WGS) has become the method of choice, providing both ST and superior information about genetic relatedness, and is today indispensable in surveillance and outbreak investigations (Moran-Gilad et al., 2015; Khodr et al., 2016; Raphael et al., 2016; Krøvel et al., 2022; Ricci et al., 2022). Analyses like core or whole genome multi locus sequence typing (cgMLST/wgMLST) and single nucleotide polymorphism (SNP) analysis have higher discriminatory power than SBT and would provide more information to aid the identification of the source of an outbreak.

A challenge in the *L. pneumophila* SBT scheme is that the *mompS* gene, which is used to determine the ST, is usually present with two copies in the genome, *mompS1* and *mompS2*. Only *mompS2* contributes to the ST (Gaia et al., 2005; Gordon et al., 2017). The two *mompS* copies are closely positioned in the genome, resulting in a *mompS*-region of about 2100 bp (see Figure 1). *L. pneumophila* SBT by Sanger sequencing amplifies *mompS2* using specific primers. Short-read WGS gives a maximum of 300 bp reads, which are too short to cover the *mompS* gene let alone the entire *mompS*-region. When the two *mompS* copies are non-identical, existing *in silico* approaches for *L. pneumophila* SBT have been shown to have limitations, related to erroneous calling of the *mompS* alleles or incorrect assembly of the *mompS*-region, which may result in incorrect ST determination and/or untypeabilty (Gordon et al., 2017).

Illumina short-read WGS has so far been the method of choice for genomic surveillance because of the higher basecalling accuracy compared to long-read sequencing technologies like Oxford Nanopore Technologies (ONT). However, challenges with untypeability and resolution of repetitive regions due to the short read length has led to exploration of long-read sequencing as an alternative (Ben Khedher et al., 2022). At the moment, hybrid-assemblies of short- and long-reads are considered the WGS gold standard over ONT-only assemblies with regards to accuracy (Wick et al., 2023).

In our laboratory, we have observed several challenges related to calling *mompS* from short-reads. In diagnostics and surveillance of *Legionella*, time and cost is of the essence and generation of hybrid assemblies for all samples is not likely to be standard procedure. Therefore, the possibility of using ONT-only data to overcome the shortcomings observed in *L. pneumophila* SBT from short-reads is highly relevant and has to our knowledge not previously been investigated. In this study, we wanted to understand the challenges we observed using short-reads, assess if other short-read methods were able to resolve the *mompS*-region and investigate the possibility of using long-reads to obtain the *mompS* allele, and thereby perform *L. pneumophila* SBT from long-reads only.

2 Material and methods

2.1 Sample collection

At the National Reference Laboratory for *Legionella*, Stavanger University Hospital, Norway, we have a collection of Illumina sequenced clinical and environmental isolates, collected between 2001 and 2022 (n=233). Of these, 35 were selected for further analysis in this study, based on discrepancies between the *mompS* result from Illumina short-read and Sanger sequencing, presence of non-identical *mompS* copies or untypeability of *mompS* (n=27). Isolates with identical copies of *mompS* were included as controls (n=8).

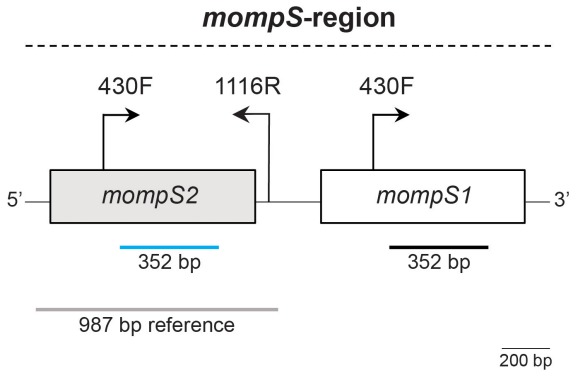

FIGURE 1
The *mompS*-region. The two *mompS* copies are closely positioned in the genome. The first step of Sanger sequencing uses primers 430F and 1116R to specifically amplify *mompS2* (Gaia et al., 2005; Gordon et al., 2017). The 352 bp conserved region used for determination of *mompS2* in *L. pneumophila* SBT is marked by a blue line. The *mompS1* gene also contains the primer binding site for primer 430F and a 352 bp conserved region, marked by a black line. A reference sequence defined by Gordon et al. (2017) to distinguish *mompS2* from *mompS1*, which is also used in our ONTmompS *in silico* approach, is indicated by a grey line. The 987 bp reference sequence covers the 5' flanking region from position 157, the entire coding sequence of *mompS2* (204-1070) and the 3' flanking region including primer 1116R. The positions refer to those in the GenBank sequence AF078136, which was used to design the original primers for Sanger sequencing. A 200 bp scalebar is indicated.

A further 46 isolates were included later in the study to validate the ONTmompS *in silico* SBT approach that we developed for assigning *L. pneumophila* ST (see section 3.3).

2.2 Whole-genome sequencing

The 35 *L. pneumophila* isolates had previously been Illumina short-read sequenced and the fastq-files deposited in our in-house sequence database. In short, DNA had been extracted using MagNA Pure 96 with the Pathogen Universal 200 4.0 purification protocol (Roche Applied Science, Penzberg, Germany). Genomic libraries were prepared using Illumina DNA library prep and sequenced to ≥30X read depth using the Illumina MiSeq platform (see Table S1 for details and BioSample accessions).

The same 35 isolates were long-read sequenced in this study. DNA was extracted using the GenFind V3 kit (Beckman Coulter, Indianapolis, United States). Genomic libraries for long-read sequencing were prepared using the Kit12 chemistry and the ligation sequencing kit (SQK-LSK112) in combination with the R10.4 MinION flow cells (n=34), or the Kit9 ligation sequencing kit (SQK-LSK-109) in combination with the R9.4.1 flow cell (n=1) from ONT (Oxford, UK). All libraries were sequenced on the ONT GridION device (GRD-X5G003), aiming for ≥100X read depth. Guppy v6.4.2 was used to basecall and demultiplex the fast5-files with the super accurate basecalling model (see Table S1 for details).

The 46 isolates used for validation of the ONTmompS *in silico* *L. pneumophila* SBT approach were ONT sequenced using Kit9/R9.4.1 (n=39) or Kit12/R10.4 (n=7) and basecalled with Guppy v6.4.2 using the super accurate model (see Table S2 for source details and BioSample accessions).

2.3 Assembly and polishing

For the short-reads we used Asmbl v0.2.0 (https://github.com/marithetland/Asmbl) to perform read trimming, *de novo* assembly and quality control: TrimGalore v0.6.7 (https://github.com/FelixKrueger/TrimGalore) was used to remove adapter-contamination and low-quality reads, prior to assembly. We generated two sets of short-read assemblies (for all 35 genomes): The first with Unicycler v0.5.0 (Wick et al., 2017), which uses SPAdes v3.15.5 (Bankevich et al., 2012) for assembly, and the second with Unicycler v0.4.8, which uses SPAdes v3.13.0, includes read error correction and short-read polishes the assembly with Pilon v1.24. In addition, we created assemblies with the following tools and parameters: 1) Unicycler v0.4.8 with SPAdes v3.13.0 without read error correction, 2) without Pilon short-read polishing, and 3) without both, 4) Unicycler v0.5.0 with SPAdes v3.13.0, 5) SPAdes v3.15.5 on its own, 6) SPAdes v3.13.0, and 7) SKESA v2.4.0 (Souvorov et al., 2018).

For the long-reads, Filtlong v0.2.1 (https://github.com/rrwick/Filtlong) was used to discard the worst 5% of read bases and any reads shorter than 1 kbp prior to assembly. We then generated long-read assemblies with three different tools: Unicycler v0.5.0 (which uses a miniasm v0.3-r179 + racon v1.5.0 pipeline), Flye v2.9 (Kolmogorov et al., 2019), and Trycycler v0.5.3 (Wick et al., 2021). Trycycler was run according to the instructions at https://github.com/rrwick/Trycycler/wiki/How-to-run-Trycycler. In short, the reads were subsampled into 12 read-sets and assembled using Flye v2.9 (Kolmogorov et al., 2019), miniasm v0.3-r179 (Li, 2016) + minipolish v0.1.3 (Wick and Holt, 2019) and raven v1.8.1 (Vaser and Šikić, 2021), providing a total of 12 assemblies per isolate, four from each assembly method. The assemblies were then clustered and reconciled. Next, a multiple sequence alignment using MUSCLE v3.8.1551 (Edgar, 2004) was performed, followed by partitioning of the reads. Lastly, a single consensus assembly was generated for each isolate. To correct any small-scale errors, the final assemblies were polished with the long-reads using Medaka v1.7.2 with model r1041_e81_sup_g610 for R10.4 flow cells and model r941_min_sup_g507 for R9.4.1 flow cells, respectively (https://github.com/nanoporetech/medaka).

To assess if ONT-only sequencing with the Kit12/R10.4 chemistry/flow cells was as accurate as the current gold-standard of using ONT data in hybrid with Illumina reads, and thus sufficient for *L. pneumophila* SBT, we also generated long-read first hybrid assemblies. This was done by further polishing the Trycyler+Medaka assemblies with short-reads using Polypolish v0.5.0 and Polca v4.0.5.

2.4 Sequence-based typing and identification of *mompS1* and *mompS2*

Sanger sequencing was the method used for SBT at the National Reference Laboratory for *Legionella* until August 2019, when it was

replaced by Illumina short-read WGS. All isolates in this study (n=35) were analyzed using Sanger sequencing according to the standardised protocol of the ESCMID Study Group for Legionella Infections (ESGLI) (Gaia et al., 2005; Ratzow et al., 2007) or the ST of the isolate was previously confirmed as part of an External Quality Assessment (EQA) programme.

For the Illumina sequenced isolates, the STs were determined with legsta v0.5.1 (https://github.com/tseemann/legsta) and/or with BLASTn v2.12.0+ (Camacho et al., 2009). Presence of non-identical copies along with the confirmation of *mompS2* were identified by examination of the assembly sequence. For investigations of the *mompS* alleles in STs 154 and 574, we created core genome alignments with RedDog v1b11 (https://github.com/katholt/RedDog) against hybrid assemblies of these STs and viewed the bam files in IGV v2.15.2 (Robinson et al., 2011).

To identify both *mompS1*/*mompS2* and the *L. pneumophila* ST from ONT-only and hybrid assemblies, we have developed ONTmompS v2.0.0 (https://github.com/marithetland/ONTmompS), see section 3.3 for details.

3 Results

3.1 Challenges with identifying *mompS2* for use in SBT from short-read sequencing data

In our in-house sequence database of *L. pneumophila* genomes (n=233), there were 62 unique STs. Of these, 18 STs (29%) carried non-identical copies of *mompS*. We included a set of 35 isolates in this study; 27 isolates representing each of the 18 STs with non-identical *mompS* copies and 8 isolates (7 STs) with identical copies. Currently, to identify *L. pneumophila* SBT at the National Reference Laboratory for *Legionella*, isolates are subjected to Illumina short-read sequencing, followed by Unicycler assembly (which uses SPAdes), and legsta, which is an *in silico* SBT tool, to determine the ST. We have encountered three main challenges with this method: 1) erroneous calling of the *mompS1* allele instead of *mompS2* by legsta, 2) misassembly of the *mompS* genes when using Unicycler v0.4.8, and 3) failure to resolve the structure of the *mompS*-region with Unicycler v0.4.8 and v0.5.0.

The first challenge occurs when an isolate contains non-identical copies of the *mompS* gene. Upon repeated runs of legsta v0.5.1 using the same input assembly-files, the *mompS2* allele called varied between the two alleles found in *mompS1* and *mompS2*. This was the case for all genomes with non-identical *mompS* copies where both alleles were defined in the database. This issue was solved by examining the assembly graph in Bandage (Wick et al., 2015) and using the *mompS2* flanking sequence in BLASTn searches to identify the correct *mompS2* allele for use in the SBT scheme.

The second challenge is more demanding as it is due to misassembly of the reads in the *mompS* region, i.e. some reads that belonged to *mompS2* assembled into *mompS1* and vice versa. This challenge applies to at least genomes belonging to ST154 and ST574 when using Unicycler v0.4.8 (Table 1). Both these STs carry *mompS1* allele 7 and *mompS2* allele 15, differing by 1 nucleotide at position 63, between A and G. For the isolates belonging to these STs (n=8), Sanger sequencing always determined the *mompS2* gene with allele 15, while the short-read assembly often determined the *mompS1* and *mompS2* genes as identical, either with allele 7 or 15. Further, the identified *mompS2* allele sometimes varied between 7 and 15 when the same isolate was re-sequenced. We investigated this with read mapping, which showed that the two nucleotides were mapped to both *mompS1* and *mompS2* at different frequencies. Further, for some of the ST154 and ST574 isolates, the short-read assemblies contained an additional copy of the *mompS* gene, resulting in three identical *mompS* copies per isolate. This misalignment and misassembly of reads occurs because of the repetitiveness of the *mompS*-region and that the Illumina read lengths are shorter (≤300 bp) than the 352 bp part of the *mompS2* gene that is used for allele determination (Gaia et al., 2005).

The third challenge; failure to resolve the structure of the *mompS*-region, which resulted in untypeability of isolates, was seen for 27 isolates when using Unicycler v0.5.0, including the eight that were misassembled with Unicycler v0.4.8 (see Table 1). Of these, 26 genomes carried non-identical *mompS* copies and one genome (ST1) had identical *mompS* copies. Investigations of the assembly graphs showed that the *mompS*-region of these isolates consisted of several short contigs that did not span the *mompS* genes and the flanking regions, which are used to distinguish *mompS2* from *mompS1*.

We investigated whether the read length or read depth affected the results we observed with the short-read assemblers. The isolates were sequenced with different Illumina kits leading to paired-end read lengths of 2x300 bp, 2x250 or 2x150 bp (see Table S1); we observed no pattern indicating that either kit was linked to typeability. Similarly, we saw no link between the read depth and typeability (the read depths ranged 32-212X). As there were differences in the short-read assemblies with the two Unicycler versions, we tested several methods of assembly with/without polishing and read error correction to see if any would consistently type the ST of all the genomes (see section 2.3). Unfortunately, none of the methods consistently typed all the isolates, however those that included short-read polishing did produce better results (for details see Table S3).

In sum, the observed misassembly or untypeability of *mompS2*, which varied with different assemblers or versions of the same tools, indicates major challenges with using short-read sequencing to identify the *mompS2* allele, and thus the ST, for several *L. pneumophila* isolates.

3.2 Long-read sequencing resolves the *mompS*-region and provides the necessary accuracy for *L. pneumophila* SBT

The combination of long-reads spanning the entire *mompS*-region (Figure 1) with the recent improvements in accuracy to the ONT sequencing technology, led us to the hypothesis that ONT reads on their own would be sufficient for calling *mompS* and

TABLE 1 Comparison of *L. pneumophila* SBT results for Sanger, Illumina short-read (assemblers with highest and lowest accuracy are shown), ONT-only and hybrid assemblies for genomes in the dataset.

Sanger sequencing or EQA		Illumina WGS (Unicycler v0.4.8, SPAdes v3.13.0)			Illumina WGS (Unicycler v0.5.0, SPAdes v3.15.5)			ONT-only WGS (Trycycler v0.5.3 + Medaka v1.7.2) and hybrid assemblies[a]			Nucleotides difference	Number of isolates
ST	*mompS2*	ST	*mompS1*	*mompS2*	ST	*mompS1*	*mompS2*	ST	*mompS1*	*mompS2*	*mompS 1/2*	
Short-read challenge 2: Misassembly of reads to the *mompS* copies												
154	15	154-1LV/154	7/15	7/15	-	–	–	154	7	15	1	4
574	15	574-1LV/574	7/15	7/15	-	–	–	574	7	15	1	4
1973	15	15	15	15	-	–	–	1973	103	15	3	1
Short-read challenge 3: Untypeability due to too short contigs												
15	26	15	3	26	-	–	–	15	3	26	1	1
20	2	20	93	2	-	–	–	20[b]	93	2	2	1
62	18	62	33	18	-	–	–	62	33	18	1	2
146	2	146	63*	2	-	–	–	146	63*	2	2	1
222	18	222	–	18	-	–	–	222	63	18	1	1
292	19	292	78	19	-	–	–	292	78	19	2	1
354	14	354	14*	14	-	–	–	354	14*	14	1	1
576	19	576	78	19	-	–	–	576	78	19	2	1
659	18	659	63	18	-	–	–	659	63	18	1	1
864	55	864	55*	55	-	–	–	864	55*	55	8	1
1328	46	1328	46*	46	-	–	–	1328	46*	46	1	1
2118	1	2118	10*	1	2118	–	1	2118	10*	1	9	1
2923	21	2923	9*	21	-	–	–	2923	9*	21	3	2
3138	2	3138	63	2	-	–	–	3138	63	2	1	1
3140	41	3140	41*	41	-	–	–	3140	41*	41	3	2
Controls: STs that were typeable with short-reads												
1	1	1	1	1	-	–	–	1	1	1	0	1
68	14	68	14	14	68	14	14	68	14	14	0	2
2110	13	2110	13	13	2110	13	13	2110	13	13	0	1
2630	12	2630	12	12	2630	12	12	2630	12	12	0	1

(Continued)

10.3389/fcimb.2023.1176182

10.3389/fcimb.2023.1176182

TABLE 1 Continued

Sanger sequencing or EQA		Illumina WGS (Unicycler v0.4.8, SPAdes v3.13.0)			Illumina WGS (Unicycler v0.5.0, SPAdes v3.15.5)			ONT-only WGS (Trycycler v0.5.3 + Medaka v1.7.2) and hybrid assemblies[a]			Nucleotides difference	Number of isolates
ST	*mompS2*	ST	*mompS1*	*mompS2*	ST	*mompS1*	*mompS2*	ST	*mompS1*	*mompS2*	*mompS 1/2*	
2454	9	2454	9	9	2454	9	9	2454	9	9	0	1
1324	6	1324	6	6	1324	6	6	1324	6	6	0	1
3142	13	3142	13	13	3142	13	13	3142	13	13	0	1

[a]The results from ONT-only (Trycycler+Medaka) and hybrid assembly (Trycycler+Medaka+Polypolish+Polca) were identical.
[b]Sequenced using Kit9 chemistry and R9.4.1 flow cell.
SBT, sequence-based typing; ONT, Oxford Nanopore Technologies; EQA, external quality assessment; WGS, whole-genome sequencing; ST, sequence type.
Inability to determine a mompS allele and thus the ST is marked by "–"; untypeability of the ST is designated with the closest matching ST as "ST-nLV"; "~" marks the closest matching allele if <100% and ≥ 80% coverage.

subsequently *L. pneumophila* SBT. We assessed three common methods of long-read assembly (Trycycler, Flye and Unicycler) to see if *mompS2* could be reliably identified using ONT-only reads. The 35 isolates were ONT sequenced, assembled and polished to create seven sets of assemblies for each isolate (Figure 2).

The *mompS*-region of all 35 isolates was resolved using either of the methods, but only Trycycler+Medaka and hybrid assemblies with long and short-read polishing assigned the correct ST in accordance with Sanger/EQA results in all isolates (Figure 2). For the Trycycler-only assemblies, one genome had an incorrect *proA* allele, which was corrected with Medaka long-read polishing. For the Flye+Medaka and Unicycler+Medaka assemblies, three and two genomes had one incorrect allele call, respectively.

To perform *in silico* SBT from long-read assemblies we first ran legsta, but this tool gave inconsistent results similar to what we observed with short-reads: some alleles were not called, even though we could identify them with BLASTn searches (most commonly *flaA* allele 11 and *neuA* allele 11), and the *mompS* allele was sometimes incorrectly called in genomes with non-identical copies.

3.3 ONTmompS for *in silico* SBT of *L. pneumophila* from long-reads

To identify the *mompS1* and *mompS2* alleles and the *L. pneumophila* ST from ONT-only and hybrid assemblies, we have developed the open source tool ONTmompS v2.0.0 (https://github.com/marithetland/ONTmompS). For each input assembly, the tool first identifies the *mompS2* allele by distinguishing it from *mompS1*: BLASTn v2.12.0+ is used to query the assembly against a 987 bp reference sequence (see Figure 1). This reference was originally defined for use in the *mompS* tool (https://github.com/bioinfo-core-BGU/mompS) developed by Gordon et al. (2017), and is a conserved sequence that covers the 352 nucleotides of the *mompS2* gene that are used for allele definition, and flanking regions, including the 430F upstream primer and the downstream primer 1116R. Next a pairwise Smith-Waterman local sequence alignment (EMBOSS v6.6.0.0) (Smith and Waterman, 1981) is performed on each *mompS* copy against the 1116R primer sequence, which is the downstream primer traditionally used in Sanger sequencing and which can be used to distinguish *mompS2* from *mompS1* (Gaia et al., 2005; Gordon et al., 2017). The copy that aligns to the primer is assigned as *mompS2* and the other copy as *mompS1*. Once the *mompS2* copy has been identified, the alleles of all seven genes in the SBT scheme and the resulting ST are determined using the same logic as that developed for *in silico* multi-locus sequence typing (MLST) of *Klebsiella pneumoniae* in Kleborate v2.3.1 (https://github.com/katholt/Kleborate) (Lam et al., 2021). ONTmompS reports two main results: 1) the allele numbers of the *mompS1* and *mompS2* copies and 2) the ST together with the alleles of the seven genes in the scheme.

Using ONTmompS v2.0.0, we were able to identify *mompS2* (and thus ST) for all Trycycler+Medaka and hybrid assemblies, in accordance with Sanger sequencing or EQA results for all 35 isolates.

This analysis showed that all the isolates that were misassembled or untypeable when using short-read assembly

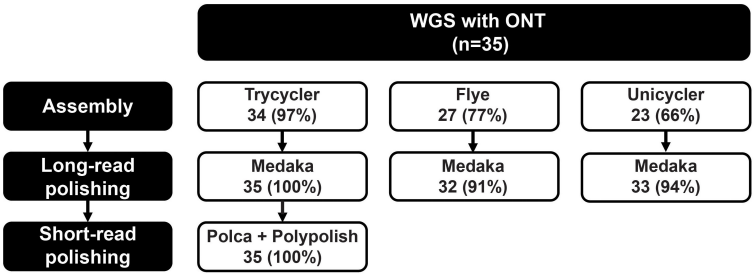

FIGURE 2
Accuracy of *Legionella pneumophila* ST calls using ONT-only or hybrid assemblies. Three assemblers (Trycycler v0.5.3, Flye v2.9, Unicycler v0.5.0) were used for long-read only assembly, followed by long-read polishing with Medaka v1.7.2. The Trycycler+Medaka assemblies were further short-read polished with Polca v4.0.5 and Polypolish v0.5.0 to create hybrid assemblies. Genomes were sequenced with Kit12/R10.4 (n=34) and Kit9/R9.4 (n=1). The number (and percentage) in each box indicates the amount of isolates whose ST was accurately called when using these tools. See also Table S1 for more details.

methods were typeable with ONT-only assemblies when using Trycycler for assembly together with Medaka long-read polishing. The controls, i.e. the isolates that had identical *mompS* copies and were typeable with short-read sequencing, were also typeable with this ONT-only workflow.

To further test and confirm that ONT-only reads are sufficient for *L. pneumophila* SBT, and potentially also from the Kit9/R9.4.1 chemistry/flow cells, we tested our workflow on an additional 46 ONT-sequenced isolates (Table S2). Of these isolates, 39 were sequenced using Kit9 chemistry/R9.4.1 flow cells, the remaining seven with Kit12/R10.4. All isolates were basecalled with the super accurate model and assembled with Trycycler+Medaka followed by analyses using ONTmompS. The *mompS*-region was resolved for all the isolates and STs were typeable and in accordance with Illumina and Sanger sequencing where that was available. This indicates that ONT-only reads (from at least Kit9/R9.4.1 and Kit12/R.10.4) are a reliable and effective solution for *L. pneumophila* SBT.

4 Discussion and conclusion

In this study, we encountered several challenges when calling *mompS2* from Illumina short-read sequences and have shown that ONT long-read sequences are a reliable alternative for determining *mompS2* and the ST of *L. pneumophila*. Today, epidemiological investigations of *L. pneumophila* and associated disease or outbreaks are mainly done by Sanger SBT and/or WGS analyses. The challenges with identifying *mompS2* due to non-identical copies of *mompS* and the short length of Illumina reads is a hindrance for routine use of short-reads in typing and surveillance. In 2015, a WGS-based cgMLST typing scheme for *L. pneumophila* was proposed, which showed high resolution of strains within the same ST/clonal complex from short-reads, but the challenge with typing due to non-identical *mompS* copies remained unsolved (Moran-Gilad et al., 2015). In 2017, the mompS tool (https://github.com/bioinfo-core-BGU/mompS) was developed to solve the challenge by accepting only reads that also flanked the *mompS2* gene and thus ensuring correct allele calls, and to ensure backwards compatibility with the Sanger SBT scheme (Gordon et al., 2017). However, in our experience the tool often failed, due to too low read coverage of the 352 bp *mompS2* sequence once the reads that do not overlap the gene and a flanking region have been filtered out. It is therefore not included as part of our standard bioinformatic analyses for *L. pneumophila* typing. In 2018, the first release of legsta was published (https://github.com/tseemann/legsta). This *in silico* approach for *L. pneumophila* SBT takes assemblies as input and uses the *L. pneumophila* SBT database together with primer sequences to identify the correct alleles for the SBT scheme. For assemblies from short-reads, long-reads, and hybrid assemblies, we unfortunately experienced that this tool was sometimes unable to call alleles or called the incorrect *mompS* allele. Neither the mompS tool nor legsta have been maintained or updated for several years.

Our analyses of short-read *L. pneumophila* genomes also showed that different assemblers or even different versions of the same assembler affected the typeability of the *mompS2* allele, adding yet another layer of complexity to the challenge of identifying STs from short-reads. When taking together the challenges of resolving the *mompS*-region from short-reads and the issues with identifying the *mompS2* allele with existing tools, it was clear that an alternative method was needed, which is why we explored using ONT long-reads and developed ONTmompS to perform *in silico L. pneumophila* SBT on ONT-only or hybrid assembled genomes.

With ONT-only reads and the ONTmompS tool, we were able to resolve the *mompS*-region and to perform *in silico L. pneumophila* SBT on all 81 genomes that were tested (the initial 35 genomes + the 46 that were used for validation). Both the Kit9/R9.4.1 and Kit12/R10.4 chemistry/flow cells with Guppy's super accurate basecalling model were used. The assemblies that were created with Trycycler followed by long-read polishing with Medaka (Trycycler+Medaka) were assigned the same ST as with Sanger SBT, EQA or hybrid assemblies for all isolates where these results were available, and an ST was assigned when using ONTmompS in all of the tested genomes. With Trycycler-only,

Flye+Medaka and Unicycler+Medaka, the STs of a few of the 35 genomes did not match with the Sanger/EQA or hybrid assemblies. There were no apparent quality issues with these genomes. We therefore recommend using Trycycler with Medaka long-read polishing for ONT-only assemblies for *in silico* SBT of *L. pneumophila*.

There was no difference in the typeability of the genomes that were ONT sequenced with Kit9/R9.4.1 and Kit12/R10.4, indicating that both chemistries/kits are appropriate for determining STs of *L. pneumophila*. This is consistent with two recent studies of ONT sequencing: Wagner et al. (2023) showed that genomic analyses of *Bordetella pertussis* from ONT-only assemblies from Kit12/R10.4 yielded results with comparable accuracy as from short-reads. Similarly, Foster-Nyarko et al. (2023) found that ONT-only reads from Kit10/R9.4, basecalled with the super accuracy model, with or without Medaka long-read polishing, were sufficient for calling ST, capsule type and AMR determinants for *Klebsiella pneumoniae*, but not sufficient for defining outbreak clusters. It was outside the scope of our study to investigate if the R10.4 ONT-only reads could be used for defining outbreak clusters of *L. pneumophila*. However, Sanderson et al. (2023) recently compared the raw read accuracy and assembly accuracy of ONT Kit10/R9.4, Kit12/R10.3, Kit12/R10.4, Kit12/R10.4 with duplex reads, and Illumina sequencing of four bacterial pathogens (not *L. pneumophila*). Sereika et al. (2022) did a similar comparison. Both studies found that ONT Kit12/R10.4 duplex reads that were basecalled with the super accuracy model could be used for complete reconstruction of bacterial genomes without the use of Illumina reads. However, Sanderson et al. (2023) noted that recovery of small plasmids was inconsistent and that hybrid assemblies still remain the most cost-effective and robust approach for bacterial whole-genome reconstruction.

By combining real-time basecalling and the ONT Kit12/R10.4, Wagner et al. (2023) recently showed that it is possible to perform highly accurate and fast high-resolution typing of bacterial pathogens while sequencing is ongoing, highlighting the time-saving potential of the ONT-technology in outbreak situations. Furthermore, the flexibility in the number of samples and/or flow cells that can be run on for instance ONT's GridIon device, makes ONT well-suited and efficient for analyzing both a single isolate and larger collections, e.g. in suspected outbreaks. In our laboratory, the cost of generating ONT-only assemblies is overall lower than for Illumina-only assemblies, with the difference being more profound for lower sample numbers. However, the cost per isolate for both technologies are dependent on the kit used, number of samples analyzed and how well the capacity of the sequencing kit/flow cell is utilized. ONT has recently launched Kit14/R10.4.1 flow cell promising an even more improved accuracy. Given our results with Kit9 and Kit12 we expect to obtain similar results with Trycycler+Medaka and hopefully even better results with the other assembly methods with the improved ONT technology.

L. pneumophila SBT is important for the characterization of *L. pneumophila* isolates and for standardized comparison of results over time and between different laboratories (Raphael et al., 2016). In source investigations, the SBT serves well to identify potential sources in the initial phase of the investigation. However, due to the lower discriminatory power of this method compared to whole genome genetic relatedness analyses, further analyses are usually needed to confirm a potential source (Raphael et al., 2016; Ricci et al., 2022). By utilizing long-read WGS, SBT can easily be assessed in an initial analysis, and in-depth whole-genome analyses may also be performed, either together with complementary short-reads to correct ONT read errors, or ONT reads on their own if and when these have been proven sufficient to use in whole genome analyses, for which evidence is starting to emerge (Sereika et al., 2022; Sanderson et al., 2023; Wagner et al., 2023).

To conclude, ONT-only sequencing is sufficient for identifying *mompS2* and *L. pneumophila* ST. Our analyses show that ONT-only assemblies provide a cost- and time-efficient solution for determining *L. pneumophila* ST from WGS, where Illumina short-reads often fail to identify *mompS2*. For the best results, we recommend basecalling with Guppy using the super accurate basecalling model, assembly with Trycycler and polishing with Medaka, before identifying the ST with ONTmompS.

Data availability statement

The datasets presented in this study are available online in the European Nucleotide Archive under accession numbers PRJEB58776 and PRJEB50383. Please see Tables S1 and S2 for individual accession numbers.

Author contributions

AK, MH, EB, and IL conceptualized the study. AK and EB performed genome sequencing. MH and MS developed ONTmompS. AK and AB analyzed Sanger and WGS results. IL provided resources and supervision. AK, MH and EB wrote the first draft of the manuscript. All authors contributed to data interpretation, reviewed and edited the manuscript, and have read and agreed to the published version of the manuscript.

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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

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Conceptual model to inform *Legionella*–amoebae control, including the roles of extracellular vesicles in engineered water system infections

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Extracellular vesicles (EVs or exosomes) are well described for bacterial pathogens associated with our gastrointestinal system, and more recently as a novel mechanism for environmental persistence, dissemination and infection for human enteric viruses. However, the roles played by EVs in the ancient arms race that continues between amoebae and one of their prey, *Legionella pneumophila*, is poorly understood. At best we know of intracellular vesicles of amoebae containing a mix of bacterial prey species, which also provides an enhanced niche for bacteriophage infection/spread. Free-living amoeba-associated pathogens have recently been recognized to have enhanced resistance to disinfection and environmental stressors, adding to previously understood (but for relatively few species of) bacteria sequestered within amoebal cysts. However, the focus of the current work is to review the likely impacts of large numbers of respiratory-sized EVs containing numerous *L. pneumophila* cells studied in pure and biofilm systems with mixed prey species. These encapsulated pathogens are orders of magnitude more resistant to disinfection than free cells, and our engineered systems with residual disinfectants could promote evolution of resistance (including AMR), enhanced virulence and EV release. All these are key features for evolution within a dead-end human pathogen post lung infection. Traditional single-hit pathogen infection models used to estimate the probability of infection/disease and critical environmental concentrations *via* quantitative microbial risk assessments may also need to change. In short, recognizing that EV-packaged cells are highly virulent units for transmission of legionellae, which may also modulate/avoid human host immune responses. Key data gaps are raised and a previous conceptual model expanded upon to clarify where biofilm EVs could play a role promoting risk as well as inform a more wholistic management program to proactively control legionellosis.

KEYWORDS

engineered water systems, environmental persistence, disinfection resistance, QMRA, monitoring

1 Introduction

Water-based (saprozoic) pathogens cost the US over $2.39 billion per year (approximately $7m per head of population), some ten-fold the cost of gastrointestinal waterborne pathogens (Collier et al., 2021). These saprozoic pathogens predominantly grow within microeukaryotes, such as free-living amoebae and their analogues, macrophages, differentiating them from saprotrophic microorganisms that live off dead and decaying matter. In Europe, legionellosis (largely caused by three species of saprozoic *Legionella* from drinking water) was identified as the fifth most significant contributor to disability-adjusted life years (DALYs) from 31 selected diseases, following influenza, tuberculosis, human immunodeficiency virus (HIV) infection/AIDS and invasive pneumococcal disease (Cassini et al., 2019). However, unlike the top four infectious diseases, legionellosis is not spread person-to-person, but largely infect humans from water/moist soil/mulch habitats *via* aerosols to lung macrophages and alveolar epithelial cells (Ashbolt, 2015) – placing management in multiple hands, given the various jurisdictions addressing water provision (treatment, distribution and premise) and its other environmental exposures. Also, our aging population and various increasing vulnerable groups to these opportunistic pathogens are escalating the disease burden from water-based pathogens globally.

In the general absence of regulations requiring saprozoic pathogen monitoring near points of potential exposures (Bartram et al., 2007; CDC, 2017; HSE, 2017; Gamage et al., 2022), along with most cases being sporadic and rarely followed up (Abu Khweek and Amer, 2018; Clopper et al., 2021), there is significant under reporting of the true impact from these pathogens (Burillo et al., 2017; Vermeulen et al., 2020). What we know most is from Legionnaires' Disease outbreaks from cooling tower aerosols, ornamental fountains and within hospitals, but these only account for some 15% of all cases, the bulk of the remainder being sporadic within the community, largely thought to be from premise plumbing systems (Falkinham et al., 2015; NAS, 2020).

Many saprozoic pathogens are transmitted to humans *via* aerosols, typically following distal disinfection processes (e.g., residual chlorine in drinking water, point-of-use UV irradiation and environmental desiccation). However, recent work has shown that extracellular vesicles (EVs) from amoebae, along with their trophozoites and cysts provide orders of magnitude resistance to disinfection for internalized pathogens compared to the freely suspended bacteria and human viruses (Storey et al., 2004; Folkins et al., 2020; Martin et al., 2020; Dey et al., 2022). From a control point of view this is important, as current guidance/regulation removal is only based on disinfection performance of the more readily inactivated freely suspended. Also relevant is *L. pneumophila*'s dual life phases (replicative & transmissive), with replicative cells (typical form used in disinfection studies) repressing, while the transmissive form induces virulence, motility and most importantly for persistence, various stress factors *via* activity of RNA-binding proteins, including CsrA and its thiamine pyrophosphate riboswitch (Sahr et al., 2017). Overall, this begs the question, has *L. pneumophila*, like other amoeba-resisting pathogens (Schmitz-Esser et al., 2010), evolved not only to grow and control host cells (using a large fraction of its genome to do so [(Burstein et al., 2016)]), but is it also adapted to persist within EVs or even generate host EVs to extend its transmissive phase? Recent transcriptomics is helping us to understand the differentially expressed *Legionella* genes within their infected amoebal hosts (Quan et al., 2020; Chauhan et al., 2023). Such techniques have yet to be applied to EVs production and within these vesicles.

Overall, identifying amoeba-associated pathogens may not only identify amplification niches in engineered systems (Thomas and Ashbolt, 2011; Thomas et al., 2014), but also pathogens more likely to be protected and deliver in an infectious dose (Shaheen and Ashbolt, 2018). No such routine amoeba-targeted monitoring is undertaken today but may well provide the proactive approach sought in water safety plans (WSPs) that are used around the world to manage waterborne enteric pathogens (WHO, 2017), but are in their infancy in being adapted to provide safe water for the management of saprozoic pathogens (Papadakis et al., 2018; De Giglio et al., 2021). Hence, the goal of this article is to summarize what is understood about the biology of *L. pneumophila* relevant to its *in-situ* biofilm hosts with the purpose of identifying promising targets to manage this critical saprozoic pathogen of engineered water systems.

1.1 Monitoring and management options for *L. pneumophila*

Current culture-based methods for known saprozoic bacterial pathogens (*Legionella* spp., nontuberculous mycobacteria etc.) take 10-days to weeks for confirmation (e.g., (ISO 11731, 2017; Pfaller et al., 2021)) – far too long for a timely response. Further, cells of these pathogens may largely be present in viable but non-culturable (VBNC) states (hence missed by traditional culture methods) yet still infectious to humans (Dietersdorfer et al., 2018). Therefore, specialized amoeba co-culture is recommended for resuscitation (García et al., 2007; Dey et al., 2019a; Dey et al., 2019b; Dey et al., 2020). While still disputed by some, molecular methods are preferred for the management of water systems, such as qPCR (Lee et al., 2011). However, determining the fraction of infectious cells by qPCR is still problematic, given uncertainties in infectivity status and the variable residual chlorine levels likely present in piped water systems (Casini et al., 2018; Donohue et al., 2019; Donohue, 2021). Flow cytometry using immunocapture (Füchslin et al., 2010) or with cell-sorting in combination with qPCR shows promise to also identify VBNC cells, particularly those missed by conventional culture (e.g., ISO 11731:2017-05 pre-treatment procedure) but capable of infecting host cells (Nisar et al., 2023).

Nonetheless, live or dead, high concentrations (in excess of 10^3 cells/100 mL, (Hamilton et al., 2021)) in distal parts of water delivery systems infer growth in the system that needs to be addressed, else risk exposure to infectious aerosols at some stage. The major amplification site for *L. pneumophila* in water systems is within biofilm amoebae (NAS, 2020). Therefore, herein a prior conceptual model for legionellae growth within pipe biofilms (Shaheen and Ashbolt, 2021) is expanded upon to include EV and possible amoeba monitoring targets and identifies research

gaps. Overall, targeting hosts prior to rapid, explosive growth of legionellae is hypothesized as a useful target within a system of checks (flushing, temp <25 or >50°C, disinfectant residual etc.) for a proactive early warning water management system.

2 Primary role of free-living amoebae supporting problematic *L. pneumophila* growth

2.1 Protist hosts for explosive growth of *L. pneumophila*

The primary hosts for *Legionella* within biofilms are various free-living protozoa, principally free-living amoebae (FLA) (Thomas et al., 2010) and ciliates (Tsao et al., 2019). While ciliates may excrete viable legionellae within fecal pellets for subsequent re-ingestion (Hojo et al., 2012; Berk and Garduño, 2013), *L. pneumophila* released following their lysis of amoebal trophozoites are more likely to lead to explosive growth cycles of phagocytosis-growth-release (Shaheen et al., 2019), given several hundred of cells that can grow per amoeba (Buse and Ashbolt, 2012).

A recent review of FLA is provided by Scheid (2019), in which he describes their diversity and the predatory heterotrophic feeding by trophozoites on biofilm microbiota and extracellular polymeric substances. Indeed, FLA are ubiquitous and instrumental to both biofilm formation and ecological successions. Important amoebal hosts supporting *Legionella* and other amoeba-resisting bacterial (ARB) pathogens include members of *Acanthamoeba*, *Naegleria, Vermamoeba* and *Willaertia* (Thomas et al., 2010). Although the term FLA has no relevance to amoebal taxonomy or phylogeny, it does separate them from the parasitic intestinal amoebae (e.g., *Entamoeba histolytica*) that have significantly reduced genomes due to their host's providing most needs (Shabardina et al., 2018). Critical to FLA success and dispersal (air, water, soils & by wildlife) is the environmentally robust cyst form, that may also encase/protect beneficial and pathogenic bacteria and their viruses (Thomas and Greub, 2010; Schulz et al., 2020; Shi et al., 2021), many of whom may carry virus-encoded auxiliary metabolic genes (vAMGs) (Yuan and Ju, 2023).

Pertinent to engineered water systems has been the increasing recognition of the importance of suspended flocs/particulates in amoebal planktonic dynamics (Anderson, 2018). While not considered biofilms of fixed surfaces, these planktonic niches may represent sloughed biofilm material or biofilm-like growth on suspended particulates that contribute to microbial activity in piped water systems (Liu et al., 2016) and ultimately the bulk of internalized pathogens transmitted *via* aerosols (Hamilton et al., 2018; Shaheen and Ashbolt, 2021).

2.2 Extracellular vesicles of FLA

Nearly all living cells may excrete EVs, which are described based on their site of origin (i.e., exosomes, ectosomes, cytonemes & nanotubes) (Vermeulen et al., 2020.). However, only recently have EVs been recognized as participating in cell-to-cell communication processes, including between predator and prey (de Souza and Barrias, 2020), and carry specific virulence factors (Cruz Camacho et al., 2023) with known immunomodulatory properties (Costa et al., 2021). Exosomes are EVs that originate from the endocytic pathway of a cell (de Souza and Barrias, 2020; Cruz Camacho et al., 2023) and FLA-EVs are of focus here because of their likely multiple connections across the life cycle of *L. pneumophila* in biofilms of engineered environments (Figure 1).

Various drinking water associated FLA have been shown to generate EVs containing tens to hundreds of *L. pneumophila* cells within the respiratory range (< 10 μm dia.) (Shaheen and Ashbolt, 2018). These EVs may provide packages of pathogens directly to the alveoli of our lungs, potentially influencing current quantitative microbial risk assessment (QMRA) models of *Legionella* risks and specifically the dose-response relationship (Hamilton et al., 2019). Hypothesized in this article are the environmental queues that may not only influence EV production but also their role leading to explosive growth of legionellae in their native biofilms (Figure 1).

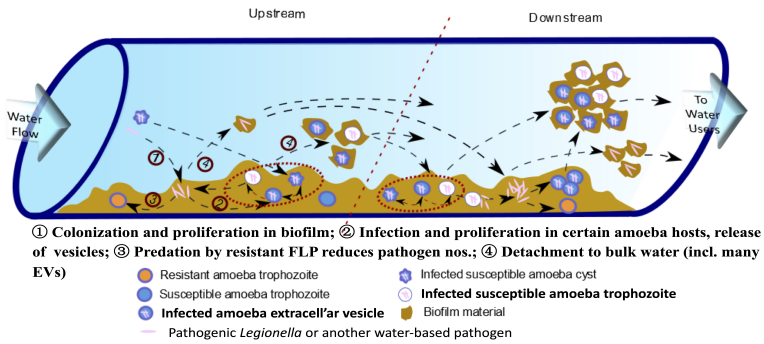

FIGURE 1
Conceptual model for explosive growth of *Legionella* in engineered water system biofilms. (Adapted from Shaheen et al. (2019), FLP - free-living protozoa [primarily amoebae and ciliates] grow on biofilm constituents, ultimately limited to feeding on less-preferred amoeba-resisting bacterial, such as *L. pneumophila*, leading to explosive growth and release in extracellular amoebal vesicles [EVs] that may enhance downstream amoebal predation over less preferred free bacteria – so increasing proliferation of pathogens.).

While speculative for FLA, in bacterial predators, such as *Myxococcus xanthus*, outer membrane vesicles (OMVs) appear to specifically carry a 'personalized' subset of proteins that only in-part are dictated by the predator's genome (Zwarycz et al., 2020). In other words, Zwarycz et al. (2020) hypothesize that the non-genome-derived proteome fraction is there to target its prey. Hence, is *L. pneumophila* really the predator not amoebae, utilizing a large fraction of its genome to not only manipulate the hosts into providing a growth niche in the form of *Legionella*-containing vacuoles (LCV) but potentially also EVs production to increase its persistence/transmission? Furthermore, as seen with other EVs, those containing legionellae may also modulate/avoid human host immune responses (e.g., triggered by phosphatidylserine lipids on the surface of EVs (Cole and Nizet, 2016)).

In bacteria, outer membrane vesicles (OMVs) have also been shown to provide a defense mechanism to phage infection (Reyes-Robles et al., 2018). Hence, next genomic then viruses specific to FLA are discussed and how they may impact on the evolution of these important hosts and ARB.

2.3 Genomic implications for *L. pneumophila* within engineered systems

As with their FLA hosts suited to the diversity of conditions in environmental biofilms, ARB maintain a larger genome than their relatives – so contradicting the expected genome reduction theory accepted for most intracellular pathogens (Moliner et al., 2010). This is likely due to the importance of gene transfer of pathogenicity factors, and the near 800 million year old arms-race between these predators and prey (Dupont et al., 2011; Shames, 2023) along with the role played by their respective viruses (Kaján et al., 2020). Of particular focus in this article is the remodeling of the *Legionella*-containing vesicle (LCV), with legionellae acquiring effector protein genes, such as Sar1/CopII that impact early secretory vesicle production genes (Robinson and Roy, 2006). As introduced in Figure 1, it is unknown how ARB may influence the release of EVs to enhance predator infection downstream, and provide a hot-spot for horizontal gene transfer (HGT). Recent work has pointed to FLA preferentially preying on non-ARB free bacteria over *L. pneumophila* (Shaheen and Ashbolt, 2021). What is unknown, but hypothesized here is that legionellae within EVs may not only avoid environmental stressors, but also overcome negative selective feeding on *L. pneumophila* by FLA. Indeed, are EVs preferential prey for FLA and an enhanced mechanism for prey to counter predators' sensing of preferred food?

The role played by quorum sensing molecules (such as LAI-1 [3-hydroxypentadecane-4-one]) involved in legionellae changing from a reproductive to a transmissive phase (Schell et al., 2016), could also be part of the evolutionary arms race between predator and prey (Simon et al., 2015). It is currently unknown if LAI-like molecules are activated within EVs, as known to occur within trophozoites. Other potential changes within trophozoites and EVs that HGT may also impact could include environmental antimicrobial resistance (AMR) (Mortensen et al., 2021). AMR has generally not been viewed as important with pathogens that do not spread zoonotically or by person-to-person (such as with *L. pneumophila*). However, AMR within amoebal ecosystems increasingly impacted by AMR (e.g., cooling towers or irrigation systems receiving AMR-laden wastewater) could present a 'perfect storm' for enhance AMR within *L. pneumophila*-FLA ecosystems.

2.4 FLA evolution and their nucleocytoplasmic large DNA viruses

FLA evolved some 800-850 My ago (Cavalier-Smith, 2009), but have and continue to have exchange with bacteria/archaea and related viruses. A particular feature of FLA are their nucleocytoplasmic large DNA viruses (NCLDV), first described in 2003 (Abrahao et al., 2014) as *Acanthamoeba polyphaga* mimivirus (APMV) (now in the genus *Mimivirus*). In a recent whole genome analysis of *A. polyphaga* various NCLDVs (*Marseillevirus, Mimivirus, Mollivirus, Pandoravirus*, *Pithovirus* and a yet to be described family) were identified - inferring that NCLDV may have been domesticated during the evolution of amoebae (Schulz et al., 2020). Hence, though considered rather unusual, NCLDVs are not just a result of recent horizontal gene transfer (HGT) but in fact are associated with most major eukaryotic lineages, so impacting all ecosystems on earth (Schulz et al., 2020).

Another feature, potentially relevant to *L. pneumophila*'s human infectivity, has been described for human enteric viruses. A good exemplar is seen with human *Norovirus*, which change commensal gut bacteria's EV production, likely impacting human host responses to infection (Mosby et al., 2022). While speculative for human infection by amoeba-legionellae material, it is intriguing that various enveloped and non-enveloped viruses generate a cytopathic effect in a range of amoebae and are released in amoebal EVs (Folkins et al., 2020; Dey et al., 2021). Hence, what roles may viruses play within amoebae (possibly in concert with internalized bacteria) regarding their expression of EVs and in modulating/avoiding human host immune responses? Important first insights to such mechanisms have been reported by Dey et al. (2022), who first described amoebic mitochondrial impacts by RNA viruses that appear to initiate and regulate apoptotic cell death. Overall this work points to research gaps that could be important to understand from an evolutionally perspective as well as to better describe human dose-response relationships for ARB pathogens.

Taking the above findings of NCLDV evolution, their likely (but not documented) presence within EVs and that specific genes from *Legionella* may be packaged within EVs – illustrates the is much to understand in the role that EVs may play in predator-prey interactions of ARB pathogens – and possibly much more to learn as to what actually leads to *Legionella* infection in human macrophages and alveolar epithelial cells.

3 Considerations for pro-active management of *L. pneumophila*

Managing emerging hazards has taken a more wholistic perspective in the last decade, and such an approach maybe particularly pertinent to legionellosis management. Stepping back to a broader view, we have an increasingly urbanized world

population with its pollution becoming a dominant public health concern (via what is call the 'pollutome') (Pini et al., 2023). For chemical exposures there is also an increasing use of omics informed decisions about the exposome (Vlaanderen et al., 2021). Hence, it seems timely to also provide a One-Health lens to include microbial exosomes given their relevance to approximately one-third of community acquired (atypical) pneumonias (CAPs); which for those over 50 years of age is focused on healthcare settings, but recent work, reported for middle-eastern countries, has exposed CAP prevalent amongst young adults (20-40 years) within the broader community where legionellosis is also a major disease (Alhoufie et al., 2022).

As discussed above, molecular methods provide near real-time ways to target markers of pathogens, and in the current study the major vehicles for *L. pneumophila* amplification (FLA) and means for its dispersal and potential transmission (EVs). Clinical studies should also focus on the role amoeba trophozoites, EVs and fragments thereof may modulate human infection (including Pontiac fever), so such water monitoring targets could also be useful in clinical investigations. In the absence of being able to directly sample biofilm materials (such as though use of pipe wall coupons etc.), first flush tap samples are probably most informative of the last few meters where most problematic concentrations of ARB develop prior to aerosol exposures (Schoen and Ashbolt, 2011; Proctor et al., 2018). Hence, in concert with proactive management of flow conditions to keep cold waters below 25°C and hot water > 50°C, flushing of distal parts of water systems etc. (CDC, 2017), monitoring for amoebal aspects should provide the first hint of an increased potential for legionellae growth. Various qPCR protocols already exist for the major water-associated FLA and research should next focus on developing a more nuanced understanding of ARB growth conditions and targets.

4 Conclusions

While EVs seem to permeate all aspects of biology, surprisingly little is known about *L. pneumophila* and other amoeba-resisting pathogens' role in driving EVs associated with FLA and engineered water systems. Seeking to provide proactive, rather than reactive management of legionellosis, by targeting management at the starting point for rapid amplification of *L. pneumophila*, i.e., a biomass of supportive amoebae, and promising EV telltales of their activity needs further examination. Molecular markers within EVs and of FLA would seem likely areas to first develop potential monitoring targets. Major data gaps identified during this study include: What role do internalized bacteria/viruses play in generating EVs and in modulating their behavior; Are amoebae induced to prey upon EVs; and, how FLA/EVs act in the gene pool leading to synchronized behavior within biofilm microbiomes and in human infection.

Author contributions

One author contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Stagnation arising through intermittent usage is associated with increased viable but non culturable *Legionella* and amoeba hosts in a hospital water system

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Hospital water systems are a significant source of *Legionella*, resulting in the potentially fatal Legionnaires' disease. One of the biggest challenges for *Legionella* management within these systems is that under unfavorable conditions *Legionella* transforms itself into a viable but non culturable (VBNC) state that cannot be detected using the standard methods. This study used a novel method (flow cytometry-cell sorting and qPCR [VFC+qPCR] assay) concurrently with the standard detection methods to examine the effect of temporary water stagnation, on *Legionella* spp. and microbial communities present in a hospital water system. Water samples were also analyzed for amoebae using culture and *Vermamoeba vermiformis* and *Acanthamoeba* specific qPCR. The water temperature, number and duration of water flow events for the hand basins and showers sampled was measured using the Enware Smart Flow® monitoring system. qPCR analysis demonstrated that 21.8% samples were positive for *Legionella* spp., 21% for *L. pneumophila*, 40.9% for *V. vermiformis* and 4.2% for *Acanthamoeba*. All samples that were *Legionella* spp. positive using qPCR (22%) were also positive for VBNC *Legionella* spp.; however, only 2.5% of samples were positive for culturable *Legionella* spp. 18.1% of the samples were positive for free-living amoebae (FLA) using culture. All samples positive for *Legionella* spp. were also positive for FLA. Samples with a high heterotrophic plate count (HPC $\geq 5 \times 10^3$ CFU/L) were also significantly associated with high concentrations of *Legionella* spp. DNA, VBNC *Legionella* spp./*L. pneumophila* ($p < 0.01$) and *V. vermiformis* ($p < 0.05$). Temporary water stagnation arising through intermittent usage (< 2 hours of usage per month) significantly ($p < 0.01$) increased the amount of *Legionella* spp. DNA, VBNC *Legionella* spp./*L. pneumophila*, and *V. vermiformis;* however, it did not significantly impact the HPC load. In contrast to stagnation, no relationship

was observed between the microbes and water temperature. In conclusion, *Legionella* spp. (DNA and VBNC) was associated with *V. vermiformis*, heterotrophic bacteria, and stagnation occurring through intermittent usage. This is the first study to monitor VBNC *Legionella* spp. within a hospital water system. The high percentage of false negative *Legionella* spp. results provided by the culture method supports the use of either qPCR or VFC+qPCR to monitor *Legionella* spp. contamination within hospital water systems.

KEYWORDS

Legionnaires' disease, water safety plan, building plumbing systems, free-living amoebae, potable water

1 Introduction

Legionella is an opportunistic premise plumbing pathogen and etiological agent of Legionnaires' disease (LD), a potentially fatal pneumonia like infection (Cunha et al., 2016). *Legionella* is ubiquitous in natural and engineered water systems and transmitted through aspiration or inhalation of *Legionella* contaminated water or aerosols (Schwake et al., 2021). Globally the incidence of LD has been increasing. In 2021, the US Centers for Disease Control and Prevention (CDC) reported 8260 confirmed cases of LD in USA (Centers for Disease Control and Prevention, 2022). In Australia, 524 confirmed cases of legionellosis were reported in 2020 (Australian Government Department of Health and Aged Care, 2021). According to the European Centre for Disease Prevention and Control (ECDC) 11,298 confirmed cases of LD were documented across European countries in 2019. However, in 2020 the number decreased to 8,372; this reduction may be associated with COVID-19 pandemic lockdown restrictions or a decrease in focus on LD. In 2021, 10,723 confirmed cases of LD were documented of which 5.4% were nosocomial infections (The European Legionnaires' Disease Surveillance Network, 2022). The actual number of legionellosis cases is understated, because in the majority of cases Pontiac fever remains unnoticed and the etiological agent of pneumonia remains unrecognized (Cassell et al., 2019). There are at least 60 distinct species of *Legionella*, with *L. pneumophila* sg.1 being the most common cause of outbreaks (Khodr et al., 2016; Miyashita et al., 2020). Initially, cooling towers were considered to be the main source of *Legionella* spp., but subsequent investigations have identified that engineered water systems are a major source of LD (Kanarek et al., 2022). Those at greatest risk of infection are the elderly and immunocompromised individuals, and as such nosocomial outbreaks associated with hospital engineered water systems are of significant concern (Bartram et al., 2007).

A range of factors influence the survival and persistence of *Legionella* spp. in hospital water systems including: biofilms, nutrients, disinfectants, protozoa hosts, water temperature, flow dynamics and stagnation (Abdel-Nour et al., 2013; Whiley et al., 2017; Nisar et al., 2020b). Naturally, *Legionella* spp. infects and survives within a wide range of polyphyletic protozoan hosts, with *Acanthamoeba* and *Vermamoeba* the most commonly identified hosts in potable water (Boamah et al., 2017; Best and Abu Kwaik, 2018; Nisar et al., 2020a). Intracytoplasmic *Legionella* spp. are protected from adverse environmental conditions (Best and Abu Kwaik, 2018), with *Legionella* spp. released from host protozoa more virulent and pathogenic in nature (Fields et al., 2002; Boamah et al., 2017). Additionally, *Legionella* spp. intrinsically tolerate water disinfection treatments by entering into a metabolically inactive but highly resistant and potentially pathogenic "viable but non-culturable" (VBNC) state (Kirschner, 2016). Under suitable environmental conditions, and in the presence of protozoa hosts, VBNC *Legionella* spp. can resuscitate back into a culturable state (Dietersdorfer et al., 2018). VBNC *Legionella* spp. are a significant challenge to water quality management as they cannot be detected using the standard culture-based method (International Organization for Standardization, 2017; Standards Australia, 2017). *Legionella* spp. specific quantitative PCR (qPCR) assay is an alternative method typically used to detect the genomic load of *Legionella* spp. (International Organization for Standardization, 2019); however, it cannot distinguish between culturable, dead and VBNC *Legionella* spp. (Kirschner, 2016). As such, there are currently limited studies that investigate the survival of VBNC *Legionella* spp. in engineered water systems.

Water stagnation in engineered water systems is categorized into two different types; permanent, and temporary stagnation (Peter and Routledge, 2018; Nisar et al., 2020b). Permanent stagnation is complete stagnation of plumbing structures, such as dead-ends and dead-legs (Nisar et al., 2020b). However, in engineered water systems, water can also stagnate in storage tanks, plumbing piping network, and within components at the water outlets for a few hours to weeks and even months (Bartram et al., 2007; Manuel et al., 2009). This type of water stagnation is known as intermittent or temporary stagnation (Manuel et al., 2009; Peter and Routledge, 2018). The relationship between *Legionella* spp. and permanent stagnation is well characterized (Totaro et al., 2018; Nisar et al., 2020b). However, less is known about the relationship between *Legionella* spp. and temporary stagnation. Therefore, this study examined the role of temporary stagnation arising through intermittent water usage on the persistence of *Legionella* spp. and free-living amoebae (FLA) within a hospital water system.

This study was the first study to utilize a novel method to enumerate VBNC *Legionella* spp. and *L. pneumophila* from environmental water samples and investigate relationships with protozoan hosts. This study utilized the Enware Smart® Flow monitoring system to examine the relationships between water flow (arising through water outlet usage) and temperature with *Legionella* spp., *L. pneumophila* and amoeba hosts. The specific aims of this study were as follows, to: (1) determine the prevalence of *Legionella* spp./*L. pneumophila* and FLA in a hospital water system; (2) examine the relationship between *Legionella* spp. and potential protozoan hosts; and (3) monitor the effect of sampling phases (months), water temperature, flow dynamics and stagnation on persistence of *Legionella* spp. in the hospital water system. To our knowledge, this is the first comprehensive study that has quantified VBNC *Legionella* spp. and FLA from a hospital water system under dynamic flow and temperature conditions.

2 Materials and methods

2.1 Sample collection and processing

From March 2021 to June 2022, water (n = 120) and biofilm (n = 46) samples were collected from the engineered water system of an Australian hospital located in New South Wales, Australia. The sampling was done in different phases, where the categorization was: March 2021 as phase 1, April 2021 as phase 2, November 2021 as phase 3 and June 2022 as phase 4. All water and biofilm samples were collected, transported and stored as recommended by standard guidelines (International Organization for Standardization, 2018; Centers for Disease Control and Prevention, 2019). For the water samples, 1 L first flush hand basin or shower water was collected in sterile wide-mouth screw capped plastic bottles (2105-0032, Nalgene™). For the biofilm samples, visible biofilm was scraped from the inside of tap faucet or shower head using sterile polyurethane-tipped swabs (CleanFoam® TX751B, Texwipe®), then 5 to 10 mL of water was added and placed with the swab in a sterile screw capped tube. For both the water and biofilm samples, 0.5 mL 0.1 N sodium thiosulfate (124270010, ACROS Organics™) was added to neutralize pre-existing chlorine-based chemical disinfectants. All samples were transported and kept at 5 ± 2°C and processed within 72 hours. The samples were vacuum filtered through 47 mm diameter 0.22 µm polycarbonate membrane (GTTP04700, Isopore™). The filtered residues were resuspended in 3 mL sterile distilled water. This sample suspension was used for further microbiological and molecular testing.

2.2 Water flow and temperature data

Parameters related to water temperature and flow dynamics were monitored in the hospital water system using the Enware Smart® Flow monitoring system. Briefly, this monitoring system measures water system delivery temperatures using temperature probes located at the hot water inlet, cold water inlet, and outlet of the thermostatic mixing valves (TMV) and the hot water inlet and cold water inlet of hand basin faucets. Water flow was measured using flow switches located at the hot water inlet and cold water inlet of both the TMVs and hand basin faucets (Whiley et al., 2019). The temperature data of the hot water supply, cold water supply and outlet was collected for the entire duration of the sampling period. For analysis these measurements were separated into a period one week and one month prior to a water sampling event. In terms of flow regime, the total duration (hours) and number (counts) of flushing events for a period of one week and one month prior to sampling were recorded. The total duration (hours) of flushing events were divided into low and high flow regimes with categorization as: low flow regime; 0 to < 2 hours per month, and high flow regime; ≥ 2 to 40 hours per month.

2.3 Molecular analysis

Quantification of *Legionella* spp. (16 rDNA gene) and *L. pneumophila* (*mip* gene) was performed using ISO/TS12869:2019 quantitative polymerase chain reaction (qPCR) assays (International Organization for Standardization, 2019). The 18S rDNA gene was amplified for the quantification of *Acanthamoeba* and *Vermamoeba vermiformis* (Qvarnstrom et al., 2006; Scheikl et al., 2016). *Legionella* spp. (GenBank Acc CP021281), *L. pneumophila* (GenBank Acc KR902705), *Acanthamoeba castellanii* (GenBank Acc U07413) and *V. vermiformis* (GenBank Acc KT185625) gBlocks gene fragments (IDT™) were used as a positive control and for the preparation of a standard curve using ten-fold serial dilutions. Using the Aquadien™ kit (3578121, BIO-RAD Laboratories Ltd.), genomic DNA was extracted from each water and biofilm sample before being subjected to a qPCR assay. The qPCR reaction mixture consisted of microbe-specific primers (Bio-Rad Laboratories Ltd.), 1X PCR reaction buffer (2X SsoAdvanced™ universal probes supermix:172-5281, Bio-Rad Laboratories Ltd.), and DNA template. To detect the potential presence of environmental inhibitors of the qPCR assays, both the purified and a one in ten dilution of extracted DNA was used as template (Hayes-Phillips et al., 2019; Nisar et al., 2022). Using a Rotor-Gene Q thermal cycler (Qiagen Ltd.), each template DNA was subjected to the qPCR assay in triplicate (Nisar et al., 2022). All fluorescence labelled probes and primers used in this study are presented in Table S1 (Supplementary Material).

2.4 Microbiological analysis

Isolation of culturable *Legionella* spp. and *L. pneumophila* was performed in accordance with the standard guidelines (International Organization for Standardization, 2017; Standards Australia, 2017). Briefly, samples were heat treated (50 ± 1°C for 30 ± 2 minutes) and/or acid treated (HCl-KCl buffer treatment for 5 ± 0.5 minutes) to reduce the contamination of interfering microbes. An aliquot of treated sample was then spread on *Legionella* agar (CM1203, Oxoid Ltd.) supplemented with GVPC (glycine, vancomycin, polymyxin B and cycloheximide: SR0152, Oxoid Ltd.) and *Legionella* growth supplement (α-ketoglutarate,

buffer/potassium hydroxide, ferric pyrophosphate, and L-cysteine: SR0110C, Oxoid Ltd.). The inoculated plates were incubated at 37 ± 1°C for 7 days and examined every day. Suspected *Legionella*-like colonies were counted from each plate and evaluated by *Legionella* latex agglutination test kit (DR0800, Oxoid Ltd.). This kit identifies genus *Legionella* and further characterizes various species and serogroups with overall 99% sensitivity and 100% specificity. Furthermore, all *Legionella*-like colonies were confirmed through *Legionella* spp. specific qPCR assays. To determine the heterotrophic plate counts (HPC), an aliquot from each sample was inoculated on R_2A agar (CM0906, Oxoid Ltd.) and incubated at 35 ± 1°C. The colonies were counted after 2, 5 and 7 days of incubation. The results for *Legionella* spp. and heterotrophic bacteria were expressed in colony forming units (CFU)/L for water samples and CFU for the biofilm samples. Isolation of culturable FLA was performed by inoculating an aliquot of each sample on heat-inactivated (57°C for 45 minutes) *Escherichia coli* American Type Culture Collection 700891™ supplemented 1.5% non-nutrient agar (Eco-NNA: CM0003, Oxoid Ltd.) (Nisar et al., 2022). The plates were incubated at 25 ± 1°C for 14 days and amoebal growth was examined daily using an inverted light microscope (AMEFC4300, EVOS™ FL, Thermo Fisher Scientific). All monoxenic amoebae cultures were characterized by microscopic examination and sequence analysis of 18S rDNA gene.

2.5 Quantification of VBNC *Legionella* spp. and *L. pneumophila*

VBNC *Legionella* spp. and *L. pneumophila* were detected and quantified by flow cytometry-cell sorting and qPCR (VFC+qPCR) assay (Nisar et al., 2023). Briefly, 300 µL sample suspension was resuspended in 200 µL of filter sterilized staining buffer (0.01% Tween-20 and 1 mM EDTA in 1X PBS, pH 7.4 ± 0.1), followed by addition of 48 µM propidium iodide (PI) and 420 nM thiazole orange (TO) dyes (cell viability kit Cat # 349480, Becton Dickinson, Franklin Lakes, USA). The mixture was incubated at 5°C for 15 minutes. Then, using a FACS Aria Fusion instrument, (Becton Dickinson, Franklin Lakes, USA) cells were analyzed and segregated into dead (PI/TO), alive (potentially culturable: TO), and injured (potentially VBNC: PI/TO) cell populations. From each sample, the injured cell fraction was isolated and subjected to DNA extraction and quantification of *Legionella* spp. and *L. pneumophila* gene markers (Nisar et al., 2023).

2.6 Data analysis

The data are described in logarithmic form with base 10 ($\log_{10}$). The percentage of both bacterial and amoebae isolates was determined based on phases or contamination levels and plotted in Microsoft® Excel®. Statistical calculations were made using R studio (version 4.2.2) and graphically presented by using "ggplot2 (version 3.3.6)" package (Wickham, 2016). Briefly, the Shapiro-Wilk test was used to assess normality of analyzed quantitative parameters. For comparison of the means of the quantitative parameters (i.e., either GU or CFU of *Legionella* spp./*L. pneumophila*, HPC, *Acanthamoeba* and *V. vermiformis*), a non-parametric Kruskal-Wallis test was used. Finally, the non-parametric Spearman's correlation (ρ) test was used to evaluate relationships among different variables (i.e., *Legionella* spp./*L. pneumophila*, HPC, *Acanthamoeba* and *V. vermiformis*). A statistically significant difference among the quantitative parameters was defined by p values of less than 0.05.

3 Results

3.1 Occurrence of *Legionella* spp. and *L. pneumophila*

Table 1 presents an overview of the percentage of samples identified as positive for *Legionella* spp. and FLA using the different detection methods. All samples that were qPCR positive for *Legionella* spp. were also positive for FLA and VBNC *Legionella*. Specifically, 21.7% (n = 36/166) of total samples were positive for *Legionella* spp. DNA (16S rDNA gene) with a concentration range of 9×10^2 to 1.5×10^6 GU (Tables 1, 2). *L. pneumophila* DNA (*mip* gene) was present in 21% samples (n = 35/166) with a concentration ranging from 3.5×10^2 to 9×10^4 GU (Tables 1, 2). All *L. pneumophila* positive samples were also positive for *Legionella* spp. During phase 1, 58.06% (n = 18/31) of the samples tested positive for *Legionella* spp. DNA, whereas in the 2nd phase 7.31% (n = 3/41), and 4th phase 28.84% (n = 15/52) of the collected samples tested positive for *Legionella* spp. DNA (Figure 1). However, in phase 3 none of the samples were positive for either *Legionella* spp. or *L. pneumophila* DNA. Standard culturing demonstrated that only four samples (two in phase 1 and two in phase 4) were positive for culturable *Legionella* spp., which were identified as non-*pneumophila Legionella* using serology and qPCR. The VFC+qPCR assay demonstrated that all samples positive for either *Legionella* spp. or *L. pneumophila* DNA (according to the qPCR assay) also contained VBNC cells (Figure 1; Table 1). Therefore, of the 36 samples that were positive for VBNC *Legionella* spp., the standard microbiological culturing assay returned a false negative result for 32 of them (88.9%). For analysis, the VBNC *Legionella* spp. and *L. pneumophila* samples were categorized into three groups based on concentration i.e., low ($< 10^3$ GU/L), medium ($\geq 10^3$ to 10^4 GU/L) and high ($> 10^4$ GU/L) contamination. Based on this grouping it was found that in phase 1, 14.3% (n = 4/31) of the water samples were positive for high VBNC *Legionella* spp. contamination, whereas in the 4th phase 33.3% (n = 10/52) water samples were positive for high VBNC *Legionella* spp. contamination (Supplementary Material, Figure S1A). It was found that the lower level of VBNC *L. pneumophila* occurred more frequently in the samples collected during phase 1 (53.5%, n = 15/31) and 4 (23.3%, n = 7/52). Only 6.7% (phase 4: n = 2/52) water samples contained high levels of VBNC *L. pneumophila* contamination (Supplementary Material, Figure S1B). Based on sampling sites it was found that in hand basin water, 13.4% (n = 9/

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TABLE 1 Prevalence of *Legionella* spp., *Vermamoeba vermiformis*, *Acanthamoeba* and total free-living amoeba in a hospital water system using different detection methods.

Sample (n)	*Number of Legionella* positive samples (%)					Number of free-living amoeba positive samples (%)		
	qPCR assay		VFC+qPCR		Culture assay	qPCR assay		Culture assay
	Legionella	*L. pneumophila*	*Legionella*	*L. pneumophila*		*Acanthamoeba*	*V. vermiformis*	
Sampling phase 1 (March 2021)								
Hand basin water (n = 16)	11	11	11	11	1	3	15	9
Shower water (n = 12)	4	4	4	4	1	0	9	6
Tap faucet biofilm (n = 3)	3	3	3	3	0	1	2	1
Total (n = 31)	**18 (58.06%)**	**18 (58.06%)**	**18 (58.06%)**	**18 (58.06%)**	**2 (6.45%)**	**4 (12.9%)**	**26 (83.87%)**	**16 (51.61%)**
Sampling phase 2 (April 2021)								
Hand basin water (n = 17)	2	2	2	2	0	0	11	8
Shower water (n = 13)	1	1	1	1	0	0	4	2
Tap faucet biofilm (n = 11)	0	0	0	0	0	0	5	0
Total (n = 41)	**3 (7.31%)**	**3 (7.31%)**	**3 (7.31%)**	**3 (7.31%)**	**0**	**0**	**20 (48.78%)**	**10 (24.39%)**
Sampling phase 3 (November 2021)								
Hand basin water (n = 18)	0	0	0	0	0	1	0	1
Shower water (n = 14)	0	0	0	0	0	0	0	0
Tap faucet biofilm (n = 10)	0	0	0	0	0	0	1	1
Total (n = 42)	**0**	**0**	**0**	**0**	**0**	**1 (2.38%)**	**1 (2.38%)**	**2 (4.76%)**
Sampling phase 4 (June 2022)								
Hand basin water (n = 16)	8	7	8	7	0	1	10	0
Shower water (n = 14)	5	5	5	5	0	0	7	0
Tap faucet biofilm (n = 22)	2	2	2	2	2	1	4	2
Total (n = 52)	**15 (28.84%)**	**14 (26.92%)**	**15 (28.84%)**	**14 (26.92%)**	**2 (3.84%)**	**2 (3.84%)**	**21 (48.38%)**	**2 (3.84%)**

67) samples were positive for high VBNC *Legionella* spp. contamination, whereas in shower water, 9.4% (n = 5/53) samples were positive for high VBNC *Legionella* spp. contamination (Supplementary Material, Figure S2A). However, the majority of hand basin (22.4%, n = 15/67) and shower (15.1%, n = 8/53) water samples contained low levels of VBNC *L. pneumophila* contamination (Supplementary Material, Figure S2B). Overall, both qPCR and VFC+qPCR assays clearly demonstrated that the standard culturing assay is frequently unable to detect *Legionella* spp./*L. pneumophila* present in the hospital water system.

3.2 Occurrence of heterotrophic bacteria and FLA

Both the hand basin and shower water samples contained HPC counts ranging from 10 to 1.5×10^5 CFU/L (Table 2). In the biofilm samples the HPC load ranged from 15 to 7.5×10^4 CFU/sample (Table 2). In case of FLA, the *V. vermiformis* gene marker was present in 40.9% (n = 68/166) of samples with concentrations ranging from 7.5×10^2 to 7.5×10^7 GU (Tables 1, 2). The *Acanthamoeba* gene marker was detected only in hand basin

TABLE 2 The minimum and maximum microbial concentrations present in the positive water and biofilm samples.

Microbes	Minimum concentration	Maximum concentration
Hand basin water (n = 67)		
Legionella DNA (GU/L)	1×10^3	1.5×10^6
VBNC *Legionella* (GU/L)	2.5×10^2	6.5×10^5
L. pneumophila DNA (GU/L)	3.5×10^2	9×10^4
VBNC *L. pneumophila* (GU/L)	6×10^2	8.5×10^4
Vermamoeba vermiformis (GU/L)	7.5×10^2	7.5×10^7
Acanthamoeba (GU/L)	1×10^3	5×10^3
Heterotrophic plate count (CFU/L)	10	1.5×10^5
Shower water (n = 53)		
Legionella DNA (GU/L)	9×10^2	7×10^4
VBNC *Legionella* (GU/L)	3.5×10^2	2.5×10^4
L. pneumophila DNA (GU/L)	3.5×10^2	9.5×10^3
VBNC *L. pneumophila* (GU/L)	70	4.5×10^3
Vermamoeba vermiformis (GU/L)	1×10^3	4×10^7
Acanthamoeba (GU/L)	0	0
Heterotrophic plate count (CFU/L)	10	1.5×10^5
Tap faucet biofilm (n = 46)		
Legionella DNA (GU)	1×10^4	3.5×10^5
VBNC *Legionella* (GU)	1.5×10^2	3×10^4
L. pneumophila DNA (GU)	7.5×10^2	1.5×10^4
VBNC *L. pneumophila* (GU)	1×10^2	1×10^4
Vermamoeba vermiformis (GU)	1×10^3	1×10^6
Acanthamoeba (GU)	4.5×10^3	8×10^3
Heterotrophic plate count (CFU)	15	7.5×10^4

water (3.01%, n = 5/166) and biofilm samples (1.2%, n = 2/166) with a range of 1×10^3 to 8×10^3 GU (Tables 1, 2). Culturable amoebae were identified in 18.1% (n = 30/166) samples; however, due to fungal overgrowth 13 isolates were unable to develop monoxenic cultures, of these 13, five isolates showed acrasid amoebae-like morphology. Only 17 isolates developed monoxenic cultures which were further characterized on the basis of cellular morphology and sequence analysis of 18S rDNA gene. Light microscopy revealed that isolates harbored monotactic morphotype and developed spherical cysts consisting of distinct inner and outer walls. Based on 18S rDNA sequencing, all these monoxenic isolates were identified as *V. vermiformis*.

3.3 Relationship among *Legionella* spp., HPC, and FLA

All shower and hand basin water samples were classified into two groups based on the HPC levels i.e., low (10 to $< 5 \times 10^3$ CFU/L) and high ($\geq 5 \times 10^3$ CFU/L) contamination. Kruskal-Wallis analysis demonstrated that quantity of both *Legionella* spp. DNA and VBNC *Legionella* spp. were significantly ($p < 0.001$) higher in water samples with high levels of HPC load (Table 3; Figure 2). Similarly, water samples having greater levels of HPC load harbored significantly higher concentrations of *L. pneumophila* DNA (Kruskal-Wallis test, $p < 0.01$), VBNC *L. pneumophila* (Kruskal-Wallis test, $p < 0.01$), and *V. vermiformis* (Kruskal-Wallis test, $p < 0.05$) (Tables 3; Figure 2). Furthermore, all samples characterized as positive for *Legionella* spp./*L. pneumophila* (DNA, culturable, and VBNC cells) were also positive for either the *V. vermiformis* gene marker or culturable amoebae. Furthermore, Spearman's analysis demonstrated both *Legionella* spp./*L. pneumophila* DNA and *Legionella* spp./*L. pneumophila* VBNC cells were positively correlated ($p < 0.001$) with *V. vermiformis* (Table 4). Overall, these results suggested that in hospital water system, high levels of HPC load and *V. vermiformis* are positively associated with both *Legionella* spp./*L. pneumophila* DNA and VBNC cells.

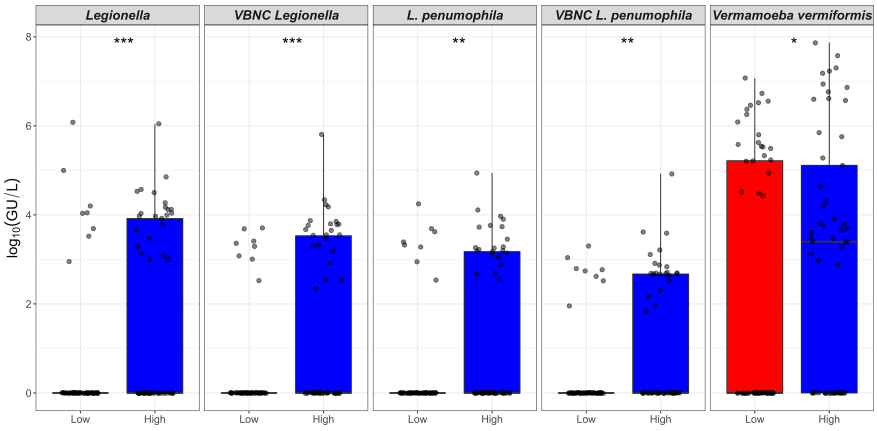

FIGURE 1
Prevalence (%) of *Legionella* spp. and free-living amoeba in a hospital water system. A total of 166 water (hand basin and shower) and biofilm (tap faucet) samples were collected in March 2021 (Phase 1), April 2021 (Phase 2), November 2021 (Phase 3), and June 2022 (Phase 4). Total amounts of *Legionella* spp./*L. pneumophila*, *Acanthamoeba*, and *Vermamoeba vermiformis* were detected and quantified by qPCR assays. Culturable *Legionella* spp. and amoebae were detected by standard microbiological culturing procedures. VBNC *Legionella* spp. and VBNC *L. pneumophila* were detected and quantified by flow cytometry-cell sorting and qPCR assay. The p values are: *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

TABLE 3 Effect of flow regime (flushing duration one-month prior to sampling) and heterotrophic plate counts of hand basin and shower water microbes.

Microbes in hand basin and shower water	Flow regime (one-month prior sampling) *		Heterotrophic plate count **	
	Low (0 to < 2 hours/ month)	High (≥ 2 to 40 hours/ month)	Low (10 to < 5×10^3 CFU/L)	High (≥ 5×10^3 to 1.5×10^5 CFU/L)
Total *Legionella* (GU/L)	1.783 ± 2.043	0.637 ± 1.559	0.532 ± 1.443	1.604 ± 2.015
VBNC *Legionella* (GU/L)	1.560 ± 1.771	0.551 ± 1.366	0.413 ± 1.100	1.455 ± 1.839
Total *L. pneumophila* (GU/L)	1.472 ± 1.671	0.495 ± 1.284	0.429 ± 1.148	1.326 ± 1.698
VBNC *L. pneumophila* (GU/L)	1.206 ± 1.372	0.416 ± 1.107	0.344 ± 0.921	1.106 ± 1.442
***Vermamoeba vermiformis* (GU/L)**	3.578 ± 2.835	1.755 ± 2.508	1.983 ± 2.763	2.952 ± 2.755
Heterotrophic plate count (CFU/L)	3.742 ± 1.010	3.656 ± 0.855	–	–

Data is log transformed and shown as mean ± standard deviation.
*Microbial loads (except heterotrophic plate count) in the low flow regime are significantly higher than in the high flow regimes (Kruskal-Wallis analysis, $p < 0.05$).
**Microbial loads in high heterotrophic plate counts are significantly higher than low heterotrophic plate counts (Kruskal-Wallis analysis, $p < 0.05$).

3.4 Influence of flow regimes and on *Legionella* spp., HPC, and FLA

The total duration (hours) of flushing events for one month prior to sampling, was categorized into: low (0 to < 2 hours/month) and high (≥ 2 to 40 hours/month) flow regimes. The Kruskal-Wallis analysis indicated that the concentrations of *Legionella* spp. DNA ($p < 0.01$), *L. pneumophila* DNA ($p < 0.01$), VBNC *Legionella* spp. ($p < 0.001$), VBNC *L. pneumophila* ($p < 0.001$) and *V. vermiformis* DNA ($p < 0.05$), were all higher in low flow regimes compared with high flow regimes (Table 3; Figure 3). When the total duration (hours) of flushing events for only one week prior to sampling was examined, no association was observed with any of the microbial concentrations measured. The HPC load did not show any measurable difference in the low vs high flow regimes either one month or one week prior to sampling (Table 3). In contrast with the total flow duration, the total number of flow counts (number of flushing events) for either one week or one month prior to sampling was not associated with any significant change in any of the microbial concentrations measured. In conclusion, a month of reduced usage (< 2 hours water flushing per month) supports the proliferation of *Legionella* spp./*L. pneumophila* and *V. vermiformis* in hospital water system.

3.5 Influence of water temperature and on *Legionella* spp., HPC, and FLA

The water outlets (hand basins and showers) of the hospital water system received water from both the cold water supply and hot water supply (Supplementary Material; Figures S3, S5). The temperature data for each sample location were averaged over one week and one month prior to sample collection (Supplementary Material; Figures S3, S5). The average temperatures (mean ± SD)

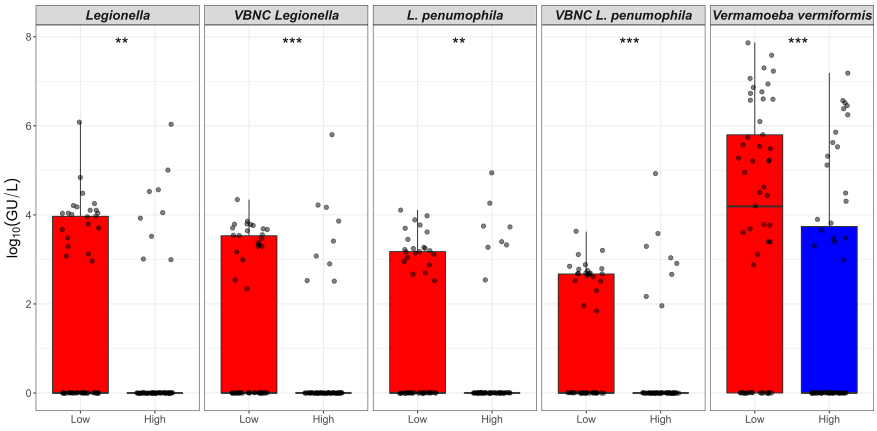

FIGURE 2
Relationship between the heterotrophic plate count and *Legionella* spp./*Vermamoeba vermiformis*. X-axis represents HPC level that is categorised into low (10 to < 5 × 10^3 CFU/L) and high (≥ 5 × 10^3 to 1.5 × 10^5 CFU/L) contamination. Y-axis represents log_{10}(GU/L) of *Legionella* spp., VBNC *Legionella* spp., *L. pneumophila*, VBNC *L. pneumophila*, and *V. vermiformis*. The p values are: $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

measured from the cold water supply were (21.78 ± 1.98°C per week and 22.01 ± 1.69°C per month), hot water supply (23.64 ± 3.15°C per week and 23.69 ± 3.08°C per month) and outlet water (23.74 ± 2.43°C per week and 23.76 ± 1.95°C per month). No relationships between microbial concentration and water temperatures were observed. This is likely due to the average water temperature being similar for both hot and cold water supplies, with increases in hot water temperature occurring through hot water usage having a limited effect on the overall average temperature due to the periods of stagnation and inactivation occurring in between usages (Supplementary Material, Figures S4, S6).

4 Discussion

In this study, it was identified that 31.3% (n = 21/67) hand basin water, 18.9% (n = 10/53) shower water and 10.9% (n = 5/46) biofilm samples were positive for either *Legionella* spp. or *L. pneumophila* gene marker (Table 1). According to the literature, the majority of engineered water systems of hospital and healthcare facilities are contaminated with *Legionella* spp. or *L. pneumophila*. In Poland, 74.7% of water samples from hospitals and other large building structures tested positive for *Legionella* spp., and *L. pneumophila* sg2-14 was the most prevalent serogroup (Sikora et al., 2015). A similar study conducted in Hungary that examined water samples from healthcare facilities and other buildings showed that 60% samples were positive for *Legionella* spp. (predominantly *L. pneumophila* sg2-14) (Barna et al., 2016). A study conducted in 20 different hospitals in Spain reported that 37.2% of water samples were colonized with *L. pneumophila* sg1 and *L. pneumophila* sg2-14 (Sabrià et al., 2004). In Taiwan, 63% of samples collected from hospital water systems tested positive for *Legionella* spp. and *L. pneumophila* sg1 (Yu et al., 2008). Comprehensive national surveillance studies conducted in 13 different states of the USA reported that 70% of hospital water systems were contaminated with *Legionella* spp. (Stout et al., 2007). A recent study conducted in Australia detected 41% samples of water and biofilms from hospital and residential buildings were colonized with *Legionella* spp. (Nisar et al., 2022). The lower *Legionella* spp. prevalence in this study could be due to the fact this was a case study of a single hospital that has been proactive in their water quality risk management compared with other hospitals.

All previous studies on *Legionella* spp. in engineered water systems have either used standard culturing or a qPCR assay to detect *Legionella* spp, and none have screened for the presence of VBNC *Legionella* spp. In the present study, VFC+qPCR assay showed that all water and biofilm samples positive for *Legionella* spp./*L. pneumophila* gene marker also contained VBNC cells. The quantity of total *Legionella* spp. detected by qPCR assay was greater than VBNC cells, which clearly highlights that the hospital water system harbored both dead and VBNC *Legionella* spp. (Figure 1). Our findings suggest that the standard *Legionella* spp./*L. pneumophila* guidelines should include quantification of VBNC cells.

Currently, there is still much debate around the exact infective dose for *Legionella* spp. (Bartram et al., 2007). An analysis by Sikora et al. (2015) estimated that legionellosis outbreaks may occur sporadically when water is contaminated with 10^3 to 10^5 CFU/L

TABLE 4 Correlation between *Legionella* spp. and *Vermamoeba vermiformis* in hand basin and shower water samples.

	Spearman's rank correlation			
	Legionella DNA	VBNC *Legionella*	*L. pneumophila* DNA	VBNC *L. pneumophila*
Vermamoeba vermiformis	ρ = 0.5819 $p < 0.001$	ρ = 0.5833 $p < 0.001$	ρ = 0.5955 $p < 0.001$	ρ = 0.5826 $p < 0.001$

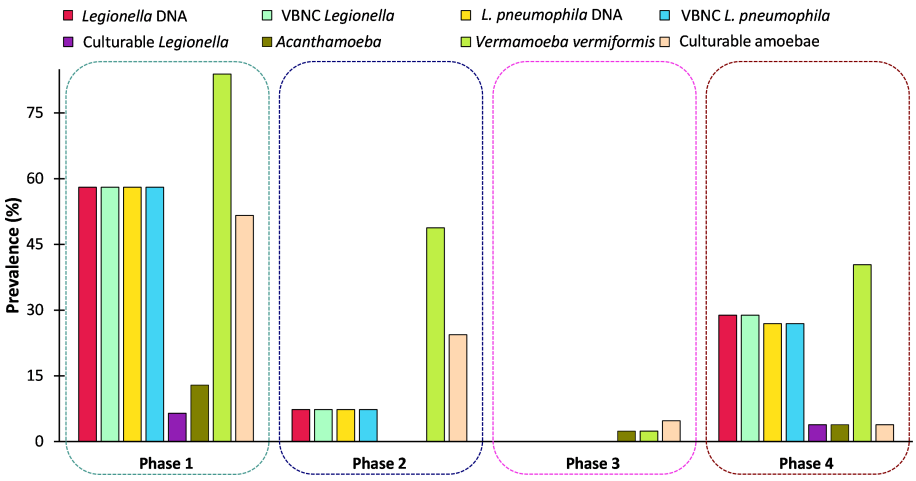

FIGURE 3
Relationship between intermittent water usage and the presence of *Legionella* spp./*Vermamoeba vermiformis*. X-axis represents total duration (hours) of flushing events recorded for one-month prior to sampling. Flushing was categorized into low; 0 to < 2 hours, and high flow regime; ≥ 2 to 40 hours. Y-axis represents $\log_{10}$(GU/L) of *Legionella* spp., VBNC *Legionella* spp., *L. pneumophila*, VBNC *L. pneumophila*, and *V. vermiformis*.

and when *Legionella* spp. counts exceed 10^5 CFU/L an outbreak of legionellosis can occur (Sikora et al., 2015). However, these estimates are based on the number of culturable cells (CFU/L), so it is challenging to determine the relative risk associated with the concentrations of VBNC cells (GU/L). In contrast with culturable *Legionella* spp., VBNC cells infect with lower pathogenicity and take a longer time to infect amoebae (Nisar et al., 2023). Therefore, future research is needed to determine the infectious dose of VBNC *Legionella* spp. to understand the role of VBNC *Legionella* spp. in nosocomial infections and the public health risk posed by this concentration of VBNC *Legionella* spp. in engineered water systems.

In hospital water systems, *Legionella* infects and survives within protozoan hosts, including *Acanthamoeba* and *V. vermiformis* (Nisar et al., 2020a). In this study, it was identified that *V. vermiformis* (gene marker and culturable) was the most commonly identified amoebae associated with *Legionella* spp. prevalent in the water and biofilm samples (Table 4). This is supported by previous studies that have demonstrated *V. vermiformis* to be widely present in potable water (Kuiper et al., 2006; Nisar et al., 2020a). Similarly, microbiome analysis of potable water also showed *V. vermiformis* as the most prevalent protozoa (Delafont et al., 2013; Delafont et al., 2016). Water samples of dental units of Italian hospitals were found to be highly contaminated with *V. vermiformis* (60%) (Spagnolo et al., 2019). Similarly, a study conducted in hospitals of South Africa identified that 69% of samples were positive for *V. vermiformis* and 30.6% for *Acanthamoeba* (Muchesa et al., 2018). A recent study conducted in Australia examined water and biofilm samples from hospital and residential buildings showed the presence of FLA in 69% of the samples. It was also found that in all tested samples, *V. vermiformis* (55%) was the more frequently detected FLA (Nisar et al., 2022). In comparison with *Acanthamoeba*, *V. vermiformis* is more sensitive to disinfection treatments (Nisar et al., 2020a). Therefore, high levels of *V. vermiformis* could be attributed to decay and lower levels of residual chemical disinfectants in the hospital water system.

To our knowledge HPC loads have not been linked to any known legionellosis outbreak and the relationship between HPC levels and opportunistic premise plumbing pathogens is still unclear (Bartram et al., 2003). The SA Health, (2013) use HPC load as an indicator of water quality and recommend that if HPC load is ≥ 10^2 CFU/mL in warm water systems then the disinfection procedures for engineered water system should be considered. In this study it was found that the water samples with high HPC loads (≥ 5×10^3 CFU/L) contained high quantities of both *Legionella* spp. and *L. pneumophila* (Figure 2; Table 3). These results are in accordance with a previous study conducted on engineered water systems of residential buildings, which also showed a positive relationship between HPC levels and concentrations of *Legionella* spp. (Ley et al., 2020). Similarly, it was also found that samples with high HPC loads harbored high levels of *V. vermiformis* (Figure 2; Table 3). The relationship between bacteria and FLA consists of three major types of interactions i.e., mutualism, parasitism, and predation (Shi et al., 2021). Generally, FLA are considered natural predators of bacteria, which could account for the high levels of *V. vermiformis* observed in the presence of high levels of HPC (Rodriguez-Zaragoza et al., 2005). It was also identified that all samples positive for *Legionella* spp./*L. pneumophila* were also positive for FLA. Furthermore, Spearman's analysis demonstrated a strong positive correlation between *Legionella* spp./*L. pneumophila* and *V. vermiformis*. In engineered water systems, FLA exist in both trophozoite (metabolically active) and cyst (dormant) states (Zhang and Lu, 2021). The trophozoites support intracellular proliferation of *Legionella* spp. and transformation of VBNC *Legionella* spp. into a culturable state (Solomon et al., 2000; Shadrach et al., 2005; Berk et al., 2008; Watanabe et al., 2016; Boamah et al., 2017). Amoebae cysts protect intracellular *Legionella* spp. from prolonged chemical and physical disinfection treatments (Dobrowsky et al., 2016;

Boamah et al., 2017). The significant role amoebae play in *Legionella* spp. survival in potable water systems suggests that guidelines for the control of *Legionella* spp. must consider acceptable limits of amoeba within these systems as a measure to control *Legionella* spp. concentrations.

Water stagnation within building distribution systems promotes the accumulation of biomass, decay of chemical disinfectants, and alters the water quality (Bedard et al., 2018). Therefore, this study investigated the effect of temporary stagnation induced by intermittent flushing and water usage on *Legionella* spp., with a special focus on VBNC *Legionella* spp. It was found that an increase in temporary stagnation once a month prior to sampling significantly ($p < 0.01$) increased the quantity of total *Legionella* spp./*L. pneumophila* and VBNC *Legionella* spp./*L. pneumophila* population; however, increased stagnation one week prior to sampling was not associated with increased risk (Figure 3; Table 3). This supports guidelines that recommend routine flushing of outlets to manage *Legionella* spp. within engineered water systems (Enhealth, 2015). To our knowledge, this is first study in which the effect of temporary stagnation, HPC load, and *V. vermiformis* on VBNC *Legionella* spp./*L. pneumophila* in hospital water systems has been investigated. This study averaged water temperatures across one week or one month prior to sampling for both the hot and cold water pipelines/outlets. As a result water temperatures were more similar to each other than anticipated. This is likely to explain the lack of a statistically significant difference in *Legionella* concentrations associated with different temperatures. Future research with a larger dataset is needed to explore the temperature relationship further.

5 Conclusion

In building plumbing systems, temporary stagnation arising through intermittent usage causes water quality to deteriorate. This study identified that temporary stagnation for over a month promotes the persistence of VBNC *Legionella* spp./*L. pneumophila*. Similarly, FLA and heterotrophic bacteria present in this temporary stagnant environment positively interact with *Legionella* spp./*L. pneumophila*. Therefore, temporary stagnation, FLA and heterotrophic bacteria must be managed for the proper control and prevention of LD. This study also showed that the standard microbiological culture method used to detection *Legionella* spp. returned a false negative result for 88% of the VBNC *Legionella* spp. positive samples. As all samples positive for VBNC *Legionella* spp. were also qPCR positive, this suggests that qPCR may be a more appropriate detection method for routine surveillance. However, future research is needed to investigate the concentrations of VBNC *Legionella* spp. that pose a risk to public health to enable interpretation of these results to inform improved *Legionella* spp. guidelines.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

MN and HW conceived and designed research. MN performed the experiments. GB assisted in the flow cytometry. KR, MB, HW and RB provided technical assistance. JX and JH assisted in sampling and data collection. MN and HW drafted and edited the manuscript. HW, KR, MB, RB, GB, JX, and JH corrected and contributed to the manuscript. All authors approved of the final manuscript.

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Conflict of interest

Authors JH and JX are employed by Enware Pty Ltd.

The remaining 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1190631/full#supplementary-material

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Bacterial persistence in *Legionella pneumophila* clinical isolates from patients with recurring legionellosis

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Bacterial persisters are a transient subpopulation of non-growing, antibiotic-tolerant cells. There is increasing evidence that bacterial persisters play an important role in treatment failure leading to recurring infections and promoting the development of antibiotic resistance. Current research reveals that recurring legionellosis is often the result of relapse rather than reinfection and suggests that the mechanism of bacterial persistence may play a role. The development of single-cell techniques such as the Timer^{bac} system allows us to identify potential persister cells and investigate their physiology. Here, we tested the persister forming capacity of 7 pairs of *Legionella pneumophila* (*Lp*) clinical isolates, with isolate pairs corresponding to two episodes of legionellosis in the same patient. We distinguished non-growing subpopulations from their replicating counterparts during infection in an amoeba model. Imaging flow cytometry allowed us to identify single non-growing bacteria within amoeba cells 17 h post-infection, thus corresponding to this subpopulation of potential persister cells. Interestingly the magnitude of this subpopulation varies between the 7 pairs of *Lp* clinical isolates. Biphasic killing kinetics using ofloxacin stress confirmed the persister development capacity of ST1 clinical isolates, highlighting enhanced persister formation during the host cell infection. Thus, persister formation appears to be strain or ST (sequence type) dependent. Genome sequence analysis was carried out between ST1 clinical isolates and ST1 Paris. No genetic microevolution (SNP) linked to possible increase of persistence capacity was revealed among all the clones tested, even in clones issued from two persistence cycle experiments, confirming the transient reversible phenotypic status of persistence. Treatment failure in legionellosis is a serious issue as infections have a 5-10% mortality rate, and investigations into persistence in a clinical context and the mechanisms involved may allow us to combat this issue.

KEYWORDS

***L. pneumophila*, virulence, bacterial persistence, antibiotic tolerance, recurring legionellosis, amoeba**

1 Introduction

Antibiotic resistance poses a serious public health threat as the emergence of new resistance mechanisms and multi-drug resistant bacteria render current treatment methods ineffective. Although there is already a large body of antibiotic resistance research exploring associated genes and mutations (Nnadozie and Odume, 2019), the role of bacterial physiology during infection and its implication in resistance remains largely unstudied. Complex host-pathogen interactions and the formation of microbial communities produce populations of genetically identical, yet physiologically distinct bacteria. This reversible phenomenon, also known as phenotypic heterogeneity, is of major clinical importance as it favours the formation of transiently non-replicative, potential "persister" cells. Furthermore, persister subpopulations display high antibiotic tolerance compared to their growing counterparts without any genetic resistance determinants (Rocha-Granados et al., 2020).

There is increasing evidence that bacterial persisters play an important role in treatment failure leading to recurring infections and promoting the development of antibiotic resistance (Fauvart et al., 2011; Harms et al., 2016; Windels et al., 2019). Persisters are defined as a subpopulation of non-replicative bacteria that are able to tolerate the presence of otherwise bactericidal concentrations of antibiotics (Balaban et al., 2019; Michaux et al., 2021). Historically, persister subpopulations were identified by a biphasic killing kinetic following the addition of bactericidal antibiotics; the sensitive population is killed rapidly while the persisters remain unaffected or are killed at a much slower rate (Harms et al., 2016). Compared to antibiotic resistance, persistence is a result of phenotypic changes rather than heritable genetic mutations (Rocha-Granados et al., 2020). Persistence can be further differentiated from tolerance as it only occurs in a subpopulation of bacteria.

Bacterial persistence is of clinical relevance as persisters have been identified in a variety of pathogenic bacteria including *Escherichia coli, Staphylococcus aureus, Salmonella enterica* and more recently *Legionella pneumophila* (*Lp*) (Claudi et al., 2014; Fisher et al., 2017; Personnic et al., 2019a; Personnic et al., 2019b). *Lp* is a gram-negative, ubiquitous environmental bacterium and opportunistic human pathogen. Interestingly, *Lp* displays a dual extracellular-intracellular lifecycle. In freshwater and human-made water systems *Lp* can live as a free-living bacterium or in eukaryotic phagocytes, such as amoeba. Inside eukaryotic phagocytes, the bacteria transition to a metabolically active but less infectious, replicative form. Subsequently, the depletion of nutrients in the host cell triggers another morphological change, whereby *Lp* reverts to its virulent and motile transmissive form (Oliva et al., 2018).

Lp is also able to colonise and replicate within various phagocytic cells including human alveolar macrophages, resulting in a severe pneumonia known as Legionnaires' disease. Immunocompromised and elderly individuals are especially susceptible to infection (Samuelsson et al., 2023) and treatment failure is a serious issue as infections have a 5-10% mortality rate (Herwaldt and Marra, 2018). Studies into antibiotic resistance in *Lp* demonstrated that *in vitro* evolution can lead to the emergence of macrolide-resistant clones carrying mutations in 23S RNA and ribosomal proteins L4 and L22 (Herwaldt and Marra, 2018). Several clones also displayed increased expression of macrolide efflux systems present in a few sub-groups of *Lp* strains (Massip et al., 2017; Vandewalle-Capo et al., 2017). However, a retrospective genomic study of *Lp* strains isolated from patients with recurring Legionnaires' disease showed that the resistance markers and genetic evolution characterised *in vitro* were not present *in vivo* and that recurring Legionnaires' disease is often the result of relapse rather than reinfection (Pouderoux et al., 2020). These observations suggest that the mechanism of persistence may play a role. Additionally, the existence of *Legionella* persisters has recently been identified in both amoeba and macrophage models using a laboratory adapted strain of *Lp*, JR32 (Personnic et al., 2019a). It is important to note that the strain JR32 is a *Legionella pneumophila* Philadelphia-1 derivative adapted for a high rate of amoeba and macrophage infection, and is thus genetically evolved compared to the clinical progenitor (Marra and Shuman, 1989; Rao et al., 2013; Maita et al., 2016). Therefore, it is still unknown whether clinical strains can form bacterial persisters, and whether the mechanism of persistence could explain recurring disease.

Research into bacterial persistence is complicated due to the transient nature of persister populations. Furthermore, standard population-level analysis provides average measures which are insensitive to population heterogeneity and to differences in minority, potential persister subpopulations (Helaine and Kugelberg, 2014; Balaban et al., 2019). The development of single-cell techniques has thus opened new possibilities in the field of bacterial persister research, allowing researchers to both identify potential persisters and investigate their physiology. Single cell fluorescence techniques take advantage of growth rate differences to identify and isolate non-growing cells (potential persisters) from their replicating counterparts (Claudi et al., 2014; Personnic et al., 2019a; Personnic et al., 2019b). For example, the Timer^{bac} system uses a DsRed S197T variant called the TIMER protein: a stable fluorescent reporter that changes from green to red fluorescence as it matures (Claudi et al., 2014). The initial green form predominates in growing bacteria as the mature red form is diluted during replication, while the red mature form accumulates in non-growing or slow-growing bacteria (Figure 1A). Fluorescence is measured on a single-cell level via flow cytometry and thus the green/red fluorescence, or Timer colour ratio, is correlated to the growth rate of individual bacteria.

The purpose of this study is to investigate the potential bacterial persister forming capacity of 7 pairs of *Lp* clinical isolates. These 7 isolate pairs represent 7 different sequence types (ST), and pairs correspond to two episodes (early and late) of legionellosis in the same patient (recurring legionellosis). Indeed, up to now, this phenomenon has only been described in *Legionella* laboratory strains. Thus, we set up the Timer^{bac} system in the *Lp* Paris reference strain and all clinical isolates, demonstrating that this system can be adapted to numerous strains of *Lp*. Additionally, we provide further evidence for the validity of this single-cell fluorescence technique in investigating potential persisters and reveal possible further applications when coupled with imaging flow cytometry, highlighting the sensitivity of this system in the detection of single bacteria, even inside a host cell. Biphasic killing

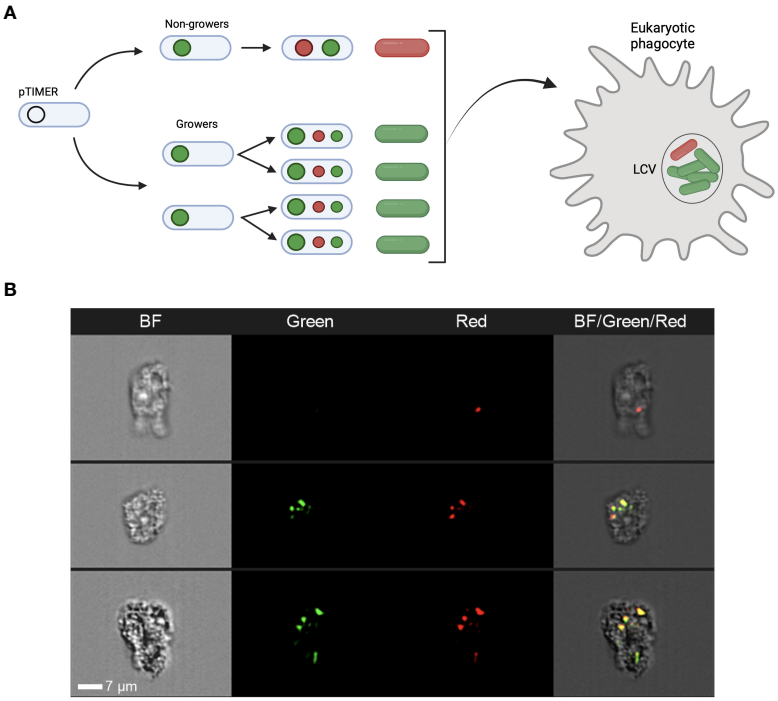

FIGURE 1
The TIMER^{bac} system in *Legionella pneumophila*. **(A)** Schematic detailing the TIMER^{bac} system adapted from Claudi et al. (2014). In non-growing cells both the green and red forms of the TIMER protein accumulate resulting in red-orange fluorescence, while in growing cells the green form predominates. Fluorescent "TIMER" bacteria can be visualised intracellularly during infection. **(B)** Representative images of TIMER bacteria after 12 hours of amoeba infection. Panels represent brightfield (BF), green fluorescence and red fluorescence channels, as well as a composite image of all three channels. Imaging flow cytometers, such as the Amnis® ImageStream®X, can distinguish both single red non-growing and multiple green replicating *Lp* ST1 Paris-timer bacteria within *A. polyphaga*. White scale bar represents 7µm.

kinetics upon ofloxacin exposure confirmed the ability of both clinical *Lp* isolates and the *Lp* Paris reference strain to form bacterial persisters as historically defined. Interestingly, these experiments also revealed an increase in *Lp* persister proportions during host infection. Finally, genome sequence analysis, carried out on clinical isolates from recurring legionellosis and isolates issued from persistence assays, confirmed the physiologic status of persistence as a phenotypic change rather than a genetic evolution.

2 Materials and methods

2.1 Bacterial strains and eukaryotic cell lines

Bacterial strains used in this study and relevant antibiotic resistances are detailed in Table 1. Concerning *Legionella pneumophila* clinical isolates, except for patient 11 (ST87) who was infected by *Lp* serogroup 3, all the other isolates were from *Lp* serogroup 1 as mentioned in Table 1. *Escherichia coli* (*E. coli*) DH5α strains were grown at 37°C in lysogeny broth (LB) or on LB agar supplemented with appropriate antibiotics. *Lp* strains were gro n at 37°C in ACES yeast extract medium (AYE) or on charcoal yeast extract (BCYE) agar plates buffered with ACES. Chloramphenicol (7.5 µg ml^{-1}) was added as required. Axenic *Lp* cultures for biphasic curves and flow cytometry were inoculated at an optical density (OD_{600nm}) of 0.1 and grown at 37°C to exponential phase growth. *Lp* cultures for amoeba infections were inoculated at OD_{600nm} 0.1 and incubated at 37°C overnight to stationary phase growth.

Acanthamoeba polyphaga strain Linc AP-1 (*A. polyphaga*) was grown in protease-yeast-glucose medium (PYG) at 30°C. Prior to infection, amoebae were plated at a concentration of 2x10^6 cells per well in PYG media lacking protease, yeast extract, glucose (PYS) and incubated at 30°C for 2 hours to allow for adherence. Bacterial cultures were resuspended in PYS with appropriate antibiotics and incubated at 30°C for 2 hours to promote infectivity and synchronise the infectious state of bacterial cells. Amoeba cells infected with *Lp* at a multiplicity of infection (MOI) of 1 and centrifuged at 500xg for 10 minutes to synchronise infection. Plates were incubated at 30°C for 2 h before wells were rinsed and culture medium was replaced to remove extracellular bacteria and promote a synchronised infection kinetic.

2.2 Construction of timer strains

Bacterial strains and plasmids used in this study are listed in Table 1. The TIMER plasmid was amplified in *Escherichia coli* (*E. coli*) DH5α, extracted and then confirmed via digestion. Electrocompetent *E. coli* were mixed with 10-100 ng of TIMER plasmid DNA, transferred to a cold electroporation cuvette and subject to an electric field (2500 V, 10 µF, 600 Ω). 900 µl of LB was

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TABLE 1 Bacterial strains and plasmids used in this study.

Escherichia coli strains		
Strain	Characteristics	Reference
DH5α	recA1, endA1, lacZΔM15	Lab collection
DH5α-timer	recA1, endA1, lacZΔM15, pTIMER (pNP107)	Lab collection
Legionella pneumophila strains		
Strain	Characteristics	Reference
Lp Paris	Clinical wild-type isolate	Cazalet et al. (Cazalet et al., 2004)
ST1.1	LP1 (ST1), early isolate patient 10	Samples provided by CNRL (Centre National de Référence des Légionelles, Lyon) (Pouderoux et al., 2020)
ST1.2	LP1 (ST1), late isolate patient 10	
ST20.1	LP1 (ST20), early isolate patient 12	
ST20.2	LP1 (ST20), late isolate patient 12	
ST23.1	LP1 (ST23), early isolate patient 6	
ST23.2	LP1 (ST23), late isolate patient 6	
ST40.1	LP1 (ST40), early isolate patient 4	
ST40.2	LP1 (ST40), late isolate patient 4	
ST48.1	LP1 (ST48), early isolate patient 3	
ST48.2	LP1 (ST48), late isolate patient 3	
ST87.1	LP3 (ST87), early isolate patient 11	
ST87.2	LP3 (ST87), late isolate patient 11	
ST62.1	LP1 (ST62), early isolate	Samples provided by CNRL (Centre National de Référence des Légionelles, Lyon)
ST62.2	LP1 (ST62), late isolate	
Lp Paris-timer	Clinical wild-type isolate, pTimer	This work
ST1.1-timer	ST1.1 with pTimer *Chl*^{R}	This work
ST1.2-timer	ST1.2 with pTimer *Chl*^{R}	This work
ST20.1-timer	ST20.1 with pTimer *Chl*^{R}	This work
ST20.2-timer	ST20.2 with pTimer *Chl*^{R}	This work
ST23.1-timer	ST23.1 with pTimer *Chl*^{R}	This work
ST23.2-timer	ST23.2 with pTimer *Chl*^{R}	This work
ST40.1-timer	ST40.1 with pTimer *Chl*^{R}	This work
ST40.2-timer	ST40.2 with pTimer *Chl*^{R}	This work
ST48.1-timer	ST48.1 with pTimer *Chl*^{R}	This work
ST48.2-timer	ST48.2 with pTimer *Chl*^{R}	This work
ST87.1-timer	ST87.1 with pTimer *Chl*^{R}	This work
ST87.2-timer	ST87.2 with pTimer *Chl*^{R}	This work
ST62.1-timer	ST62.1 with pTimer *Chl*^{R}	This work
ST62.2-timer	ST62.2 with pTimer *Chl*^{R}	This work
Plasmids		
Name	Characteristics	Reference
pTimer (pNP107)	pMMB207-C, ΔlacIq, Ptac-timer (constitutive timer expression), *Chl*^{R}	Personnic et al., 2019 (Personnic et al., 2019a)

then added and tubes were incubated at 37°C for 1 hour for cells to express resistance genes. Finally, suspensions were spread onto LB agar supplemented with 5 µg/ml chloramphenicol to select for Timer-transformants. The TIMER plasmid was extracted from DH5α-timer cultures via mini prep with the EZNA Plasmid DNA Mini Kit (Omega Biotek) and confirmed via digestion.

Electrocompetent *Lp* were prepared from colonies on BCYE agar after a 3-day incubation at 37°C. Using a 10 µl inoculation loop, bacteria were collected and resuspended in 1 ml cold sterile water. Bacterial suspensions were then centrifuged at 6000 xg for 10 minutes at 4°C and washed in 1 ml cold sterile water. This step was repeated 3 times. Cells were resuspended in an appropriate volume of sterile water, mixed with approximately 600 ng of plasmid DNA, and then transferred to a cold electroporation cuvette. Cells were subjected to an electric field (2500 V, 25 µF, 400 Ω). 900 µl of AYE was then added and tubes were incubated at 37°C for 2 h. Finally, suspensions were spread onto BCYE agar supplemented with 7.5µg/ml chloramphenicol.

Initial verification of the TIMER^{bac} system as growth reporter in axenic *Lp* Paris cultures was performed by fluorescence microscopy. Axenic cultures were transferred onto agar pads (1% ultrapure agarose (Invitrogen) in sterile water) and imaged at 100x magnification with the EVOS FL Life Technologies digital inverted microscope. Fluorescence parameters were as follows: green fluorescence 470 nm excitation, 525 nm emission; red fluorescence 530 nm excitation, 593 nm emission.

2.3 Imaging flow cytometry

12 hours post-infection (hpi), amoeba infected with TIMER *Lp* strains were fixed in 4% Paraformaldehyde (Sigma Aldrich) for 20 min. Amoeba were then collected, washed in PBS and quenched in 0.1 M glycine (Roth) for 20 min. Finally, samples were resuspended in 1 ml PBS and kept at 4°C until analysis by flow cytometry. At least 20 000 events were acquired with the ImageStream X Mark II (Amnis) imaging flow cytometer. 5100 infected amoebae were identified and visualised using the IDEAS 6.2 software. Aspect ratio vs area was used to gate single amoeba, while intracellular bacteria were identified using green (Ex 488 nm, Em 528/65nm) and red fluorescence (Ex 561 nm, Em 610/30 nm) parameters. Images were acquired at 60x magnification using extended depth of field (EDF) and show brightfield, green fluorescence and red fluorescence channels as well as composite images of all three channels.

2.4 Flow cytometry

At the relevant time points post-infection, amoebae were lysed in 0.1% Triton X-100 (Sigma) 150 mM NaCl. Samples were then centrifuged and washed in Dulbecco's Phosphate Buffered Solution (PBS) (Gibco), before fixation for 20 min. in 4% Paraformaldehyde (Sigma Aldrich). Following fixation, samples were washed in PBS and quenched in 0.1 M glycine (Roth) for 20 min. Finally, samples were centrifuged and resuspended in PBS. Fixation of axenic cultures for flow cytometry followed the same protocol without the cell lysis steps.

Samples were analysed using both the BD™ LSR Fortessa 4L and Attune CytPix flow cytometers and the following spectral parameters: side scatter, green fluorescence (Ex 488 nm, Em 515-545 nm) and red fluorescence (Ex 561 nm, Em 600-620 nm). The gating strategy was performed as shown in Figure 2A. To be able to accurately compare bacterial counts between conditions a defined resuspension volume and acquisition time was used. The software FlowJo™ was used to process populations and define the percentage of growing and non-growing subpopulations for each strain. As per *Personnic et al.* growth rate was determined at a single-cell level by calculating the TIMER colour ratio: $\log_{10}$(green fluorescence 525 nm/red fluorescence 610 nm) and subsequent frequency distribution histograms of bacterial populations were produced by GraphPad Prism for Mac.

2.5 Persister assays

Axenic biphasic kill curves: Axenic pre-cultures were grown as previously mentioned. AYE was inoculated with 2x10^6 mid-exponential phase bacteria with or without ofloxacin 30 µg mL^{-1} (>20 x MIC) and incubated at 37°C. At given time points bacteria were collected, washed twice in PBS, serially diluted, and plated onto BCYE to quantify CFUs. Colonies were taken from the last time-point plates and used for "repeat" experiments. Additionally, these colonies were re-isolated and conserved in 20% glycerol at -80°C for subsequent follow-up experiments. Results are displayed as survival frequency transformed using the formula freq*((n-1)+0.5)/n, where n is the number of observations, to avoid 0 and 1 values.

Post-infection biphasic kill curves: *A. polyphaga* were infected as described above. Infected cells were lysed at 17h post-infection, or mid-replicative phase, using 0.1% Triton X-100 (Sigma) 150 mM NaCl and bacteria were resuspended in AYE supplemented or not with ofloxacin 30 µg mL^{-1}. Bacterial suspensions were incubated at 30°C in continuity with the host cell environment. At given time points bacteria were collected, washed twice in PBS, serially diluted, and plated onto BCYE to quantify CFUs. Colonies taken from the last time-point plates were used for "repeat" experiments. Additionally, these colonies were re-isolated and conserved in 20% glycerol at -80°C for subsequent follow-up experiments. Results are displayed as survival frequency transformed using the formula freq*((n-1)+0.5)/n, where n is the number of observations, to avoid 0 and 1 values.

2.6 Comparative genomic analysis

Long-read sequencing: For the clinical reference isolates ST1.1 and ST1.2, long-read sequencing technology was used for genome closing purposes. Libraries were prepared using the V14 chemistry rapid barcoding kit and sequencing was performed on a GridION sequencer using live super-accurate basecalling (Nanoporetech, Oxford, UK). Genome assembly and polishing were performed

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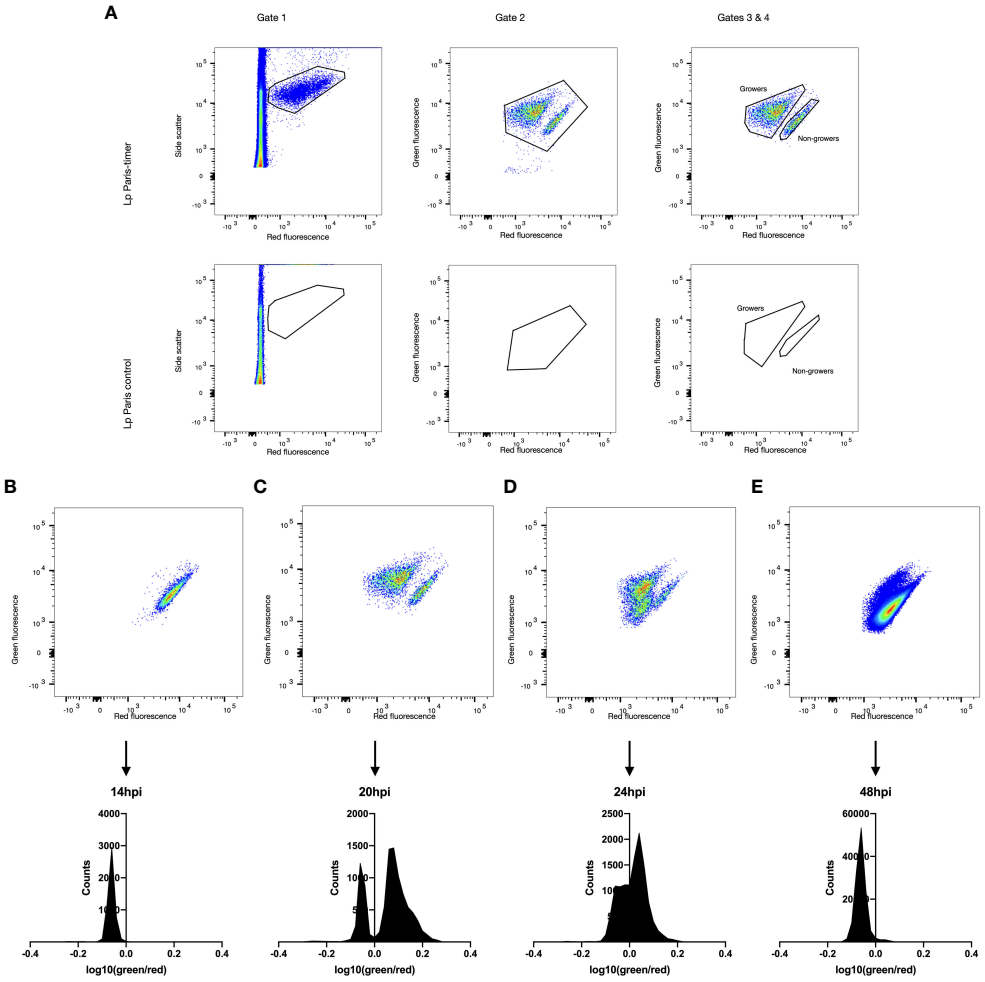

FIGURE 2
Growth rate heterogeneity of *Lp* during *A. polyphaga* infection. *A. polyphaga* amoebae were infected at MOI 1 with *Lp* ST1 Paris-timer. At the relevant time points amoebae were lysed and bacteria fixed in 4% PFA. Bacteria were then analysed by flow cytometry and their TIMER colour ratio $\log_{10}$(green fluorescence 525nm/red fluorescence 610nm) was determined. **(A)** The spectral properties of the timer protein were used to distinguish fluorescent, TIMER-producing bacteria (Gate 1, upper panel) from autofluorescence and cell debris (Gate 2, lower panel). Subsequently, Gate 2 eliminates any remaining debris associated with fluorescent bacteria while Gates 3 and 4 define the growing (green) and non-growing (red) populations. **(B–E)** Flow cytometry scatter plots and frequency distributions of *Lp* ST1 Paris-timer at 14hpi (hours post infection), 20hpi, 24hpi and 48hpi respectively.

with flye 2.9.1 and medaka 1.7.3, respectively (Kolmogorov et al., 2019) (https://github.com/nanoporetech/medaka). The obtained genomes were annotated with bakta 1.7.0 (Schwengers et al., 2021).

Short-read sequencing: Whole-genome sequencing (WGS) was performed using Illumina DNAprep kit for library preparation followed by paired-end 2x150 bp sequencing using a Nextseq 550 sequencer (Illumina®, San Diego, USA). WGS was performed on stock clones prior to biphasic testing as well as on last time point clones from post-initial killing kinetics and clones post-repeat killing kinetics for both axenic and post-infection conditions. Consequently, 23 ST1 Paris clones were sequenced corresponding to: 6 pretest stock clones; 3 clones at 6h and 1 clone at 24h of initial axenic killing kinetics; 3 clones at 24h of repeat axenic kinetics; 10 clones at 24h of initial post-infection killing kinetics. In addition to the closed ST1.1 and ST1.2 reference genomes, 62 clinical ST1 clones were sequenced corresponding to: 8 isolates from the same ST1.1/ST1.2 patient (sampled during recurring legionellosis); 5 ST1.1 and 6 ST1.2 pretest stock clones; 9 ST1.1 and 9 ST1.2 clones at 24h of initial axenic killing kinetics; 3 of each at 24h of repeat axenic killing kinetics; and 10 ST1.1 and 9 ST1.1 clones at 24h of initial post-infection killing kinetics.

Genetic variant identification: Genetic variants (SNPs/Indels) in persister assay isolates were identified with snippy 3.2 which does both mapping and variant calling. Sequences of ST1 Paris persister isolates from axenic and post-infection conditions were mapped against the Paris reference genome (Chromosome and plasmid: accession NC_006368.1 and CR628338.1, respectively). As no differences were identified between ST1.1 and ST1.2 reference genomes, sequences of ST1.1 and ST1.2 persister isolates from axenic and post-infection conditions were mapped against the assembled genome of the ST1.1 reference strain obtained from long read sequencing.

2.7 Statistical analysis

Firstly, survival frequencies were transformed to standardise the data on a 0-1 scale, removing 0 and 1 values using the formula freq*((n-1)+0.5)/n where n is the number of observations (Smithson and Verkuilen, 2006) (http://www.jstatsoft.org/v34/i02/). Then, the LOGIT function was applied to data as visualised in Supplementary Figure 5B to account for left skewed data (Cribari-Neto and Zeileis, 2010). These transformed data were analysed using a linear and nonlinear mixed-effects model with the NLME R package (Pinheiro J and Core Team, 2023) (https://CRAN.R-project.org/package=nlme).

3 Results

3.1 Timer^{bac} system in *Legionella* axenic cultures and during host infection

3.1.1 Timer signal kinetics in during axenic growth

To equate the red/green fluorescence signal with bacterial growth rate, axenic cultures of *Lp* ST1 Paris (sequence type 1) were analysed using flow cytometry at different time points (Supplementary Figures S1, S2). The first 4h time point showed a range of fluorescence ratios from red, through orange to green, corresponding to a heterogenous resumption of growth in AYE liquid media from a BCYE plate culture. Between 13 h and 20 h or the exponential phase of growth, the TIMER ratio switched to majority green, with almost all bacterial cells demonstrating a green replicative signal. Finally, from 24 h to 48 h the TIMER signal shifted back to red corresponding to the stationary growth phase. The evolution of the TIMER colour ratio $\log_{10}$(green/red) for each event (bacterium) analysed by flow cytometry closely follows bacterial growth.

3.1.2 Timer signal of bacterial cells within amoebae

To assess the functioning of the Timer^{bac} system (Figure 1A) during host infection, imaging flow cytometry (Amnis® ImageStream®X) was implemented. The *A. polyphaga* cells were fixed, 12 hours post-infection (hpi) with *Lp* Paris prior cytometry analysis (Figure 1B). This technology allowed us to visualise bacteria cells expressing TIMER protein inside amoebae and to clearly distinguish red non-growing and multiple green replicating bacteria. In some cases, bacteria detected within an amoeba were all associated with either red or green fluorescence thus meaning homogenous bacteria population inside host cell, non-growing (a unique red bacteria cell; upper panel Figure 1B) or growing (green bacteria cells; lower panel Figure 1B). However, in most cases, we were able to distinguish both green and red bacterial subpopulations within a single amoeba as presented in the middle panel (Figure 1B), which may be the result of an amoeba double infection (two bacteria in a physiological distinct behaviour: grower and persister) or of appearance of persister subpopulation during infection cycle of *Legionella* within the host cell.

3.1.3 Timer signal kinetics during amoeba infection

To precisely quantify each bacterial subpopulation, TIMER signal kinetics were analysed during infection using an *A. polyphaga* amoeba model. Amoeba were lysed at varying points post-infection and the bacteria were recovered and analysed by flow cytometry. A comparison of ST1 Paris and ST1 Paris-TIMER bacteria at 20 hpi enabled the definition of a gating strategy to identify fluorescent bacteria and differentiate them from amoeba debris (growing; Figure 2A Gates 1 & 2). Additionally, analysis of TIMER bacteria post-infection led to the identification of two distinct subpopulations, corresponding to red, non-growing and green, growing bacteria (Figure 2A, Gate 4). Furthermore, the ST1 Paris infection kinetic revealed the evolution of intracellular *Lp* growth: the dominant population moves from red (Figure 2B), to green (Figure 2C) and progressively back to red (Figures 2D, E) at the end of the infection cycle. Interestingly, in contrast to the results seen during axenic growth, a subpopulation of red, non-growing bacteria was characterised throughout the infectious cycle and are especially distinct at time point 20 hpi, revealing the presence of non-growing, potential persister cells. The accuracy of bacteria events gating was confirmed by images taken with the Attune CytPix flow cytometer as shown on Figure 3A. Each cytometry event was clearly associated with a single bacterium, therefore validating the accuracy of this method in identifying green/growing and red/non-growing subpopulations.

3.2 Clinical isolate subpopulations during amoeba infection

The growth rates of the 7 clinical isolate pairs (early and late isolates) were analysed by cytometry at time points 15 hpi, 17 hpi or 20 hpi in amoeba, corresponding to the time of optimal subpopulation differentiation. All clinical samples displayed two subpopulations: growers and non-growers. Furthermore, the relative proportions of these subpopulations were conserved between isolate pairs (i.e. between early and late isolates), with the exception of ST48 clinical isolates (Figure 3B; Supplementary Figure S3) where there appears to be a slight increase in the late isolate non-grower subpopulation compared to the early isolate. Additionally, the ST1 clinical isolates displayed similar grower/non-grower subpopulation proportions to the reference ST1 Paris strain, while subpopulation levels appear to vary from one sequence type (ST) to another, suggesting that the proportion of non-growers during the infection cycle might be ST rather than isolate specific.

3.3 Non-growing cells *vs.* persister cells

Historically, the presence of persister subpopulations has been identified using the biphasic killing kinetic technique, whereby bacterial numbers are measured following the addition of bactericidal antibiotics (Figure 4A). Persisters are defined by their non-replicative state and antibiotic tolerance, flow cytometry

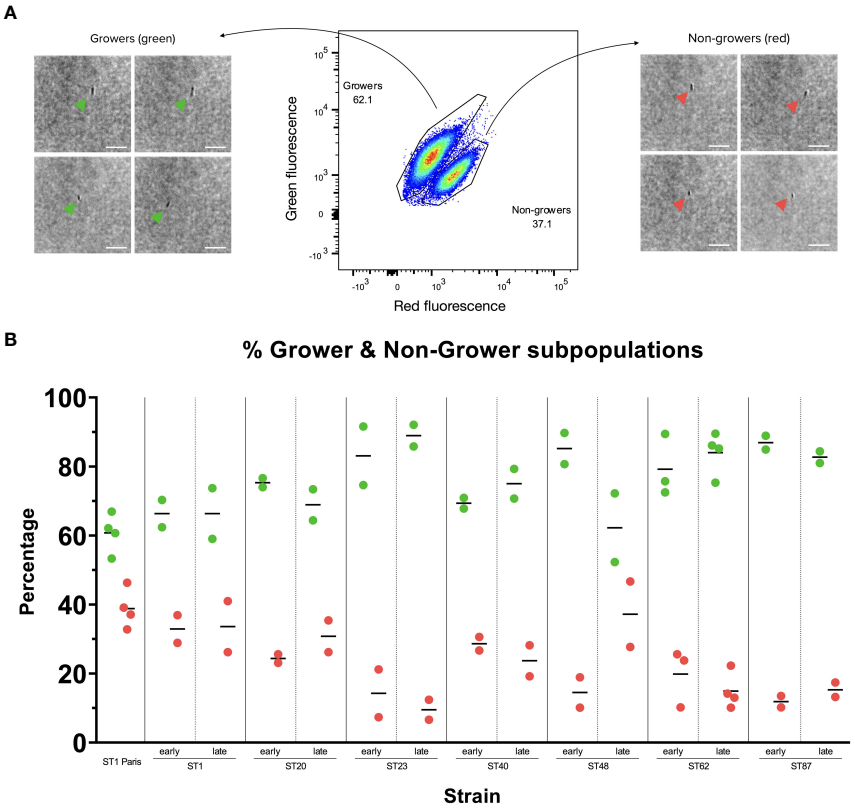

FIGURE 3
Growing and non-growing subpopulations in *Lp* Paris-TIMER and clinical *Lp* strains **(A)** *Lp* ST1 Paris-timer sub-populations 17 hours post-infection (hpi) in *A. polyphaga*. The TIMER^{bac} system, coupled with flow cytometry, allows us to differentiate green, growing bacteria from red, non-growing bacteria. Bright-field images taken with the Attune CytPix flow cytometer confirm that each event on the scatter plot corresponds to an individual bacterium. White Scale bar represents 10µm. **(B)** Growing (green) and non-growing (red) subpopulations in clinical *Lp* isolates post-*A. polyphaga* infection. *A. polyphaga* were infected at MOI 1 for 17 to 20 h with pairs of clinical *Lp* isolates from patients with recurring LD. Measures represent 2 to 4 repetitions, corresponding to 2 to 4 red and green dots. Isolates were named according to their sequence type (ST) and whether they were isolated at the time of first infection (early) or recurring infection (late). Grower and non-grower population percentages were calculated in FlowJo.

analysis revealed that clinical isolates are able to form distinct non-growing subpopulations, however this technique was not able to determine whether these cells are antibiotic tolerant. Consequently, killing kinetics were carried out using the ST1 Paris reference strain as well as several clinical isolates to confirm the persister forming capacity of clinical isolates. Killing kinetics were performed using axenic bacterial cultures and bacterial samples post-*A. polyphaga* infection. As described in Figure 4B, ofloxacin at 30 µg mL^{-1} (>20 x MIC) was added and surviving bacteria were quantified by growth resumption of BCYE agar plates after 0, 1, 3, 6 or 24 h exposure to the antibiotic.

3.3.1 Persistence of *Lp* ST1

The killing curves of *Lp* ST1 Paris and the pair of ST1 clinical isolates (early: ST1.1 and late: ST1.2) are shown in Figure 5. When bacteria were grown in axenic conditions (AYE medium), a typical biphasic killing curve was observed for all three ST1 strains (Figure 5A).

The same experiments were conducted with the ST1 strains recovered from *A. polyphaga* during infection (17hpi) and similar biphasic killing curves were observed (Figure 5B solid lines). The curves were nearly identical for the 3 strains with the same number of surviving bacteria at the end of the experiment (24 h exposure to ofloxacin 30 µg mL^{-1}). Interestingly, the proportion of surviving bacteria in the second phase of the curve, the persister population, was noticeably greater post-infection compared to axenic culture.

Another key characteristic of persistence is that the antibiotic tolerance demonstrated by this subpopulation is reversible. Thus, to assess the reversibility of this phenotype, persistent bacteria of each strain were collected from last time-point plates of killing kinetics and subject to the same killing kinetic experiment (Figure 5 dotted lines). For all strains, ST1 Paris, ST1.1 and ST1.2, identical biphasic curves were obtained using the collected persisters (repeats). These data confirmed the reversibility of the phenomenon, corresponding to a temporary physiological state: phenotypic heterogeneity.

3.3.2 Statistical analysis of *Lp* ST1 strains' persistence behaviour

As with many biological experiments, variations may occur from one experiment to another. In the case of the biphasic killing kinetics post-ofloxacin exposure, a major concern was the variation in bacterial concentration at the beginning of experiment, especially in lysed amoeba samples. To overcome this limitation, surviving bacterial subpopulations were compared using survival frequencies:

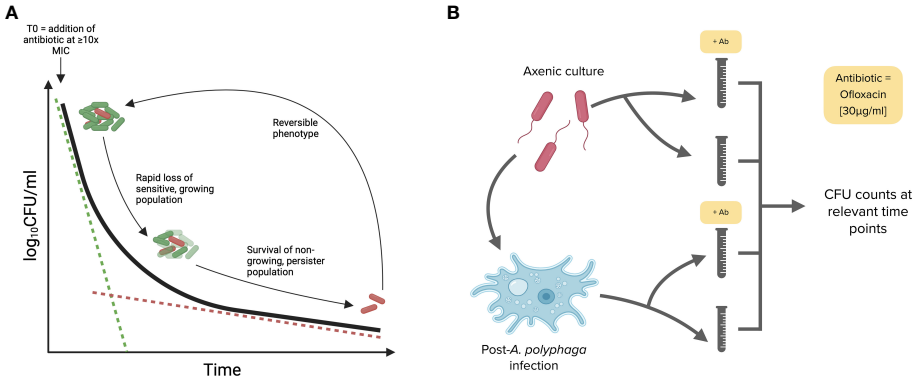

FIGURE 4
Killing kinetic and biphasic curve methodology used to identify persister subpopulations. **(A)** A model biphasic killing kinetic profile characteristic of persister populations. Following the addition of a bactericidal antibiotic the sensitive bacteria are killed rapidly while the tolerant persister cells are killed more slowly. **(B)** Workflow for killing kinetics. The ability of *Lp* strains to form persisters was tested in both axenic conditions (14 h or mid-exponential phase cultures) and post-*A. polyphaga* infection (17 hpi or during the replicative phase). Bacteria were exposed to ofloxacin at [30 µg mL^{-1} = 20 times MIC] for 24 h in AYE broth.

the relative proportion of bacteria surviving at each time point (i.e. persisters) compared to the initial population at 0 h (Figure 6).

The evolution of persister subpopulations over time, between strains and between conditions was analysed using a linear and nonlinear mixed-effects model (NLME R package) of transformed survival frequencies at 1, 3, 6 and 24 h time points of the killing kinetic (Supplementary Figure 5). As expected, the persisters population decreased over time of exposure to ofloxacin, which is statistically verified. But no statistical difference was identified between survival rates of strains ST1.1, ST1.2 and ST1 Paris irrespective of time point and condition (ST1.2 vs Paris 1.477499e-01; ST1.2 vs ST1.1 1.537929e-01). Additionally, a dynamic analysis of survival rate evolution over time showed that this evolution is statistically identical from 1 h to 24 h for clinical ST1 strains. Interestingly, this analysis also revealed that while there is a significant difference in the survival dynamic of clinical ST1 isolates vs ST1 Paris from 1 h to 3 h, post-3 h the survival dynamic of the latter evolves as per clinical ST1 strains and was not statistically different. In contrast, the experimental condition (axenic vs post-infection) was shown to have a significant effect on bacterial survival rates, *i.e.* persister subpopulations, for all strains (p-value 7.775878e-15). Specifically, bacterial survival rates and thus persister proportions are significantly greater in bacterial populations following infection as shown in Figure 6. Therefore, persistence capacity seems inherent to ST1 but dependent on environmental conditions, *ie.* increased during the *Lp* infection cycle.

3.3.3 Persistence of *Lp* ST48

The cytometry analysis of ST48 isolates (early and late) highlighted a difference in non-grower levels during the infection cycle. Therefore, killing kinetics were performed on both isolates, ST48.1 (early) and ST48.2 (late), to analyse their capacity to form persisters in axenic conditions and post-infection in *A. polyphaga* (Supplementary Figure S4). Biphasic killing curves were observed for both isolates and were identical between isolates in each environmental condition. However, as previously observed with ST1 strains, the proportion of recovered persisters cells was higher

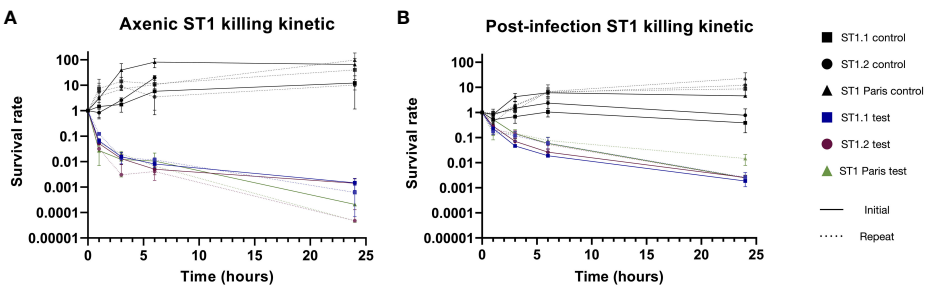

FIGURE 5
Biphasic killing kinetics of ST1 clinical isolates and ST1 Paris. The formation of *Lp* persisters in axenic conditions **(A)** and in infected *A. polyphaga* **(B)**. After pre-culture in AYE broth or amoeba lysis 17hpi, bacteria were exposed to ofloxacin (30µg mL^{-1} = 20 times MIC; solid colour lines) or cultivated without antibiotics (solid black lines) for 24 hours. Survivors of the 24h antibiotic treatment were reisolated, and their biphasic killing profile was retested as above (dotted lines). In both initial (solid lines) and repeat (dotted lines) experiments, antibiotic treatment led to similar biphasic killing kinetics, confirming the presence of persisters. Data represent the mean survival rate ± SEM of three biological replicates (n=3), expect for initial post-infection ST1.1 and Paris where data represent two biological replicates (n=2). The pair of ST1 clinical isolates originate from a patient with recurring LD, corresponding to the early (.1) and late (.2) isolates.

when the assay is performed with bacteria extracted from infected amoeba.

3.4 Genetic evolution of ST1 persister clones

Inherent in the definition of persistence is the notion that non-growing and antibiotic tolerant characteristics of persister bacteria are a result of phenotypic changes rather than genetic modifications. To establish the potential role of persisters in recurring legionellosis, early (ST1.1) and late (ST1.2) clinical isolates (8 isolates) from the same patient as well as multiple clones from stock used in killing kinetics were sequenced and analysed to identify microevolutions. Similarly, ST1 Paris stock clones were sequenced and compared to the NCBI *Lp* Paris reference genome to identify pre-existing differences between the laboratory stock and reference strain. Additionally, to confirm the absence of genetic modifications in *Lp* clinical subpopulations following killing kinetic experiments, multiple clones of each ST1 strain (Paris, ST1.1 and ST1.2) were collected from plates at the end of killing kinetics in both axenic and post-infection conditions, and their genomes were sequenced. Consequently, a total of 87 genomes were sequenced and analysed to identify potential point mutations (SNPs), deletions or insertions.

Among a total of 23 sequences from ST1 Paris, no differences were identified between pre-killing kinetic stock clones and the reference strain. Similarly, 64 clones were analysed for the ST1 clinical isolates. Analysis of ST1.1 and ST1.2 reference genomes, the 8 isolates from the patient as well as 5 ST1.1 and 6 ST1.2 pre-killing kinetic stock clones showed these genomes to be 100% identical, revealing that no genetic microevolutions occurred during successive legionellosis episodes or during conservation in the laboratory. Given the identical nature of these strains, the closed genome of ST1.1 was used as reference for variant calling in both ST1.1 and ST1.2 clones.

Regarding analysis between reference strains and post-killing kinetic clones, four different SNPs were found in three ST1 Paris clones; two in the same gene of the same clone and the other two in different genes of different clones (Table 2). Similarly, three different SNPs were found in three ST1.1 or ST1.2 post-killing kinetic clones (one SNP per clone) (Table 2). Limited number of SNPs not retained in the population is not relevant regarding genetic variation in association with persistence behaviour (Table 2). Notably, all SNPs identified in clones isolated from initial axenic killing kinetic experiments and were not found again in clones issued from repeat experiments, confirming the random status of SNP in sequenced clones with no selective pressure link (*i.e.* persistence associated).

4 Discussion

Treatment failure of legionellosis is still a serious issue today with a 5-10% mortality rate (Herwaldt and Marra, 2018). To date, natural antibiotic resistant *Lp* are rarely characterised compared to other bacterial pathogens (Pappa et al., 2020; Ginevra et al., 2022). Even though macrolide resistant clones can be easily obtained during *in vitro* evolution (Descours et al., 2017), these resistance-associated mutations are not recovered in *Lp* clinical isolates issued from patients with relapsing legionellosis (Pouderoux et al., 2020). Thus, this is ideal biological material to study the possible mechanisms involved in recurrence of the illness. One of these mechanisms may be the persistence phenomenon that has been previously identified in many pathogenic bacteria including *Lp*, using the laboratory strain JR32 (Personnic et al., 2019a; Personnic et al., 2021).

Considering all the previous data, our study focused on persistence in *Lp* clinical isolates from patients with recurring

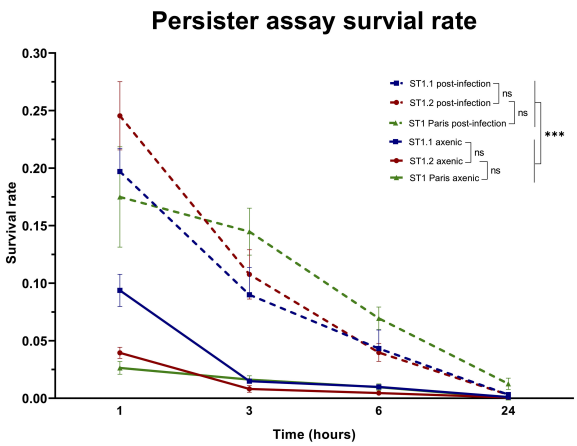

FIGURE 6
Comparison of ST1 bacterial survival rates in axenic conditions and post-*A. polyphaga* infection. The survival rates were calculated as the number of surviving bacteria at time points 1 h, 3 h, 6 h and 24 h of the killing kinetic compared to 0 h, before the addition of ofloxacin [30 µg mL^{-1}]. Each point represents the mean survival rate ± SEM of all biological replicates as follows: ST1 Paris axenic n=11, post-infection n=5; ST1.1 axenic n=6, post-infection n=5; ST1.2 axenic n=6, post-infection n=6. A linear mixed effects model (NLME R package) was used to analyse the effect of the factors strain (ST1.1; ST1.2; ST1 Paris) and condition (axenic; post-infection) on bacterial survival. This analysis determined that strain had no effect (ns (not significant) p-value >0.1), while the condition had a significant effect on bacterial survival (***p-value <0.001).

legionellosis. As a first step, the Timer^{bac} system was set up in the ST1 reference strain Paris, to optimise the infection kinetics and follow the non-growing population during the amoeba infection cycle. Notably, the non-growing subpopulation was not detectable by cytometry analysis on axenic growth in rich medium, with all the bacterial populations moving from non-growing to growing cells over time and reverting to non-growing state upon reaching the stationary phase. On the other hand, two subpopulations of *Lp* Paris bacteria were identified during amoeba infection. Likewise, the presence of a non-growing subpopulation was also characterised in all clinical isolates during amoeba infection. Except for ST48 clinical isolates, the proportion of non-growing cells was stable within each pair of isolates (early and late), which may reflect a persister-forming capacity inherent to each strain and independent of any potential adaptations during persistence in the human host. Furthermore, the proportion of non-growing bacteria appeared to be strain- (early and late) or even ST-specific as the proportion of clinical ST1 isolates (ST1.1 and ST1.2) non-growing cells were identical to the ST1 reference strain Paris. The biphasic killing kinetic following the addition of bactericidal antibiotics (20 times the MIC of ofloxacin) confirmed the persistence behaviour of non-growing *Lp* subpopulations and repeat experiments on persister clones proved the reversible nature of this physiological state. Indeed, this higher reversible tolerance toward antibiotics is a specificity of persistence, which clearly distinguishes it from the development of resistance mechanisms (Harms et al., 2016; Rocha-Granados et al., 2020). Moreover, identical biphasic killing curves were obtained with both early and late ST48 clinical isolates (ST48.1 and.2 respectively) suggesting that the behaviour of strain ST48 was conserved during infection in the patient, even if the Timer^{bac} cytometry assay appeared to indicate a slight increase in the proportion of non-growing cells between the 2 isolates (ST48.2 vs ST48.1).

Focusing on ST1 strains (Paris, ST1.1 and ST1.2), the comparison of all the ofloxacin killing assays followed by NLME analysis pointed out that (i) all strains had the same biological response towards the antibiotic: no statistical difference between the three strains regardless of the environmental condition; and (ii) the environmental condition (axenic *vs* infectious cycle) influences the level of persistence, *i.e.* the bacterial infection cycle promotes the emergence of persisters. Therefore, in agreement with the cytometry observations using Timer^{bac} system, no increase in persistence capacity was associated with the ST1 clinical isolates compared to the laboratory ST1 reference strain Paris. Furthermore, as observed with other bacterial pathogens (Helaine and Kugelberg, 2014), the infection cycle within a host results in an increase in persister proportions. This suggests that intracellular stress triggers the development of the persister subpopulation, which is in agreement with previous studies linking *Lp* quorum sensing and persister development during amoeba infection cycle (Personnic et al., 2019a). Stress-associated persistence has already been described with the HipBA toxin-antitoxin (TA) system in *E. coli* (Correia et al., 2006; Pandey et al., 2023). More specifically, the complex regulatory function of HipBA ends in alarmone (p)ppGpp synthesis, activating a stringent response and resulting in dormancy and persistence. In this case, natural *hipA* mutations were associated with an up-regulation of persister formation. HipA-like proteins were identified in numerous bacterial genomes (Gerdes et al., 2021) and can also be found in many *Lp* genomes. But no *hipA*-like gene/persistence association was identified in persistome analysis (Personnic et al., 2019a) nor in our genome analysis of persisters mutations. In fact, our data clearly showed that antibiotic tolerance is not due to genetic changes as comparative genomic analysis showed no notable genetic differences between reference ST1 clinical isolates and bacteria isolated post-persistence assays. Additionally, the limited number of SNPs identified were not fixed in the population. For example, the SNP identified in *arnT* of one bacterial clone isolated from initial killing kinetic was not found in repeat experiment isolates. Considering that the few mutations observed have no apparent link to the experimental procedure and that the genomes of ST1 clinical isolate pairs from recurring legionellosis are identical (Pouderoux et al., 2020) (ST1 sequence analysis done in this work), no *Lp* genetic evolution is associated with recurring legionellosis in the patients nor with the development of persister cells *in vitro*.

Alternatively, clear differences in persister proportions between sequence types suggest that there might be a genetic basis for persistence capacity. Thus far no genetic link has been demonstrated between specific genotypes and pathogenicity for *Legionella*. However, some particular genetic backgrounds of *Lp* (*e.g.* ST1 or ST47) appear to have different epidemiological patterns (Ginevra et al., 2009; Cassier et al., 2015) and to induce different

TABLE 2 SNPs in ST1 strains of *Legionella pneumophila* post-biphasic curve experiments.

Location	Gene	Description	Instances/clones sequenced	Strain	Condition	Substitution(s)
Chromosome	***arnT***	Glycosyltransferase	1/64	ST1.2	24h axenic	leu182phe
Chromosome	***prlC***	Oligopeptidase A	1/64	ST1.1	24h axenic	glu225ala
pLPP	***traC***	F pilus assembly protein	1/64	ST1.1	24h axenic	silent
Chromosome	***sidE***	Ubiquitinating enzyme	1/23	ST1 Paris	6h axenic	lys918stop
						tyr906asn
Chromosome	***lpp0804***	Hypothetical protein	1/23	ST1 Paris	24h post-infection	glu64asp
Chromosome	***gacS/letS***	Transmission sensor	1/23	ST1 Paris	24h post-infection	leu854stop

Colonies sampled at 6h and 24h post-antibiotic addition during biphasic curve experiments. Condition refers to experiments carried out on axenic cultures or on bacteria collected 17h post- *A. polyphaga* infection. A total of 89 genomes were sequenced: 23 ST1 Paris and 64 clinical ST1. This can be further divided based on test conditions: 10 axenic ST1 Paris; 13 post-infection ST1 Paris; 10 clinical ST1 reference genomes (10 first isolate (.1) and last isolate (.2) isolates); 15 axenic ST1.1; 12 post-infection ST1.1; 15 axenic ST1.2; and 12 post-infection ST1.2.

immune responses (Guillemot et al., 2022). Regarding the pathogenicity of isolates used in this study, clinical data and patient outcomes have been previously described for all isolates (Pouderoux et al., 2020) expecting the patient infected with ST62 whose outcomes and clinical data are not available. Given the magnitude of genome differences between clinical strains of different sequence types, it has been difficult to identify candidate genes using an unbiased approach. Another complication is the small number of clinical isolates associated with recurring legionellosis which is not yet sufficient to perform a GWAS approach that could include other clinical isolates not associated with recurring legionellosis and environmental strains. In the future, increasing the collection of *Lp* strains associated with recurring legionellosis and developing a rapid screening test to evaluate the persistence capacity of each *Lp* strain may allow us to undertake a more global approach.

Looking at infected amoebae by imaging flow cytometry allowed the observation of three distinct infection behaviours: amoeba cells harbouring non-growing bacteria (one single red bacterium), amoeba cells harbouring a homogenous growing bacterial population (green multiplying bacteria) and amoeba cells where two bacterial subpopulations coexisted, non-growing (red) and growing cells (green). Although, at this stage the latter case could be explained by a multiple infection process (at least two bacteria infecting one amoeba with one remaining in a persister state), an alternative hypothesis is that non-growing minor subpopulations emerge during the multiplication of *Lp* within the host, an important point to better understand the inducible mechanism of persistence in relation to stress generated towards intracellular bacteria (*ie.* ROS/NOS molecules). These observations highlight the need to investigate bacteria population behaviour during the infection cycle. Further experiments will be designed to clarify this hypothesis such as time-lapse microscopy on immobilised amoeba cells and the use of more accurate fluorescent reporter systems to identify persister cells within numerous growing bacteria cells.

To conclude, all clinical isolates of *Legionella pneumophila* used in this study were able to efficiently produce a subpopulation of persisters cells in a proportion that might be ST dependent. This persistence phenomenon was reversible and not associated with any genetic microevolution. Evidently, patient parameters participated in recurring status of legionellosis but no *Lp* strain associated characteristic has been identified yet. The highly variable genomes between ST groups make difficult the identification of persistence pathways involved in *Lp* working on genetic comparison. In future, better understanding the mechanisms involved in persistence is essential to revisit the medical protocols to apply in case of recurring legionellosis.

Data availability statement

The genetic and genomic data presented in the study are deposited in the European Nucleotide Archive (ENA), accession number PRJEB62570 (https://www.ebi.ac.uk/ena/browser/view/PRJEB62570).

Author contributions

XAW and CGil contributed to the conception and design of the study. XAW, CGil and CGin conducted the experiments. XAW and CGin analysed the DNA sequencing and genetic polymorphism data. All authors participated in the interpretation of the results and in the design of supplementary experiments important in the finalisation of this study. XAW and CGil wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1219233/full#supplementary-material

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Hyper-inflammatory profile and immunoparalysis in patients with severe Legionnaires' disease

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Introduction: Severe Legionnaires' disease (LD) can lead to multi-organ failure or death in 10%–30% of patients. Although hyper-inflammation and immunoparalysis are well described in sepsis and are associated with high disease severity, little is known about the immune response in LD. This study aimed to evaluate the immune status of patients with LD and its association with disease severity.

Methods: A total of 92 hospitalized LD patients were included; 19 plasmatic cytokines and pulmonary *Legionella* DNA load were measured in 84 patients on the day of inclusion (day 0, D0). Immune functional assays (IFAs) were performed from whole blood samples collected at D2 and stimulated with concanavalin A [conA, n = 19 patients and n = 21 healthy volunteers (HV)] or lipopolysaccharide (LPS, n = 14 patients and n = 9 HV). A total of 19 cytokines (conA stimulation) and TNF-α (LPS stimulation) were quantified from the supernatants. The Sequential Organ Failure Assessment (SOFA) severity score was recorded at D0 and the mechanical ventilation (MV) status was recorded at D0 and D8.

Results: Among the 84 patients, a higher secretion of plasmatic MCP-1, MIP1-β, IL-6, IL-8, IFN-γ, TNF-α, and IL-17 was observed in the patients with D0 and D8

MV. Multiparametric analysis showed that these seven cytokines were positively associated with the SOFA score. Upon conA stimulation, LD patients had a lower secretion capacity for 16 of the 19 quantified cytokines and a higher release of IL-18 and MCP-1 compared to HV. IL-18 secretion was higher in D0 and D8 MV patients. TNF-α secretion, measured after *ex vivo* LPS stimulation, was significantly reduced in LD patients and was associated with D8 MV status.

Discussion: The present findings describe a hyper-inflammatory phase at the initial phase of *Legionella* pneumonia that is more pronounced in patients with severe LD. These patients also present an immunoparalysis for a large number of cytokines, except IL-18 whose secretion is increased. An assessment of the immune response may be relevant to identify patients eligible for future innovative host-directed therapies.

KEYWORDS

Legionnaires' disease, hyper-inflammation, immunoparalysis, immune functional assays, severity, LPS stimulation, cytokines, IL-18

Introduction

Legionella pneumonia, known as Legionnaires' disease (LD), is an important cause of community-acquired pneumonia (CAP). Although LD is mainly characterized by mild lung dysfunction, it can also progress to multiple organ failure and lead to septic shock (Cecchini et al., 2017; Andrea et al., 2021). The overall mortality rate is approximately 10% but can increase to 30% according to underlying comorbidities and immune status (Chidiac et al., 2012; Cecchini et al., 2017). Non-specific inflammatory mediators have been found to be highly expressed in severe and non-surviving LD patients: C-reactive protein (CRP) level ≥500 mg/L at admission was described as related to mortality in a large cohort (Chidiac et al., 2012) and high serum procalcitonin concentration was associated with higher initial severity score (Haeuptle et al., 2009), intensive care unit (ICU) admission, and hospital death rate (de Jager et al., 2009).

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al., 2016). The host immune response to sepsis involves a first stage of cytokine overexpression. While some patients with sepsis maintain a robust immune response, a concomitant or following stage of immunoparalysis can induce poor outcomes (higher mortality, longer hospital stay, and secondary hospital infection acquisition) in a subset of patients (Van Der Poll et al., 2021). The latter have altered immune function characterized by low cytokine release after leukocyte stimulation, lymphopenia, and a low expression of monocyte human leukocyte antigen-DR (mHLA-DR), which has been described as a surrogate marker of sepsis-induced immunoparalysis. In particular, an impaired secretion of tumor necrosis factor-α (TNF-α) and other pro-inflammatory cytokines after an *ex vivo* lipopolysaccharide (LPS) challenge, named endotoxin tolerance, has been demonstrated in these patients (Hotchkiss et al., 2013; Delano and Ward, 2016; Van Der Poll et al., 2021).

In the context of COVID-19 or pneumococcal CAP, a hyper-inflammatory profile has been associated with disease severity (Fernández-Serrano et al., 2003; Burgmeijer et al., 2019; Leisman et al., 2020). Altered innate and adaptive immune responses, including severe lymphopenia as well as phenotypic and functional T-cell alterations, have also been described in critically ill COVID-19 patients (Osuchowski et al., 2021; Venet et al., 2021). Data regarding the immune response in LD are still lacking: only two studies have described an increase in T-helper 1 cytokines and interleukin (IL)-6 and IL-8, and no study has assessed the immune functional capacities of LD patients (Tateda et al., 1998; Fernández-Serrano et al., 2003).

The present work aims to assess whether patients display a dysregulated innate or adaptive immune response at the early stage of LD and to study the association between their immune status and the pulmonary injury or overall disease severity.

Materials and methods

Study design

This study included LD patients hospitalized in ICUs or conventional medical departments of 12 French hospitals between August 2017 and January 2021. Patients were enrolled within a median of 1 day (interquartile range, IQR [1–3]) after diagnosis. A total of 19 plasmatic cytokines and the pulmonary *Legionella* DNA load were measured at the day of inclusion (day 0, D0) in a group of patients (group A, n = 84). Immune functionnal assay (IFAs) were performed at day 2 (D2) in patients hospitalized at the University Hospital of Lyon, separated according to the stimulant used: samples from group B patients (n = 19 ICU patients) and from healthy volunteers (HV, n = 21) were stimulated with concanavalin A (conA), while samples from group C patients (n = 6 ICU and

8 non-ICU patients) and from HV (n = 9) were stimulated using LPS. As described in Figure S1, LD patients may belong to one or more groups. HV were matched for age and sex. For severity assessment, the Sequential Organ Failure Assessment (SOFA) score and the mechanical ventilation (MV) status were recorded at D0 for all patients; the MV status was recorded at day 8 (D8) for ICU patients only. We assumed that patients (n = 22) who were not in the ICU at D8 did not receive MV on D8. At D8, patients were classified using an ordinal severity scale (discharge, ward, ICU, ICU+MV, death) derived from the WHO scale for COVID-19 severity assessment (Marshall et al., 2020). Evidence of septic shock was recorded during ICU stay, based on the sepsis 2 definition: vasopressor requirement to maintain a mean arterial pressure of 65 mm Hg or greater (Singer et al., 2016).

Ethics and regulatory issues

Adult patients were enrolled in the national prospective cohort "ProgLegio", which aimed to identify bacterial and human biomarkers of prognostic value for severe LD (NCT03064737; project PRTS ANR/DGOS, ANR-15-CE17-0014NCT03064737), using the following inclusion criteria: (i) patients with clinical and laboratory signs of LD (positive urinary antigen test and/or *L. pneumophila* PCR on respiratory sample), and (ii) having provided written informed consent (legal representative could be used as a surrogate). Exclusion criteria were as follows: (i) LD caused by *Legionella* non *pneumophila*; (ii) patients for whom respiratory secretions could not be obtained, (iii) cases diagnosed only by serology, and (iv) outpatients. Age and immunosuppression status (IS, e.g., long-term corticosteroids, immunosuppressive therapy including anti-TNF-α and other biotherapies, active solid cancer or hemopathy, and other diseases inducing an immunosuppression) were collected for all LD patients but were not considered as exclusion criteria. This study was approved by the regional institutional review board (*Comité de Protection des Personnes Sud-Est IV, France*; ID-RCB 2016-A01021-50). It was registered with the *Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation* (DC-2008–509) and the French data protection agency (*Commission nationale de l'informatique et des libertés*). Serum samples from HV were obtained from the French national blood services (*Etablissement Français du Sang*, EFS) and used as controls; their written non-opposition to the use of donated blood for research purposes was obtained, according to the EFS standardized procedures.

Legionella pulmonary DNA load

Pulmonary samples (sputum, n = 49; tracheal aspirates, n = 30; and broncho-alveolar lavages, n = 4) were taken at D0 and stored at −20°C before DNA extraction. DNA was extracted from 200 µL of sample using the MagNA Pure Compact Instrument (Roche, Basel, Switzerland) automated system. *Legionella* DNA load was next estimated from 5 µL of DNA sample by a *mip* qPCR as already described (Mentasti et al., 2012). A calibration range was applied using Lp DNA standard reference material for quantification (Baume et al., 2013). Results were expressed in genome units (GU) per reaction and a DNA load >0.1 GU/reaction was considered as positive.

Cytokines measurement

Luminex

Plasma samples and supernatant from whole blood samples after conA stimulation were stored at −20°C before cytokine measurement. A total of 19 circulating cytokines (G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, MCP-1, IL-1α, IL-18, MIP-1β, and TNF-α) were simultaneously quantified using a Luminex technology (Bio-Plex 200, BioRad) from thawed plasma and post-conA stimulation supernatant. Results were expressed in pg/mL.

Ella automated immunoassay

Supernatant from whole blood samples after LPS stimulation were stored at −20°C before cytokine measurement. TNF-α was quantified from the thawed supernatant on Simple plex cartridges (ProteinSimple, San Jose, CA) using the Ella nanofluidic system (Biotechne, Minneapolis, MN), according to the manufacturer's instructions. Results were expressed in pg/mL.

Immune functional assays

IFAs were performed in heparinized whole blood from patients of groups B and C sampled at D2 and from HV; stimulation of white blood cells (WBCs) occurred within 3 h after whole blood collection. For group B patients (n = 19) and matched HV (n = 9), 500 µL of fresh blood was incubated for 24 h at 37°C with a Type IV conA. The latter acts as a strong and nonspecific activator of T-cell apoptosis and autophagy by binding osidic residues at the surface of lymphocyte T-cell receptors (concentration= 2.5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA). For group C patients (n = 14) and matched HV (n = 9), 1 mL of blood was incubated for 24 h at 37°C in standardized TruCulture tubes (Myriad Rbm, Austin, TX, USA) prefilled with ultrapure *E. coli* LPS (100 ng/mL, *E. coli* O55:B5). The latter is a toll-like receptor (TLR)-4 and -2 ligand, which triggers the innate immunity and induces an intense cytokine and chemokine secretion and the maturation of antigen presentation. In parallel, a negative control (NUL condition) containing only culture medium (Gibco RPMI 1640 medium, Fisher Scientific SAS, Illkirch, France) was carried out for all patients and HV samples.

mHLA-DR measurement

Expression of surface mHLA-DR was measured by flow cytometry from EDTA samples collected within 2 h at D2 or D3 for 18 patients belonging to group B. The anti-HLA-DR/Anti-Monocyte Quantibrite assay (BD Biosciences, San Jose, USA) was performed on a Navios flow cytometer and data were analyzed using Navios software (NAVIOS; Beckman-Coulter, Brea, CA, USA). Monocytes were gated based on CD14 expression. mHLA-DR expression was measured as the median of fluorescence intensity related to the entire monocyte population, as recommended by the manufacturer (Demaret et al., 2013). The fluorescence was converted to antibodies bound per cell (Ab/C)

using a calibrated standard curve determined with phycoerythrin (PE)-beads (BD QuantiBrite™ - PE Beads, Becton Dickinson). Results are expressed as number of sites per cell (Ab/C). The usual values are 13,500–45,000 Ab/C and the threshold value for immunosuppression is <8,000 Ab/C (Venet et al., 2022).

Statistical analyses and cluster building

Results are expressed as median and IQR for continuous variables. Statistical analyses were conducted using GraphPad Prism Software 8 (San Diego, CA, USA). Non-parametric Mann–Whitney tests were used for rank comparisons between two unpaired groups, and non-parametric Kruskal–Wallis tests were used for comparisons between three or more paired or unpaired groups. Unpaired *t*-tests with Welch's correction were used to compare the means of mHLA-DR expression and post-LPS TNF-α secretion levels. R^2 (eta-squared) coefficients were calculated to measure the effect size between patients and normal value, patients and HV values, and D0 and D8 MV status. The effect size was considered small for $0.01 \leq r^2 \leq 0.09$, medium for $0.09 \leq r^2 \leq 0.25$, and large for $r^2 > 0.25$. Non-parametric chi-squared tests were used for proportion comparisons between groups A, B, and C, and among the entire cohort for MV/no-MV comparisons. Principal component analysis (PCA) was carried out using the PCA formula from FactoMineR package (R software version 3.5.1). A heatmap was generated by scaling and centering log10-transformed cytokines concentrations, and the dendogram was drawn based on hierarchical clustering analysis (Euclidean distance matrix with Ward's method) using the heatmap3 package (R software version 3.5.1). A *p*-value <0.05 was considered significant.

Results

Patient characteristics

A total of 92 patients from the ProgLegio trial were enrolled. D0 MV patients (n = 36) were more severe than no-MV patients (n = 56). The D0 MV patients were more often admitted to the ICU; had a higher median SOFA score and a lower median blood pressure; were more frequently under vasopressors, hemofiltration, and MV at D8; more frequently developed septic shock during their stay; and had longer hospital and ICU stays (Table 1). D0 MV patients had higher WBC count, lower lymphocyte count, and higher pulmonary *Legionella* DNA load. In contrast, the proportion of LD risk factors, including immunosuppression, and the D28 mortality rate were not significantly different between D0 MV and no-MV patients. A higher proportion of D0 MV patients received a combination therapy for LD (fluoroquinolones and macrolides; Table 1), but there was no significant difference in combination therapy administration according to the inclusion hospital.

Different immune parameters were evaluated in three subgroups of patients (Figure 1; Figure S1): A (n = 84), B (n =

TABLE 1 Clinical and laboratory data of LD patients according to D0 mechanical ventilation status.

Criteria	LD patients (n = 92)	No-MV (n = 56)	MV (n = 36)	p-value
LD risk factors				
Smoking, n (%)	45 (49)	27 (48)	18 (50)	1
COPD, n (%)	9 (10)	5 (9)	4 (11)	0.73
Alcoholism, n (%)	9 (10)	3 (5)	6 (17)	0.15
Diabetes, n (%)	10 (11)	4 (7)	6 (10)	0.18
Immunosuppression, n (%)	25 (27)	14 (56)	11 (31)	0.63
Immunosuppressive therapy, n (%)	16 (17)	11 (20)	5 (13)	0.42
- corticosteroids, n (%)	12 (13)	9 (16)	3 (8)	0.35
- other, n (%)	3 (3)	1 (2)	2 (6)	0.56
Cancer/hemopathy, n (%)	5 (5)	3 (5)	2 (6)	1
Other immunosuppressive conditions, n (%)	6 (7)	3 (5)	3 (8)	0.68
≥ 1 risk factor	69 (75)	42 (75)	27 (75)	1
Inclusion data (D0)				
ICU admission, n (%)	60 (65)	24 (43)	36 (100)	**<0.0001**
SOFA score, median [IQR]	3 [1–7]	1 [0–3]	8 [4–9.8]	**<0.0001**
Fluoroquinolone and macrolide combination therapy (n = 81), n (%)	57 (70)	26 (54)	31 (94)	**0.0001**

(Continued)

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TABLE 1 Continued

Criteria	LD patients (*n* = 92)	No-MV (*n* = 56)	MV (*n* = 36)	*p*-value
D0 laboratory parameters				
White blood cells (G/L), median [IQR]	12.7 [8.9–15.8]	11.2 [8.4–15]	14.2 [10.0–21.9]	**0.04**
Polynuclear neutrophils (G/L), median [IQR]	10.8 [7.9–14.6]	10 [7.1–13]	12.7 [8.5–20]	0.06
Lymphocytes (G/L), median [IQR]	0.80 [0.48–1.1]	0.93 [0.64–1.23]	0.55 [0.35–1.13]	**0.03**
CRP (mg/L), median [IQR]	310 [197–421]	296.7 [207–418.3]	309 [185.3–392.3]	0.96
Creatininemia (µmol/L), median [IQR]	87.5 [71.5–160.3]	81 [69–114]	97 [73–203]	0.06
Pulmonary *Legionella* DNA load (GU/reaction), median [IQR]*	48.5 [0.9–731.2]	12.86 [0.90–89.9]	455.8 [31.24–7,460]	**0.0006**
Clinical data				
Mean blood pressure (mmHg), median [IQR]	100 [87–110]	103.8 [89.63–111.5]	92.50 [84–105]	**0.01**
Temperature (T°C), median [IQR]	39 [38.5–40]	39 [38–40]	39.9 [39–40]	0.19
Intensive care during ICU stay (*n* = 60)				
Vasopressor, *n* (%)	20 (22)	2 (4)	18 (50)	**<0.0001**
Hemofiltration, *n* (%)	8 (9)	1 (2)	8 (22)	**0.002**
Corticosteroids (sepsis management), *n* (%)	8 (9)	5 (9)	3 (8)	1
Evolution				
Septic shock, *n* (%)	21 (23)	0 (0)	20 (56)	**<0.0001**
D8 MV, *n* (%)	23 (25)	0 (0)	23 (35)	**<0.0001**
Hospitalization duration (d), median [IQR]	10 [6–26]	7.5 [5–10.75]	20 [10–31]	**<0.0001**
ICU duration (d), median [IQR]**	10 [5–16]	5 [2–7.25]	13.5 [8.5–25.75]	**<0.0001**
D28 mortality, *n* (%)	4 (4)	1 (2)	3 (8)	0.29

COPD, chronic obstructive pulmonary disease. Alcoholism corresponds to an alcohol consumption >3 glasses per day for men and >2 glasses per day for women for at least 1 year. Other immunosuppressive therapies include anti-TNFα therapy (Etanercept, Infliximab, Certolizumab, and Adalimumab) and anti-inflammatory biotherapy (Methotrexate). Other immunosuppressive conditions included rheumatoid polyarthritis, solid organ transplantation, and psoriatic arthritis.
MV, mechanical ventilation; IQR, interquartile range; ICU, intensive care unit; LD, Legionnaires' disease; SOFA, Sequential Organ Failure Assessment; CRP, C-reactive protein; GU, genome unit.
The p-value column indicates the p-values of Mann–Whitney tests used for rank comparisons between MV and no-MV patients.
*no data n = 9.
**n = 58.
Inclusion data, D0 laboratory data, ICU stay data, and outcomes were collected.
Bold values are statistically significant results, and normal values (non-bold) are non significant.

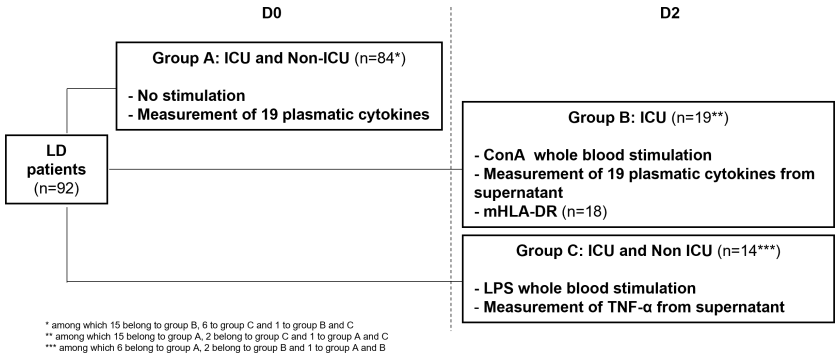

FIGURE 1
Study design and measurement of immune parameters according to patient group. LD, Legionnaires' disease; ICU, intensive care unit; ConA, Concanavalin A; mHLA-DR, monocytic human leukocyte antigen DR; LPS, lipopolysaccharide.

19), and C (n = 14). Most demographics, LD risk factors (including immunosuppression), clinical, and laboratory parameters were common between groups A, B, C, and the entire cohort (Table S1). However, patients in group B, who were only ICU patients, had a higher D0 SOFA score and were more often under MV at D8 compared to groups A, C, and the entire cohort.

Initial hyper-inflammatory profile in severe patients

We first measured 19 plasmatic inflammatory markers in patients from group A at D0 and compared their median expression between patients with (n = 32) or without (n = 52) MV at D0. Pro-inflammatory cytokines (IL-6, IL-8, and TNF-α), chemokines (MCP-1 and MIP1-β), T-helper (Th)-1 (IL-2 and IFN-γ), and Th-17 (IL-17) cytokines were significantly overexpressed in MV vs. no-MV patients (Figure 2A). In contrast, levels of IL-7, a lymphocyte growth factor, was reduced in MV patients. Cytokine levels did not differ significantly according to IS status, underlying immunosuppressive therapy, and corticosteroid administration for sepsis management. The median *Legionella* pulmonary DNA load was higher in D0 MV vs. no-MV patients (455.8 [31.24–7,460] GU/reaction vs. 12.86 [0.90–89.93] GU/reaction, $p < 0.0001$) and D8 MV vs. no-MV patients (1,357 [171.8–25,328] GU/reaction vs. 14.28 [0.835–126.5] GU/reaction, $p < 0.0001$; Figure S2).

The cytokine levels were then assessed according to the ordinal severity scale (discharge, ward, ICU, ICU+MV/death) at D8. There was a significant difference in IL-6, IL-8, TNF-α, MCP-1, MIP-1β, IL-17, IFN-γ, IL-2, and IL-18 secretion levels according to severity (Figure 2B).

A PCA was next performed in patients from group A to visualize the expression of plasmatic cytokine secretion, pulmonary *Legionella* DNA load, and D0 SOFA score using a single representation (Figure 3A). The variables projected onto the first two principal components showed an overall variance of 45.1%. Eigenvalues and individual values are presented in Figure S3. A main group of seven cytokines (MCP-1, MIP1-β, IL-6, IL-8, TNF-α, IL-17, and IFN-γ) in addition to the D0 SOFA score contributed the most to axis 1 (29.3%). In contrast, IL-12, IL-2, and IL-4 formed a smaller group, which mostly contributed to axis 2 (15.8%). Other cytokines (IL-13, IL-7, IL-1β, IL-10, GM-CSF, G-CSF, IL-1α, and IL-5) and the pulmonary DNA load did not appear to have a major contribution to axis 1 or 2.

We then used an unsupervised hierarchical clustering to assess the profile of each patient according to the expression of the seven cytokines contributing to axis 1, IS, D0 and D8 MV status, D0 SOFA score, septic shock, Extracorporeal Membrane Oxygenation (ECMO) and hemofiltration status, the severity scale, and the D28 mortality (Figure 3B). The heatmap divided the patients into two clusters (1, n = 56 and 2, n = 28) and two subgroups within cluster 1 (1a, n = 8 and 1b, n = 48). Patients from cluster 2 had high cytokine secretion compared to patients from cluster 1 who had low (cluster 1a) or intermediate (cluster 1b) cytokine expression. While IS status was not associated with any cluster, 64% (18/28) of patients from cluster 2 had a D0 MV, and 54% (15/28) had a D8 MV compared to 25% (14/56) and 9% (5/56), respectively, for patients from cluster 1. In cluster 2, 54% (15/28) of patients had septic shock compared to 5% (3/56) in cluster 1 ($p < 0.0001$). Overall, 82% (23/28) of the patients from cluster 2 were D8 ICU or D8 ICU + MV/death according to the severity scale. The median D0 SOFA score was higher in patients from cluster 2 compared to cluster 1 (8 [3–10] vs. 2 [0–4], $p < 0.0001$). Cluster 2 also grouped all ECMO patients (n = 4), all patients with hemofiltration (n = 7), and all D28 non-survivors (n = 4). These results suggest that patients with non-severe LD (cluster 1) may be differentiated from patients with severe LD (cluster 2) based on their cytokine profile.

Impaired cytokine secretion post-conA stimulation in LD patients

To study the immune functional response of LD patients in relation to disease severity, we performed an IFA at D2 in patients from group B (n = 19 ICU patients). Among them, 32% (6/19) had a septic shock during their stay (Table S1). We compared WBC function between LD patients and HV (n = 21) after stimulation with the non-specific lymphocyte activator conA. A total of 16 out of 19 mediators were significantly less released in LD patients (Figure 4): IL-1α and IL-1β inflammasome cytokines, MIP-1β chemokine, pro-inflammatory cytokines (IL-6, IL-8, and TNF-α), GM-CSF growth factor, Th1 (IL-2, IFN-γ, and IL-12), Th2 (IL-4, IL-5, and IL-13), and Th17 (IL-17) cytokines, IL-7 lymphocyte growth factor, and IL-10 anti-inflammatory cytokine. In contrast, the inflammasome cytokine IL-18 and the chemokine MCP-1 were increased. The cytokine levels did not differ significantly according to IS status. As LD patients had a higher leukocyte count (median [IQR]: 12.8 [6.9–15.0] vs. 6.4 [5.3–7.2], p = 0.0015) and a lower lymphocyte count (median [IQR]: 0.97 [0.40–1.75] vs. 1.82 [1.62–2.32] G/L, p = 0.0010) than HV, the cytokine concentration was normalized to the lymphocyte count. After normalization, the secretion of 16/19 mediators still varied in the same way, but the secretion of TNF-α and MIP-1β was no longer significantly different between HV and LD and G-CSF was increased in LD.

We next assessed whether the low leukocyte response was associated with the severity of the disease, expressed by D0 or D8 MV. There was no significant difference between severe and non-severe group B patients according to D0 MV status for 18 of the 19 measured parameters (Figures S4A, S4B). However, the median IL-18 secretion was higher in patients with a D0 MV (126.9 [69.3–279.1] vs. 25.5 [9.2–40.4] pg/mL, p = 0.0009, Figure S4A) and in those with a D8 MV (88.7 [36.6–650.6] vs 30.6 [10.1–58.2] pg/mL, p = 0.029, Figure S4B) than in no-MV patients. The secretion variations had a similar pattern when using hemofiltration or septic shock as severity criteria.

mHLA-DR expression compatible with an immunoparalysis status in ICU patients

Furthermore, we measured the expression of mHLA-DR at D2 or D3 in 18 patients from group B. There was a medium effect size in the mean ± SD mHLA-DR expression between the patients and

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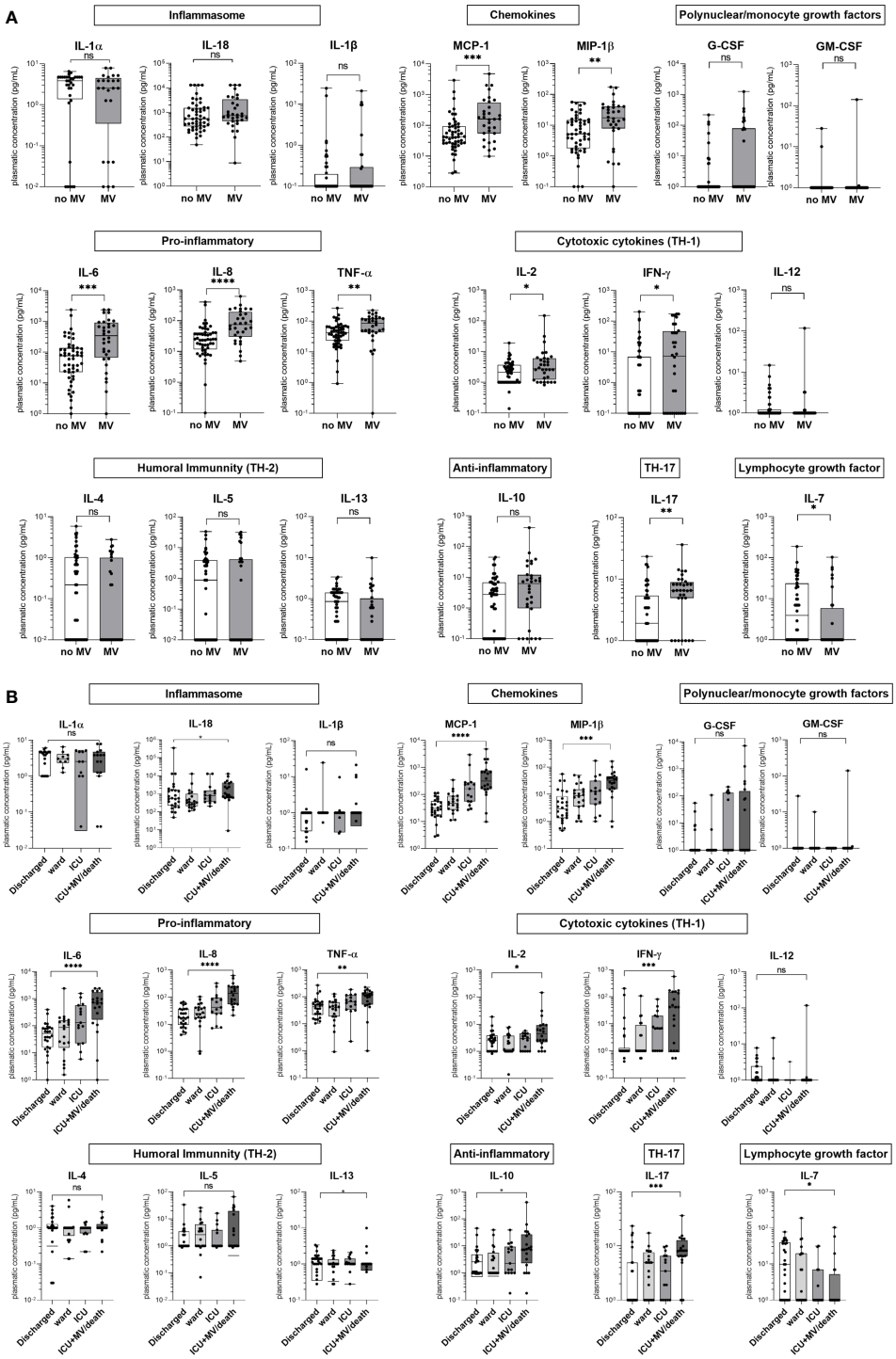

FIGURE 2
Concentrations of 19 plasmatic cytokines for group A patients at D0 according to mechanical ventilation (MV) status at D0 and to a severity scale at D8. **(A)** Concentrations for D0 MV (n = 36) vs. D0 no-MV (n = 48). **(B)** Concentrations at D8 according to severity: discharged (n = 26), ward (n = 20), ICU (n = 15), and ICU+MV/death (n = 20). We assumed that patients who were not in the ICU at D8 (n = 22) did not receive MV at D8. The patient who died between D0 and D8 was excluded from the analysis. Data are represented as boxplots illustrating the median, interquartile range, and range; Mann–Whitney **(A)** or Kruskal–Wallis **(B)** comparison tests: ns, non significant; *p < 0.05, **p < 0.01, ***p < 0.01, ****p < 0.0001.

the normal value (difference between means −3,447 ± 7,856 Ab/C, 95% CI [−6,690; −204], r^2 = 0.17), suggesting an immunosuppressed status for these patients. Among the 18 patients, 72% (13/18) had MV at D0 and 61% (11/18) had MV at D8. There was a medium effect size in mHLA-DR expression between D0 MV and D0 no-MV patients (−5,692 ± 7,155 Ab/C, 95% CI [−12,847; 1,464], r^2 = 0.17) and a small effect size for D8 MV status (−4,044 ± 6,434 Ab/C, 95% CI [−10,478; 2,390], r^2 = 0.07).

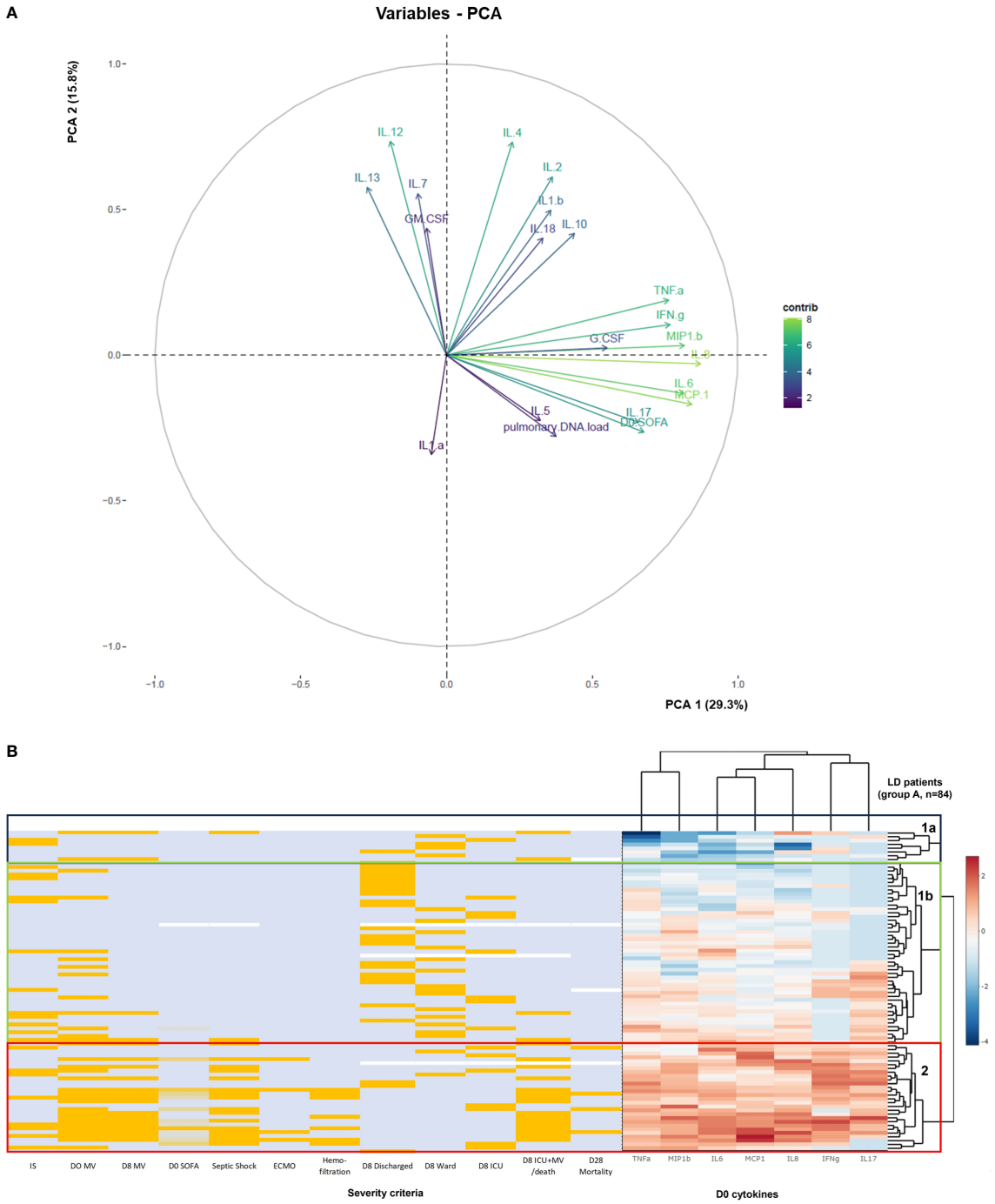

FIGURE 3
Analysis of inflammatory response in association with *L. pneumophila* DNA and clinical severity criteria in patients from group A. **(A)** Principal component analysis (PCA) performed with 19 cytokines, D0 SOFA score, and pulmonary *Legionella* DNA load. Principal component 1 and 2 (PC1 and PC2) contributed to 29.3% and 15.8% of the variation, respectively. The contribution of the variables to axes 1 and 2 is indicated by the color gradient of the arrows. **(B)** The heatmap of cytokine expression profiles from the supervised analysis (Euclidean distances matrix with Ward's methods) was generated by scaling and centering the log10-transformed of the values of the seven D0 cytokines that significantly contributed to axis 1. Immunosuppression status, D0 and D8 mechanical ventilation (MV) status, D0 SOFA score, septic shock, ECMO, hemofiltration, severity scale, and the D28 mortality were added. Patients with positive results for previous criteria are shown in orange and patients with negative results are shown in light blue. For SOFA score, light blue corresponds to SOFA ≤ quartile 1 (Q1, SOFA = 1), light orange corresponds to Q1 < SOFA ≤ median (SOFA = 3), and orange corresponds to SOFA > median. Missing values (n = 1 for D0 SOFA score and septic shock, n = 3 for the severity scale, n = 4 for D28 mortality) are shown in white. Blue box corresponds to cluster 1a, green box corresponds to cluster 1b, and red box corresponds to cluster 2.

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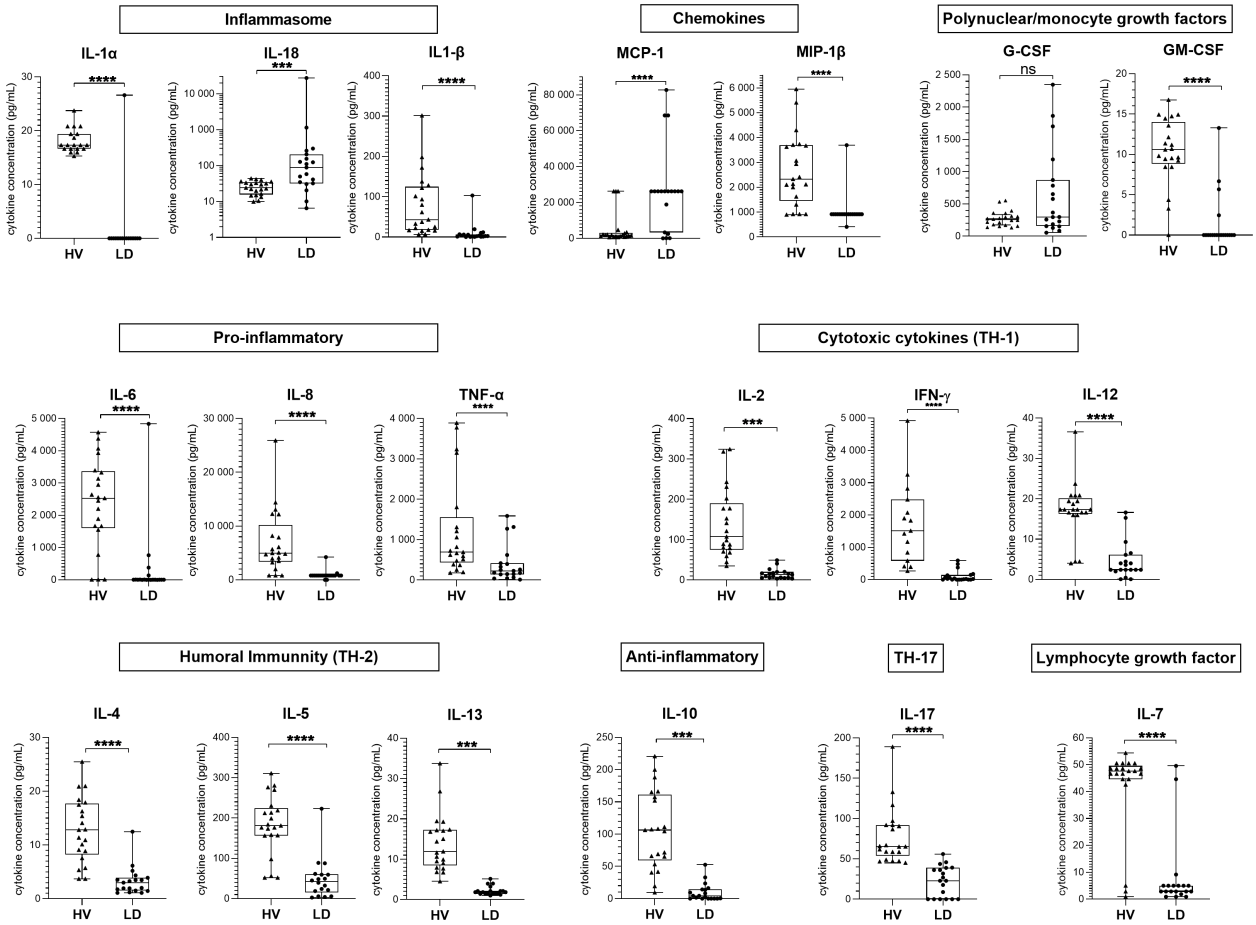

FIGURE 4
Cytokine concentration in supernatant after conA whole blood stimulation in patients from group B compared to healthy volunteers. The supernatant concentration of 19 cytokines from samples of group B patients (LD, *n* = 19) compared to those of healthy volunteers (HV, *n* = 21), after stimulation with conA, are shown. Data are represented as boxplots illustrating the individual values, median, interquartile range, and range; Mann–Whitney comparison tests: ns, non significant; ***p < 0.01, ****p < 0.0001.

Low TNF-α secretion post-LPS stimulation in patients with D8 MV

We next assessed whether the post-stimulation low cytokine secretion could also be observed in patients with less severe LD. For this purpose, we stimulated whole blood collected at D2 from group C patients (*n* = 6 ICU patients and *n* = 8 non-ICU patients) in TruCulture tubes prefilled with LPS and compared their immune response with that observed in 9 HV. Among LD patients, 14% (3/14) had a septic shock during their stay (Table S1). There was a large effect size in the mean ± SD TNF-α secretion between HV and LD patients (4,125 ± 1,186 pg/mL, 95% CI [2,939; 5,311], r^2 = 0.84; Figure 5A). The effect size was medium between D0 no-MV and D0 MV (542.0 ± 1,043 pg/mL, 95% CI [−500.9; 1,585], r^2 = 0.14; Figure 5B) but large between D8 no-MV and D8 MV (−1,273 ± 501.8 pg/mL, 95% CI [−1,775; −771.2], r^2 = 0.76; Figure 5C) The effect size was also large between patients without and with septic shock (−1,234 ± 491.6, 95% CI [−1,726; −742.6] pg/mL, r^2 = 0.76). The TNF-α levels did not differ significantly according to the IS status.

Discussion

The study herein showed that LD patients with D0 MV had a higher systemic pro-inflammatory burst. Furthermore, at D2, after non-specific whole blood stimulation, most cytokine secretion capacities were significantly reduced in LD patients compared to HV, suggesting a leukocyte hyporesponsiveness. Interestingly, a standardized IFA with LPS underlined that the patients with the lowest TNF-α release were the ones still under MV at D8.

The present results add original data on the immune response of LD patients, highlighting an initial phase of intense pro-inflammatory mediator expression in the most severe patients. Using multi-parametric analysis, the initial SOFA score was associated with a profile of seven pro-inflammatory parameters, i.e., three pro-inflammatory cytokines (IL-6, IL-8, and TNF-α), two chemokines (MCP-1 and MIP1-β), Th-1 (IL-2 and IFN-γ), and Th-17 (IL-17) cytokines. In contrast, pulmonary *Legionella* DNA load was less associated with SOFA score than these seven cytokines. Hierarchical clustering confirmed that the severity criteria (D0 and

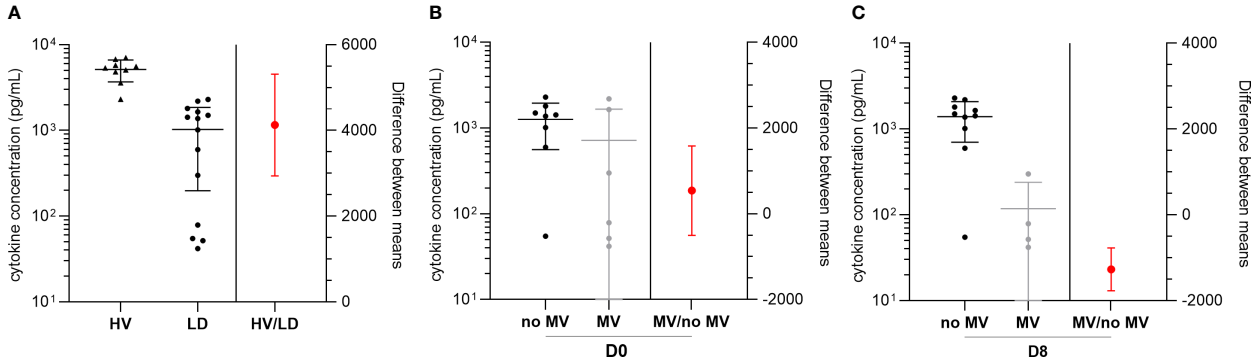

FIGURE 5
TNF-α concentration after LPS stimulation at D2 in the supernatant of group C patients according to mechanical ventilation (MV) status. **(A)** Mean TNF-α concentration in the supernatant of group C patients (*n* = 14) compared to healthy volunteer (HV) samples (*n* = 9) after stimulation with LPS and difference between means. TNF-α concentration in LD patients and differences between means according to D0 **(B)** and D8 **(C)** MV status. Data plotted on the left *Y* axis are represented as scatter dot plots illustrating the individual values, mean, and SD. Data plotted on the right *Y* axis are represented as mean and SD.

D8 MV and D0 SOFA score) were more present in a cluster of patients with initial high levels of the cytokines previously mentioned. This suggests that the severity of LD may be related to hyper-inflammation. The immune response following *Legionella* infection has mainly been studied in cellular and animal models. TNF-α, highly secreted *in vitro* and in mice models (McHugh et al., 2000; Guillemot et al., 2022), has been demonstrated to be a key cytokine for the bacterial restriction (Fujita et al., 2008). Elevated concentrations of IFN-γ and IL-6 are also found in murine models (Spörri et al., 2006; Liu et al., 2020). The present results are consistent with previous findings showing a high expression of circulating Th-1 cytokines, IL-6, and IL-8 in LD patients (Tateda et al., 1998; Fernández-Serrano et al., 2003) and complete the description of the immune profile in a larger cohort of patients. The systemic overexpression of inflammatory cytokines has also been described in other severe infectious pneumonia such as COVID-19 and pneumococcal or *Mycoplasma pneumoniae* CAP (Fernández-Serrano et al., 2003; Burgmeijer et al., 2019; Leisman et al., 2020; Hu et al., 2021), in which the excessive inflammation has been shown to lead to fatal tissue damage in the lung (Xu et al., 2023). In the present study, severe LD patients had a more pronounced lymphopenia, as previously described in LD, septic shock, and severe COVID-19 patients (de Jager et al., 2013; Girardot et al., 2017; Bermejo-Martin et al., 2020). Absolute lymphopenia may result from T-cell migration to the lungs and increased apoptosis mediated by the high levels of circulating pro-inflammatory cytokines (de Jager et al., 2013; Girardot et al., 2017).

Furthermore, the present study shows that leukocytes in LD patients are impaired in their ability to release cytokines within the first days after infection. The IFA with in-house T-cell stimulation using conA showed a significantly lower production of 16 cytokines from major innate and adaptive pathways compared to HV. These alterations were observed in severe and non-severe patients, regardless of the development of a septic shock during the hospital stay. The exception are IL-18 and MCP-1, whose production was increased upon *ex vivo* stimulation of patient WBC compared to HV. IL-18 production suggests that the caspase-1-dependent inflammasome pathway leading to the release of IL-18 is functional. In animal models of *Legionella* infection, it has indeed been proven that the NLR family CARD domain containing 4 (NLRC4) inflammasome drives pyroptosis through caspase-1 activation and IL-18 release (Case et al., 2009; Mascarenhas and Zamboni, 2017). IL-18 expression was the only elevated cytokine in patients with initial MV or an unfavorable ventilatory evolution. While no study has assessed IL-18 secretion in human LD, several studies have shown that high plasma IL-18 levels are associated with poor clinical outcome in patients with bacterial sepsis (El-Sayed Zaki et al., 2007; Feng et al., 2016). Moreover, IL-18 mRNA transcript was recently shown to be overexpressed in a cohort of patients with septic shock (Tawfik et al., 2020).

Nowadays, the use of IFA with standardized stimulant concentrations has been shown to provide robust and reproducible results (Albert-Vega et al., 2018; Mouton et al., 2020). In order to standardize IFA and obtain a stimulation of innate immune cells complementary to the one obtained with conA, we used commercial TruCulture tubes prefilled with a fixed concentration of stimulant. *Escherichia coli* LPS was selected due to its ability to induce a strong activation of innate and adaptive immunity in HV (Urrutia et al., 2016) and because it is the reference test for endotoxin tolerance (Biswas and Lopez-Collazo, 2009). As most inflammatory mediators were underexpressed following conA stimulation, we measured only one representative parameter for this standardized IFA. TNF-α was chosen because it is one of the surrogate markers of endotoxin tolerance and plays a key role in *Legionella* restriction. Through this assay, we confirmed the quantitative defect in the production of TNF-α in LD patients compared to HV. In the context of sepsis, the post-LPS stimulation cytokine release, or endotoxin tolerance, is part of the sepsis immunoparalysis (Hotchkiss et al., 2013; Albert Vega et al., 2020). A study showed a lower TNF-α response to LPS in patients with sepsis or septic shock compared to less severe post-surgical patients

(Winkler et al., 2017). In line with these findings, we showed a lower TNF-α release capacity for D8 MV compared to no-MV patients. Furthermore, ICU LD patients also displayed monocyte dysfunction with weak expression of mHLA-DR, suggesting that these patients share features of sepsis-induced immunosuppression (Hotchkiss et al., 2013; Venet and Monneret, 2018). Interestingly, LPS stimulation allowed us to identify that patients with MV at D8 had a lower TNF-α secretion at D2. However, since all D8 MV patients were under MV at D0, we could not assess whether the impaired TNF-α release was a predictor or rather a consequence of MV. Conversely, we did not show any difference with conA stimulation between MV and no-MV. This could be explained by the different pathways activated by each stimulant: conA induces a strong and non-specific activation of T-cells whereas LPS mimics a bacterial infection by activating TLR-4 and TLR-2 innate receptors. The *in vitro* LPS stimulation may thus be closer to the *in vivo Legionella* infection (Akamine et al., 2005).

The present study has several limitations. Patients were enrolled based on positive Lp1 urinary test, and therefore, patients with non-Lp1 LD [<20% of cases in Europe and the USA (Beauté and Network, 2017)] were not included. In addition, the ICU admission rate was higher than previously described (Levcovich et al., 2016) which could lead to an over-representation of severe LD and thus increase the observed cytokine variations. Patient WBC count, and particularly lymphopenia, could represent an important confounder for IFA. Although a rather similar pattern of cytokine secretion was shown after normalization to lymphocyte blood counts, some changes were observed. Moreover, this normalization could not be performed for the entire cohort. Another limitation relates to the methodological issue of requiring fresh whole blood collection for IFA and mHLA-DR measurements, allowing these parameters to be tested only for a limited number of patients, in two separate cohorts, hospitalized in the same hospital. This pre-analytical requirement complicates the routine use of such immunosuppression markers. The small number of subjects tested may have reduced the robustness of the findings regarding the association with severity, and has precluded the classification of patients according to the hard endpoint (e.g., the severity scale). Furthermore, the association between baseline cytokine release and impaired antigen-induced response is well described in sepsis and severe COVID-19 infection; the small number of patients tested herein using IFA, however, did not allow to evaluate such an association. Finally, since only TNF-α was measured using a standardized IFA, the release of other Th-1 and pro-inflammatory mediators known to be impaired in sepsis, as well as mHLA-DR expression, need to be evaluated using a similar assay in a larger multicenter LD cohort.

A study by Lettinga et al. showed that LD patients from a large outbreak maintained low IFN-γ capacity secretion following *E. coli* LPS stimulation up to 1 year after the initial infection compared to exposed but uninfected individuals (Lettinga, 2003). These data suggest either that low function may persist for some time or that a basal low cytokine production capacity may contribute to the susceptibility to *Legionella* infection. Whether these alterations persist over time in the month following the infection should be assessed. LD patients show profound alterations in TNF-α secretion after LPS stimulation, the degree of alteration being related to disease severity as it has been described in sepsis. Immunostimulatory therapies (IFN-γ and GM-CSF) have been attempted in clinical trials for septic shock (Hotchkiss et al., 2013). More recently, IL-7 immunostimulation has shown functional recovery of lymphoid cells and a reduced mortality in cellular and animal models (Venet et al., 2012; Shindo et al., 2017). Therapeutical trials showed an increase in T-cell proliferation in septic shock patients treated with IL-7 compared to placebo (Francois et al., 2018; Daix et al., 2023). As LD patients have comparable immune defects to septic shock patients, such treatment could be proposed as a personalized medicine after routine assessment of sepsis-induced IS.

In conclusion, we describe a hyper-inflammatory phase at the initial phase of *Legionella* pneumonia that is more pronounced in patients with severe LD. These patients also present an immunoparalysis for a large number of cytokines, except IL-18 whose secretion is increased. An assessment of the immune response may be relevant to identify patients eligible for future innovative host-directed therapies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Comité de Protection des Personnes Sud-Est IV, France; ID-RCB 2016-A01021-50. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

The project was conceived, planned, and supervised by SJ. SJ, FA, CA, GD, LB, MI, PD, JG, AC, and GL were involved in the design, implementation, and day-to-day management of the study. WM and ST-A were involved in the IFA selection and analysis of the results. CA-V brought post-LPS stimulation HV data. J-CR and FA brought their expertise about clinical and biological data interpretation. LA, J-CR, AF, VL, YJ, NaF, and FA included patients in the study. FV was responsible for the mHLA-DR generation. CA, HT, NoF, GD, LB, MI, and SJ were responsible for the microbiological analyses; CA and NaF were responsible for the immunological analyses. CA was involved in the statistical analyses. CA and SJ wrote the original draft of the manuscript, which was reviewed and edited by WM, ST-A, FV, JG, PD, AC, and FA and reviewed by all co-authors. All authors approve the final version of the manuscript.

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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/fcimb.2023.1252515/full#supplementary-material

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Multi-criterion analysis of the effect of physico-chemical microbiological agents on *Legionella* detection in hotel water distribution systems in Crete

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Introduction: Water distribution systems in hotels have been related to outbreaks caused by *Legionella* spp. Certain measures, including disinfection by chlorination, maintaining increased temperatures are usually undertaken to prevent *Legionella* outbreaks. However, these preventive strategies are not always effective, since there are several factors (e.g., synergistic interactions with other microbes, physico-chemical factors, biofilm formation, availability of nutrients) that promote survival and proliferation of the pathogen in water pipes., Accordingly, there is a need of a holistic approach in development of preventive models for *Legionella* outbreaks associated with water distribution systems.

Methods: Water samples were collected from hotel water systems and were tested for the presence of *Legionella*, *E. coli*, total coliforms, total mesophilic count and *Pseudomonas*. In each sample, temperature and chlorine were also tested. Other epidemiological factors were additionally recorded including number of rooms, stars, proximity of sampling point to the boiler, etc. Data were processed by generalized linear analysis, and modeling based on logistic regression analysis to identify independent predictive factors associated with the presence of *Legionella* in hotel water systems.

Results: According to the generalized linear model, temperature affected ($p<0.05$) the presence of *Legionella* regardless of the species or the water supply (hot or cold). Additionally, opportunistic (*P. aeruginosa*) or non-opportunistic (*E. coli*, coliforms) pathogens were significantly associated ($p<0.05$) with the presence of all *Legionella* species. Temperature also exhibited a positive effect to all pathogens tested except for *Pseudomonas* according to the linear model. Multivariate analysis showed that *Pseudomonas*, total coliforms, HPC and temperature had a statistically significant effect on the presence of *Legionella*. Based on a binomial model, cold water had a positive

effect on *Legionella*. Type of sampling and proximity of the sample to the boiler seemed to pose different effect on *Legionella* depending on the cfu/L. The number of hotel stars and rooms did not appear to have any effect in all tested models.

Discussion: Collectively, these results indicate the need for development of individualized water safety plans tailored by the presence of other microbiological agents, and unique physico-chemical factors, which could facilitate the survival of *Legionella*.in hotel water systems.

KEYWORDS

Legionella, water, model, physico-chemical agents, microbiological agents

Introduction

Legionella is a genus of Gram-Negative bacteria, which contains over 50 different species and is a part of the gamma proteobacteria class (Schwake et al., 2015). Legionella species are the causative agents of either Pontiac Fever, a flu like non-fatal disease, or Legionnaires' Disease (LD), an atypical form of pneumonia with much more severe symptoms which can be fatal. *Legionella pneumophila* is the commonest cause of LD, accounting for almost 85% of cases (Papagianeli et al., 2021; Kermani et al., 2022). The most common transmission route of LD is through inhaling of *Legionella* contaminated aerosols, resulting in subsequent pulmonary infection (Kermani et al., 2022) (Volker et al., 2016). As the notification rate for Legionellosis has risen to 1.8/100.000 which is the highest ever recorded in the EU, it is critical to prevent the *Legionella* contamination of artificial water systems, which pose the biggest threat for human infection (Papagianeli et al., 2021; Kermani et al., 2022).

Legionella species' natural reservoirs are freshwater and wet soil, where the bacteria survive mostly as intracellular parasites of free-living protozoans, especially amoeba species such as *Acanthamoeba* (Rogers et al., 1994; Borella et al., 2005). However, *Legionella's* most important reservoirs are man-made water environments including piped drinking water, cooling towers, fountains and humidifiers, with hot water systems being colonized more often than cold water and hotel hot water systems being related to Travel Associated LD (Borella et al., 2005; Volker et al., 2016; Rasheduzzaman et al., 2020). In artificial aquatic environments, *Legionella* colonization has been associated with the presence of biofilms (Borella et al., 2005; Bargellini et al., 2011), which protect *Legionella* from disinfectants and contain the protozoa in which *Legionella* replicates. In the biofilm environment, the abundance of nutrients allows for *Legionella* to grow extracellularly, also (Schwake et al., 2015).

Legionella in hotel water systems may pose a great risk especially to immunocompromised and debilitated individuals. Furthermore, hotels often have a high occupancy rate and a constant turnover of guests. This means that if *Legionella* is present in the water system, many people may be exposed to it over time, increasing the risk of an outbreak. Of crucial importance is the complexity of plumbing systems in the hotels, including numerous water fixtures, pipes, and tanks. *Legionella* bacteria thrive in warm water, and these systems can provide ideal conditions for their growth and spread of the pathogen. Moreover, water in some parts of the system may stagnate, creating pockets of stagnant water where *Legionella* can multiply. Since *Legionella* bacteria can become aerosolized in showers, hot tubs, water spectacles, etc., guests may get infected by inhalation of contaminated water droplets. Finally, there are always legal and reputational concerns since if a guest contracts Legionnaires' disease while staying at a hotel, it can lead to legal liability and significant damage to the hotel's reputation. Lawsuits, investigations, and negative media coverage can result from outbreaks associated with hotels.

To mitigate the risk of Legionella in hotel water systems, many jurisdictions have implemented regulations and guidelines for hotel operators to follow, including regular monitoring, water treatment, and maintenance practices to ensure the safety of their guests. Proper water management and *Legionella* prevention measures are essential to protect both guest health and the hotel's reputation. To ensure that the above-mentioned criteria are met, several factors need to be considered when trying to minimize the risk of water systems from *Legionella*.

Various studies have associated several physio-chemical factors with the survival of *Legionella* and the formation of biofilms in pipeline surfaces with the colonization of *Legionella* in an attempt to create mathematical models that predict the risk of the bacterium' s growth and human exposure, models which would help develop control strategies to prevent legionellosis (Yunana et al., 2021). Despite the fact that most "traditional" entero-pathogens, enter artificial water systems in the rare case of sewage system malfunction or greater water age (Falkinham et al., 2015; De Giglio et al., 2021), *Legionella* are considered opportunistic environmental pathogens, a distinct category of pathogens, similarly to *Mycobacterium* spp., and *Pseudomonas aeruginosa*. If certain conditions are met, these pathogens are able to survive disinfection procedures and grow in the oligotrophic environment of engineered water, even in low concentrations of oxygen, posing a health threat especially for the immunocompromised people (Lu

et al., 2017; Zhang et al., 2021; Zhang et al., 2021a). The most important risk factors identified so far include high water temperature (Rasheduzzaman et al., 2020), water stagnation, pipeline material and roughness, structure age, nutrient concentration, such as Zn and Mn, disinfectant concentration, pipeline length and corrosion and Heterotrophic Platelet Count (HPC) (Bargellini et al., 2011; Volker et al., 2016).

The aim of the current study was to implement different mathematical approaches to try and obtain conclusive results on the effect of several physico-chemical and microbiological factors on the presence of *Legionella* in water distribution systems.

Materials and methods

Hotel selection

The study took place during the period 2018-2022. The hotels that participated in the study were distributed at the four prefectures (Lassithi, Heraklion, Rethymno, Chania) of the island of Crete, southern Greece. Hotels were chosen either based on routine surveillance or following the notification of a human case of *Legionella* infection in a tourist (Travel-Associated *Legionella* Disease) from ECDC and the national ministry of health. The hotels were distributed at the four prefectures of the island based on the number of hotels accommodated in each prefecture and on the corresponding population. An effort was also made to distribute the hotels according to city- or non-city ones and on seasonal or annual operation.

For each hotel, the sorting according to stars was recorded; the number of rooms, the date of sampling, whether the sampling was the first one or not, and the mode of operation (annual or seasonal) were also recorded.

The permanent population of the region where each hotel was hosted was retrieved from the database of the Hellenic Statistical Authority (https://www.statistics.gr/en/home/).

For each hotel, the geographical coordinates were recorded to map the results at a later stage and try to come to certain conclusions on the presence of *Legionella* that could be due to the locality of the hotel, the local public water distribution system, the local climate, etc. These data are not shown for reasons that have to do with the confidentiality of results.

Sampling

The number of samples tested from each hotel were calculated based on the complexity of the water distribution system, the number of boilers and the size of the establishment. In general, one water sample (tap water) was collected from the following sites: inlet of each hotel water system, right after the main water supply tank, water returning or leaving the boiler, and the closest and the farthest, from the boiler, rooms. The samples from the rooms (room showers) were collected following a one-minute flush for the cold and a two-minute flushing for the hot water. Direct sampling, collecting samples right after opening the showers, was not carried out to avoid any misleading results that could be due to a dirty end.

Water samples were collected in sterile plastic bottles (1 liter each) that contained 20mg of sodium thiosulfate to neutralize the residual-free chlorine. All water samples were stored at 4°C and processed within 24h of collection.

Legionella isolation-detection-identification

Isolation of *Legionella* from water samples was performed by culture according to the International Standard method ISO 11731 (1998), the ISO 11731-2 (2004) and the ISO 11731:2017. Based on our experience as a reference laboratory for water quality assessment for the island of Crete, the application of the latest ISO only, may lead to false negative or false positive results.

Briefly, water samples were concentrated by filtration through a 0.22μm pore diameter sterilized polyethersulfone membrane (Sartorius, Germany). After filtration, bacteria collected on the membranes were re-suspended in 10 ml 1/40 Ringer's solution in 50ml falcon tubes and vortexed for two minutes. Two hundred microliters were spread on BCYE (buffered charcoal yeast extract with α-ketoglutarate, L-cysteine and ferric pyrophosphate), BCY (buffered charcoal yeast extract without L-cysteine) and GVPC (agar supplemented with vancomycin, polymyxin B, cycloheximide and glycine) (Oxoid, ThermoFisher Scientific) petri dishes, directly after filtration, after incubation at 50°C for 30 minutes and after addition of acid buffer. The inoculated plates were incubated for 10 days at 36 ± 1°C in 5% CO_2 with increased humidity and were checked at two, five, seven and ten days. Based on our protocol, the cut-off for considering a positive result was set at 50 cfu/L.

Suspected colonies were further processed by MALDI Biotyper (Microflex LT MALDI-TOF mass spectrometer, Bruker Daltonics, Germany) for identification of *Legionella* species. Agglutination testing (Prolex-Lab Diagnostics, Waltham, USA), was also applied to discriminate *L. pneumophila* serogroup 1 from serogroups 2-14 (and each serogroup in separate) and *Legionella* species.

Isolation-detection-identification of other microorganisms

Apart from *Legionella*, water samples were also tested for the presence of *E. coli*, coliforms, *Pseudomonas* and Total Mesophilic bacteria.

The ISO 9308 (2014) was followed for the isolation, detection and enumeration of *E. coli* and total coliforms. Briefly, 100 ml of water sample were filtered through a 0.45μm pore diameter sterilized polycarbonate membrane (Pall Corporation, Michigan, USA). The membrane was placed onto a Chromogenic Coliform Agar (CCA) and was incubated at 36 ± 2°C for 24 hours. *Escherichia coli* were identified based on color whereas coliforms were confirmed by oxidase testing. *Escherichia coli* isolates were further

confirmed by MALDI-TOF-MS analysis. The results were expressed as number of *E. coli* and/or total coliforms colonies/100ml.

The ISO 16266 (2009) was followed for the isolation, detection, and enumeration of *Pseudomonas*. Briefly, 100ml of water samples were concentrated by filtration through a 0.22μm pore diameter sterilized polycarbonate membrane (Pall Corporation, Michigan, USA). The membrane was placed onto a *Pseudomonas* agar base/CN-agar and was incubated at 36 ± 2°C for 22 ± 2 44 ± 4 hours. All plates were checked under UV light (UV λ=360 ± 20nm). Typical *P. aeruginosa* isolates and isolates positive to the Nessler's reaction were further confirmed by MALDI-TOF-MS analysis. The results were expressed as number of *Pseudomonas* colonies/100ml.

The ISO 6222 (1998) was followed for the isolation and enumeration of Total Mesophilic Bacteria. Briefly, 1ml of each water sample was spread onto a Yeast Extract Agar plate, in duplicate. The first plate was incubated at 36 ± 2°C for 44 ± 4 hours while the other at 22 ± 2°C for 68 ± 4 hours. The number of colonies was recorded, and the results were expressed as number of colonies/ml.

MALDI-TOF mass spectrometry

MALDI Biotyper (Microflex LT MALDI-TOF mass spectrometer) (Bruker Daltonics, Leipzig, Germany) equipped with a microSCOUT ion source, was used for identification of individual *Legionella* colonies against the microbial database (v3.1.2.0). Spectra were recorded using the flexControl software with the default parameters set by the manufacturer for optimization (Bruker Daltonics, Leipzig, Germany). For each spectrum, 240 laser shots were collected and analyzed (6 × 40 laser shots from different positions of the target spot. All identifications were evaluated according to the manufacturer scoring scheme. *Escherichia coli* ATCC 8739 was used as an internal control.

Physico-chemical testing

At the time of sampling free residual chlorine and temperature were recorded. The Lovibond MD 100 chlorine, LR/HR, ClO_2 instrument was used for the recording of free residual chloride. The Testo 206 (Testo, Germany) was used for the recording of temperature. Both instruments were calibrated since our laboratory is accredited for all the above-mentioned methodologies.

Statistical analysis

Quantitative data were extracted for certain biological and environmental parameters focusing on the impact of temperature, HPC and number of rooms as the environmental factors and of Chlorine, *E. coli*, Coliforms, *P. aeruginosa* and *Pseudomonas* species as the biomarkers in the search of any relationship with the presence of *Legionella*. An attempt was made to build a multivariate regression model that would predict the presence and variability in the numbers of *Legionella* bacteria in water distribution systems.

All the analyses were performed using statistics packages in R version 3.6.1 environment to build Binomial and Poissons/Quasi-Poissons models. Pscl was used to develop Zero-inflated and Hurdle models and Ade4 for the development of descriptive models. OptimalCutpoints was used to test for different values as cut-off points. Dbscan and fpc were used for clustering of data.

The Benjamini-Hochberg Procedure (B-H) correction was applied to decrease the false discovery rate and to make sure that small p-values (less than 5%) do not happen by chance (false positives).

Results

A total of 663 samples were tested over the four-year period of 2018-2022. The samples were collected from 38 different hotels from the four prefectures of the island of Crete distributed as follows: seven (7) from Lassithi (eastern Crete), nine (9) from Heraklion (central Crete), four (4) from Rethymno (western Crete) and 18 from Chania (western Crete).

Of the samples tested, 107 (16%) were positive for *Legionella* (regardless the serogroup, or species), 12 (1.8%) were positive for *E. coli*, 102 (15.4%) were positive for total coliforms, 73 (11%) were positive for P. aeruginosa and 107 (16%) for *Pseudomonas* species.

Apart from *L. pneumophila* sg 1, five (5) other serogroups were also, detected in the water samples tested; these were serogroups 3, 6, 8, 9, and 10. The dominant of all serogroups including 1, was 3 (it was detected in 39 out of the 663 samples tested; 5.9%). In fact, during the last decade, serogroups 3, 6 and 8 have dominated the isolation rate in water samples tested in Crete. Serogroup 14 has almost disappeared while several species are not frequently isolated any more. A large metagenomic analysis is undertaken to figure out the reasons hiding behind that. As regards Legionella species, *L. anisa*, *L. londiniensis*, *L. taurinensis* and *L. erythra* were isolated with their rates ranging not more than one percentage of the total samples. These results along with the range of the cfu/L are shown in Table 1.

The distribution of the water samples collected according to their temperature is shown at Figure 1. Some of the samples collected from hot water supplies were far away from being considered as hot ones since their temperatures were very low. On the other hand, very few cold-water samples fulfilled the criterion of less than 20°C.

Cut-off points

The variability in the data could affect the robustness of the models that we intended to develop. To avoid misleading data points, yet without excluding the outliers that could be crucial, optimal cut-point analysis was used as an extension of ROC analysis, to make the transition from quantitative variables to qualitative.

Certain cut-off point values were selected for either negative or positive (yes/no) test results. For the purposes of our analysis, the

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TABLE 1 Isolation rate of *L. pneumophila* and L. species.

Species/serogroup	No tested samples	Positive	% positive*	% positive^	cfu/L range
L. p. 1	**663**	35	5.3		50-8750
L. p. 2-14		92	13.9		50-5550
L. p. 3		39	5.9	42.4	550-5550
L. p. 6		27	4.1	29.3	350-4250
L. p. 8		23	3.5	25.0	200-3500
L. p. 9		2	0.3	2.2	50-250
L. p. 10		1	0.2	1.1	50
L. species		11	1.7		50-1050
L. anisa		4	0.6	36.4	50-150
L. londiniensis		3	0.5	27.3	50-350
L. taurinensis		9	0.3	18.2	300-850
L. erythra		11	0.3	18.2	550-1050

The range of colony forming units (cfu/L) for each species/serogroup is also shown. L. p. 1: *L. pneumophila* serogroup 1; L. p. 2-14: *L. pneumophila* serogroups 2-14. *: as per total number of samples tested. ^: as per intra-serogroup/species.

cost-benefit (CB) criterion was selected to calculate the slope S of ROC curve as the weight of the relative costs that multiple different predictions may have on the positive/negative result.

The following formula was used for the analysis: (McNeill et al., 1975; Metz et al., 1975; Metz, 1978)

$$S = ((1 - p)/p)*CR$$

Table 2 presents the results of OptimalCut Analysis with no categorical variable as co-variate, and with hot and cold-water systems as categorical variable respectively.

Linear model

A generalized linear model was initially used to test for the effect of temperature on the presence of *Legionella*, of *L. pneumophila* and of *L. pneumophila* sg 1. A binomial model was adjusted considering a linear effect of temperature and considering that the effect of temperature will be different depending on the water supply (hot or cold). This model was applied to estimate the effect of temperature on the probability of presence of *Legionella*, of *L. pneumophila* and of *L. pneumophila* sg 1 and the probability of detecting Total *Legionella* over the legal limit as described in the national legislation (1000 cfu/L).

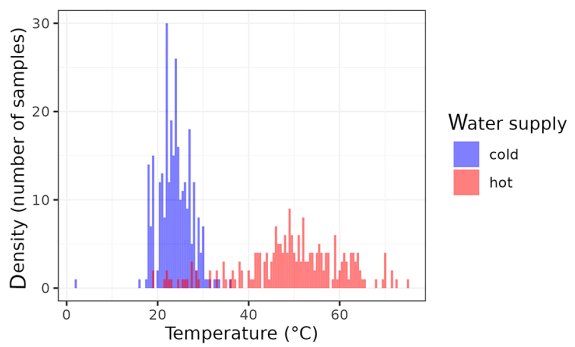

FIGURE 1
Distributions of the water temperatures of analyzed samples (red: hot water; blue: cold water).

The results presented (Table 3) indicate significant differences in the effect of temperature based on the water system supply. Specifically, at the same temperature, the estimated log-odds were significantly higher in hot water systems compared to cold water systems, as evidenced by the models Leg Tot Nor, Leg Tot Pre, L. p. Tot Pre, and L. p. sg1 Pre (with estimates of 6.58; p: 0.0016, 5.23; p <0.001, 6.06; p <0.001, 8.44 p:0.0013). Moreover, increasing temperature exhibited distinct effects on cold and hot water systems. While an increase in temperature augments the probability of *Legionella* presence in cold water systems, a corresponding increase in temperature appeared to decrease the probability of *Legionella* presence in hot water systems. These findings highlight the critical role of temperature in *Legionella* colonization and can inform strategies to mitigate *Legionella* growth in water systems.

A similar correlation analysis was carried out to test for the effect of each of the other pathogens detected (*E. coli*, Coliforms, *P. aeruginosa*, *Pseudomonas* sp). Except for *Pseudomonas* species, the other three pathogen species seem to have a positive correlation with *L. pneumophila* sg 1, *L. pneumophila* and *Legionella* in total (Table 4).

The results of the application of the linearized models are shown in Figure 2.

Univariate and multivariate analysis

The extracted cut-off points were used as inputs in a univariate and multivariate binomial model. Each cut-off point of the biological or environmental parameters was used as the factor

TABLE 2 Cut-off points using ROC and OptimalCut: a) with no categorical variable as co-variate; b) at hot water systems; c) at cold water systems.

	Variable	Breaks
No categorical variable	*Legionella*	[0, 1000], (1000, 16000]
	HPC	[0, 20], (20, 1000]
	Free chlorine (mg/L)	[0, 0.2], (0.2, 8.4]
	No of rooms	(0, 100], (100, 200], (200, 563]
	Temperature	(0, 25], (25, 50], (50, 75]
	E. coli	[0], (0, 42]
	Coliforms	[0], (0, 300]
	P. aeruginosa	[0], (0, 350]
	Pseudomonas species	[0], (0, 250]
Hot water systems	No of Stars	[0, 4], (4, 6]
	No of rooms	[0, 132], (132, 564]
	Free chlorine (mg/L)	[0, 0.11], (0.11, 9.4]
	Temperature	[0, 47.4], (47.4, 76]
	E. coli	[0, 0], (0, 43]
	Pseudomonas species	[0, 1], (0, 251]
	Coliforms	[0, 1], (0, 301]
	P. aeruginosa	[0, 1], (0, 351]
	HPC	[0, 13], (13, 1000]
Cold water systems	No of rooms	[0, 134], (134, 564]
	Free chlorine (mg/L)	[0, 0.16], (0.16, 9.4]
	Temperature	[0, 24.3], (24.3, 76]
	E. coli	[0, 1], (1, 43]
	Pseudomonas species	[0, 1], (1, 251]
	Coliforms	[0, 1], (1, 301]
	P. aeruginosa	[0, 1], (1, 351]
	HPC	[0, 21], (21, 1000]

variable of exposure, while the cut-off point for *Legionella* was used as the response variable of the outcome. Given the exposure to the variable of interest, the odds of the outcome variable were computed, indicating whether the particular exposure could be considered as a risk factor of the observing outcome.

According to the OR estimates and the associated p-value (Table 5) following the univariate analysis for each of the characteristics against *L. pneumophila* sg 1, *L. pneumophila* and Total *Legionella* showed that the exposure to *Pseudomonas* and Coliforms as biological characteristics and to temperature and HPC as environmental parameters had a statistically significant (OR>1 and p-value <0.05) impact on the presence of *Legionella* regardless of the species.

It also seems that. among all the other exposure variables, the exposure to temperature (under 25°C for cold water and over 47°C for hot water) showed a high association with *Legionella*, reaching an OR up to 5.27 (Table 6).

Multivariate model was also performed as an extension of the odds ratio analysis in logistic regression, the results of which are shown as 95% CI (confidence intervals) at Figure 3.

The importance level was set at 0 to test for the simultaneous effect of HPC, *E. coli*, Coliforms, *Pseudomonas* species and *P. aeruginosa* under the covariate of temperature. The CI corresponding to those of HPC and *Pseudomonas* indicated a statistically significant association, while their small range depicts a higher precision of the OR metric (Figure 4).

The models hiding behind the above-mentioned analyses are given below.

For the multivariate model, the presence of *Legionella* together with the other five parameters could be described as:

$$\blacktriangleright logit(\mu_i) = \beta_0 + \beta_1 \times variable1_i + \beta_2 \times variable2_i + \beta_3 \times variable3_i \ldots$$

For the univariate model, only one parameter was taken into account each time:

$$\blacktriangleright logit(\mu_i) = \beta_0 + \beta_1 \times variable\Psi 1_i, logit(\mu_i) = \beta_0 + \beta_2 \times variable2_i, logit(\mu_i) = \beta_0 + \beta_3 \times variable3_i \quad etc$$

If temperature is added into the model (multivariate model with temperature) then the odds of samples at the same level of temperature are compared:

$$\blacktriangleright logit(\mu_i) = \beta_0 + \beta_1 \times variable1_i + \beta_2 \times variable2_i + \beta_3 \times variable3_i \ldots + \beta_{temp} \times temp_i + \beta_{(hot/cold)_i} \times (hot/cold) + \beta_{interaction} \times (hot/cold)_i : temperature_i$$

When we used the univariate model for temperature, a single parameter was taken into account considering the differences due to temperature:

$$\blacktriangleright logit(\mu_i) = \beta_0 + \beta_1 \times variable1_i + \beta_{temp} \times temp_i + \beta_{(hot/cold)_i} \times (hot/cold) + \beta_{interaction} \times (hot/cold)_i : temperature_i logit(\mu_i) = \beta_0 + \beta_2 variable2_i + \beta_{temp} \times temp_i + \beta_{(hot/cold)_i} \times (hot/cold) + \beta_{interaction} \times (hot/cold)_i : temperature_i \quad etc...$$

Following all the above analyses, exposure to different water temperatures seemed to be the covariate with the greater association with *Legionella*. In terms of logistic regression, a binomial model was applied between *Legionella* as the response variables and water temperature as the binomial categorical independent variable with two values, hot water and cold water, as follows:

$$oddsi = exp(b0 + b1 * temperature)$$

Cold water seems to have positive linear relationship with *Legionella* while hot water seems to have negative linear relationship with the bacterium (Figure 4).

Using a binomial model, we supposed that under the logit scale, temperature has a linear power. In this case, the simple model

TABLE 3 Estimated log-odds and associated p-value based on a generalized linear model that estimated the effect of temperature on the odds for the presence of *Legionella* (all *Legionella* assigned as Leg Tot Pre), *L. pneumophila* (assigned as L. p. Tot Pre) *L. pneumophila* sg 1 (assigned as L. p. sg1 Pre), and for *Legionella* lying withing normal values (assigned as Leg Tot Nor) according to the national legislation (1000 cfu/L).

Variable	Leg Tot Nor		Leg Tot Pre		L. p. Tot Pre		L. p. sg1 Pre	
	estimate	*p* value	estimate	*p* value	estimate	*p* value	estimate	*p* value
(Intercept)	-6.29	<0.001	-3.61	<0.001	-4.65	0.0012	-8.60	<0.001
Temperature	0.14	0.0671	0.09	0.0379	0.10	0.0922	0.21	0.0196
Cold water	6.58	0.0016	5.23	<0.001	6.06	<0.001	8.44	0.0013
Hot water	-0.18	0.0161	-0.15	0.0011	-0.16	0.0068	-0.27	0.0040

A binomial model was adjusted considering a linear effect of temperature and considering that the effect of temperature will be different depending on the water supply (hot or cold).

TABLE 4 Correlation between presence of *Legionella* (*L. pneumophila* serogroup 1, *L. pneumophila* sgs 1-15 designated as L. p. total and all *Legionella* including *pneumophila* and species) and other bacteria using hi-squared test.

Variable	L. p. sg1			L. p. total			*Legionella* total		
	chi2	*p* value	adj p	chi2	*p* value	adj p	chi2	*p* value	adj p
E. coli	13.11	0.0095	0.024	5.21	0.0440	0.0733	10.75	0.0025	0.0031
Coliforms	13.50	<0.001	0.005	35.79	<0.001	0.0025	43.21	<0.001	0.0025
P. aeruginosa	6.05	0.0270	0.045	12.61	0.0025	0.0062	14.07	<0.001	0.0025
Pseudomonas sp	0.08	0.8051	1.000	1.83	0.1614	0.2018	12.23	0.0015	0.0025

The Benjamini-Hochberg Procedure (B-H) correction was applied at p-values to decrease the false discovery rate. Adj p: adjusted p-value.

would be as follows:

$$logit(\mu_i) = \beta_0 + \beta_1 \times temperature_i$$

which could also be written as:

$$odds_i = \exp(\beta_0 + \beta_1 \times temperature_i)$$

making, however, the model more complicated since the parameter cold and hot water comes into play.

To deal with the excessive zero count data that may affect the robustness of our model, zero inflated and hurdle count regression analysis were performed using R pcsl package.

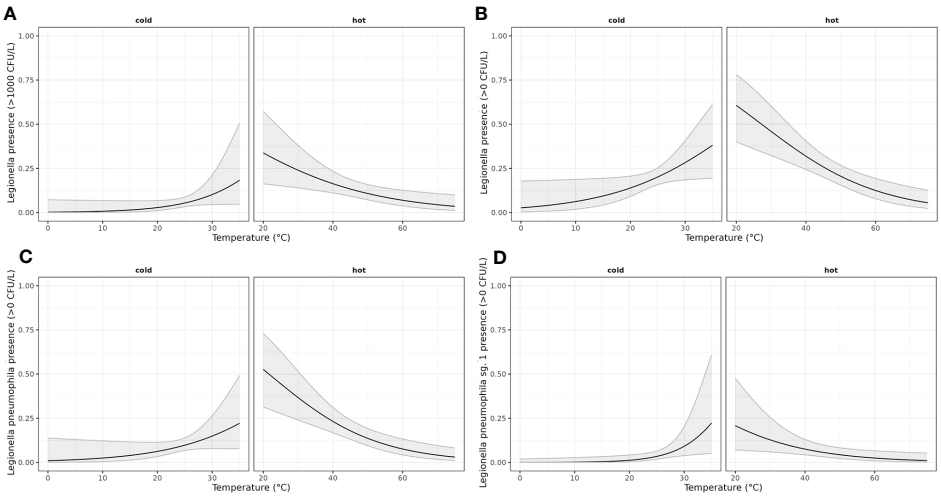

FIGURE 2
Generalized linear model estimating the effect of temperature on the odds of presence of LEGTOT, LPT, LP01 and LEGTOTNORM. A binomial model was adjusted considering a linear effect of temperature and allowing for a different effect of temperature depending on the water supply (hot or cold). **(A)** Estimated probability (with confidence interval of 95%) of LegTot being over norm depending on water supply (hot/cold) and temperature; **(B)** Estimated probability (with confidence interval of 95%) of LegTot being detected depending on water supply (hot/cold) and temperature; **(C)** Estimated probability (with confidence interval of 95%) of LPT being detected depending on water supply (hot/cold) and temperature; **(D)** Estimated probability (with confidence interval of 95%) of LP01 being detected depending on water supply (hot/cold) and temperature.

TABLE 5 Univariate analysis for 10 variables against each of *L. pneumophila* sg 1 (L. p. sg 1), *L. pneumophila* and Total *Legionella* (*Legionella* species).

Variable	Parameter	Odds Ratio	p-value
Pseudomonas species	L. p. sg 1	3.42	0.09
	L. pneumophila	3.96	0.00
	Legionella species	4.58	0.00
P. aeruginosa	L. p. sg 1	0.75	1.00
	L. pneumophila	1.92	0.13
	Legionella species	1.72	0.16
Coliforms	L. p. sg 1	3.76	0.07
	L. pneumophila	4.40	0.00
	Legionella species	2.94	0.00
E. coli	L. p. sg 1	0.00	1.00
	L. pneumophila	0.98	1.00
	Legionella species	1.24	0.68
HPC	L. p. sg 1	6.65	0.01
	L. pneumophila	2.12	0.05
	Legionella species	2.70	0.00
Free chlorine (mg/L)	L. p. sg 1	0.46	0.44
	L. pneumophila	0.63	0.68
	Legionella species	0.16	0.00
Temperature	L. p. sg 1		0.09
	L. pneumophila		0.00
	Legionella species		0.00
12 month operation	L. p. sg 1	0.00	1.00
	L. pneumophila	1.25	0.59
	Legionella species	1.55	0.45
Hotel rooms	L. p. sg 1		0.25
	L. pneumophila		0.00
	Legionella species		0.02
Hotel stars	L. p. sg 1		0.08
	L. pneumophila		0.00
	Legionella species		0.00

A ZI model assumed that zeros originated from two groups, "structural zeros" driven by the distribution itself and "sampling zeros" produced by the outcomes that were never reported. On the other side, a hurdle model assumed that all zeros are "structural", as part of a Poisson or a truncated negative binomial distribution (Feng, 2021).

Despite the different handling of zero occurrences between the two models applied (Count and Hurdle models), the results confirm the strong association between *Legionella* and water temperature as shown at the trend lines in Figure 5.

TABLE 6 Univariate analysis for the effect of temperature against each of *L. pneumophila* sg 1 (L. p. sg 1) and Total *Legionella* (*Legionella* species). Cut-offs of 1000 and 50 cfu/L were chosen based on the recommendation of the national legislation and the LOD of the laboratory, respectively.

Parameter	Cut-off (cfu/L)	Odds Ratio	p-value
Legionella species	1000	2.66	0.00
	50	2.94	0.00
L. p. sg 1	1000	5.27	0.04
	50	4.83	0.00

As regards the effect of chlorine, different models were applied considering chlorine either as a linear or a qualitive parameter. The different models were tested for each of Total *Legionella* or for *L. pneumophila* sg 1, using as cut-off values either the 1000 or the 50 cfu/L. Only Total *Legionella*, dis-regarding the cut-off value, revealed a statistically significant value and only when chlorine was treated as a qualitative parameter (Table 7).

We also tested the data for potential differences among the samples in terms of the presence of other bacteria, that could be attributed to the presence or absence of chlorine. None of the parameters did not seem to produce any statistically significant result (Figure 6).

Further processing of the data by multivariate analysis with mixed quantitative variables revealed a strong correlation with temperature. *Pseudomonas aeruginosa* seems to prefer lower temperatures than *Legionella* for its survival and multiplication (data not shown).

The qualitative data of HPC, *Pseudomonas*, *E. coli*, *Legionella* against the physico-chemical parameters tested were further processed using Principal component analysis (PCA); the corresponding results are shown in a two-dimension scale. Cold water seems to positively affect *Pseudomonas*, whereas hot water exerts a negative effect at all pathogens tested, when chlorine and temperature are not considered as variables. Free chlorine seems to exert a similar effect on all pathogens. On the other hand, temperature acts in a different way based on whether the water is described as hot or cold (data not shown).

Apart from the microbiological and physico-chemical agents, the potential effect of other epidemiological factors (type of sample, number of stars and rooms of the hotel, number of hotels in the area tested) was also tested.

When testing for the effect of direct or indirect sampling, no relationship was calculated at a cut-off of 1000 cfu/L for *Legionella* and of 50 cfu/L for *L. pneumophila* sg 1. A negative effect was calculated at a cut-off of 50 cfu/L for *Legionella* and a positive effect at a cut-off of 1000 cfu/L (Figure 7A).

As regards the effect of proximity of the sample to the boiler, no effect was calculated when testing for *Legionella* regardless of the cut-off used. On the other hand, a positive effect was calculated for *L. pneumophila* sg 1 when testing the samples that were collected from sites far from the boiler, and a negative effect for samples close to the boiler. In both circumstances, the cut-off point did not seem to play any role (Figure 7B).

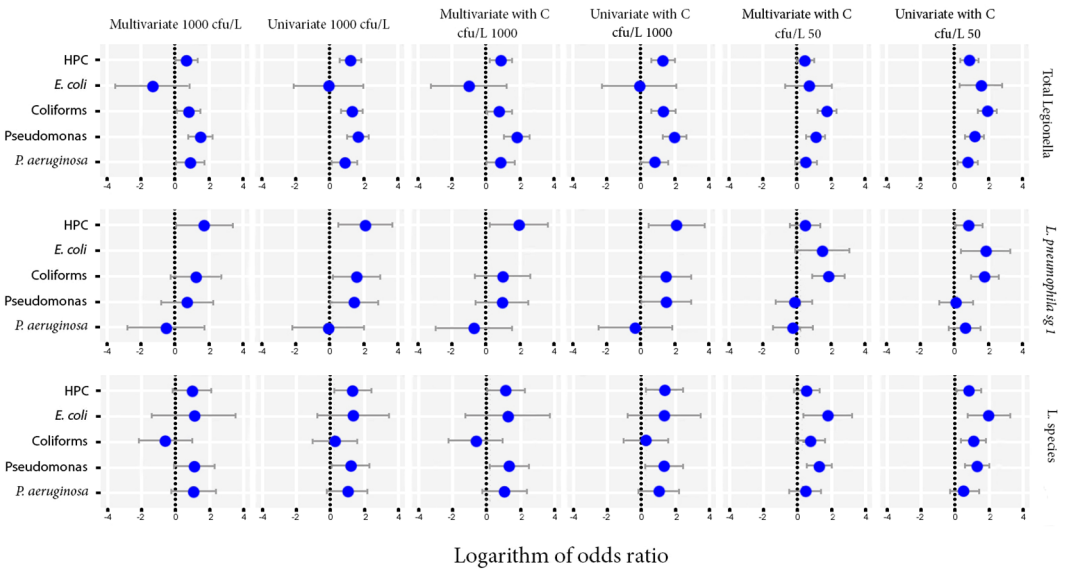

FIGURE 3
Odds ratios for the microbiological parameters of HPC, *E. coli*, Coliforms, *Pseudomonas* species and *P. aeruginosa* using univariate and multivariate models against Total *Legionella*, *L. pneumophila* sg 1 and *Legionella* species. Cut-offs of 1000 and 50 cfu/L were chosen based on the recommendation of the national legislation and the LOD of the laboratory, respectively.

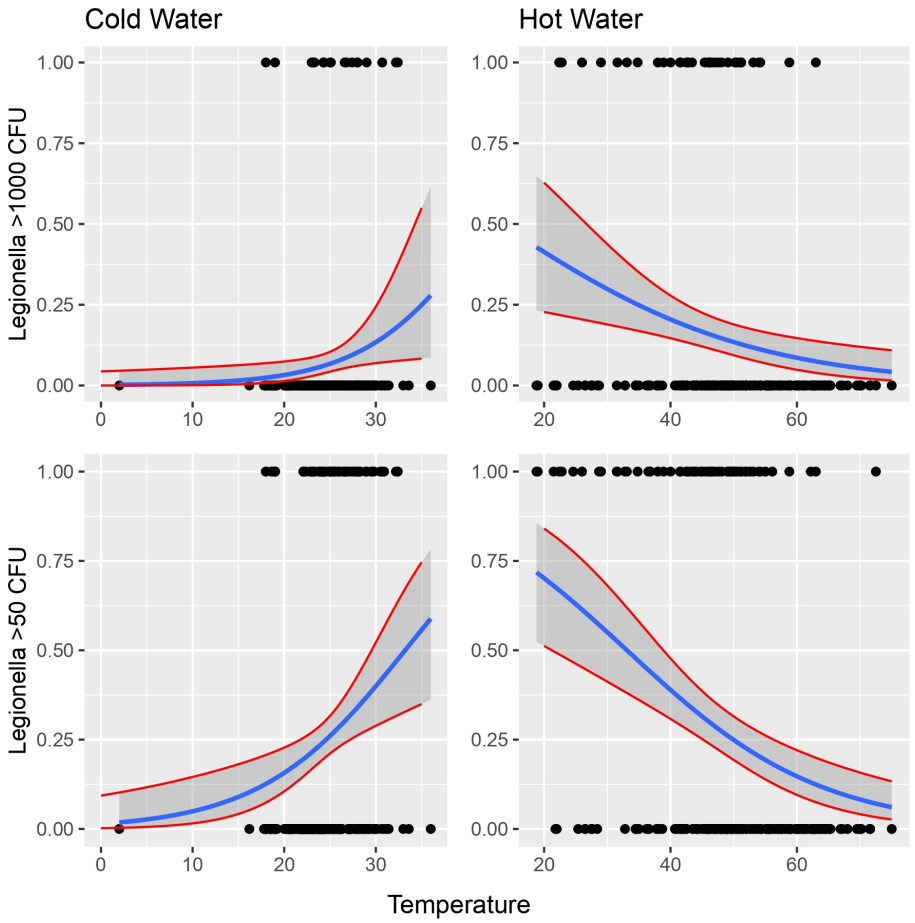

FIGURE 4
Possibility of detecting *Legionella* in the water according to temperature. Cut-offs of 1000 and 50 cfu/L were chosen based on the recommendation of the national legislation and the LOD of the laboratory, respectively.

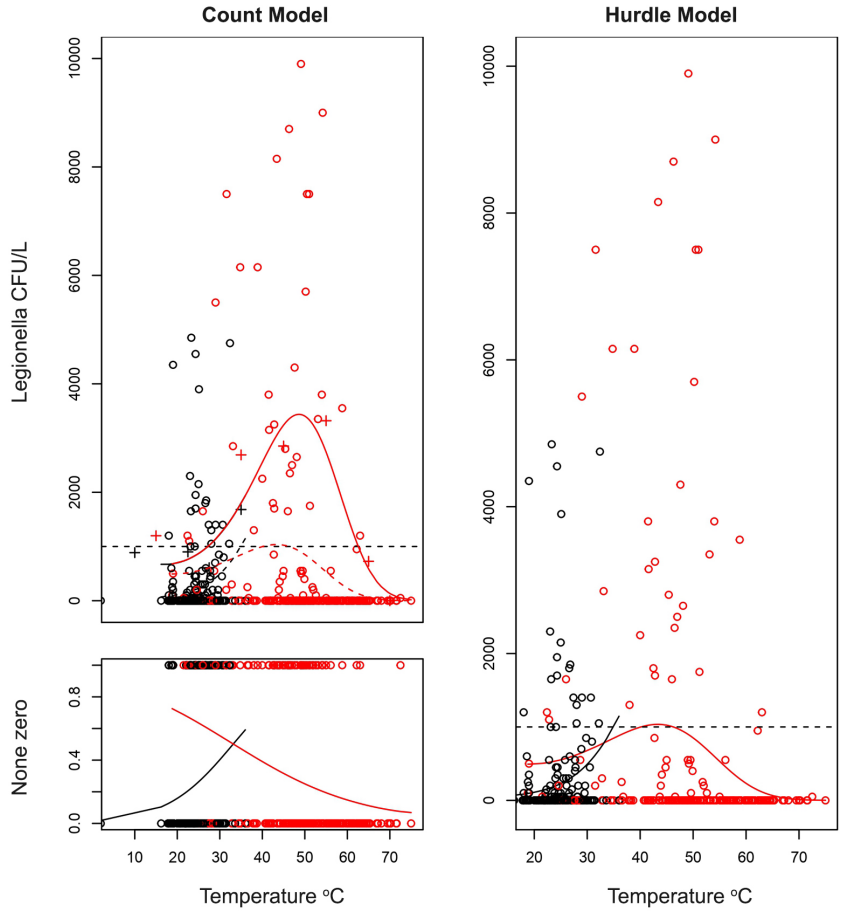

FIGURE 5
Estimations of quantitative effect of temperature on the count of *Legionella* CFU/L depending on the temperature of water. A hurdle model was adapted with the zero-prediction model and a polynomial model for the count data (coefficients on Table 6). On the first graph (top left) the fit of non-zero count data is presented. *Legionella* has an optimal temperature at which it best proliferates and can give very high CFU counts. However, as described in previous sections, the higher the temperature the more probable it is for the sample of being negative as described on the second figure (bottom left). Combining these two models gives the hurdle model on the right. Confidence intervals were not plotted, and the lines only represent the best fit. The red and black points are the samples with red corresponding to hot water and black to cold water samples.

The odds ratio analysis for the presence of *Legionella* compared to the number of stars and rooms of the hotel tested and of the number of hotels situated at the region of sampling did not seem to reveal and statistically significant effect irrespective of the model (binomial or Poisson) or the analysis (univariate or multivariate) chosen Figure 8).

On the contrary, when focusing strictly on the number of hotels tested in the area from which the samples were collected, a statistically significant p-value was calculated regardless the type of analysis followed (uni- or multi-variate) (Table 8).

Discussion

In this study we evaluated the effect of several factors that may influence the presence of *Legionella* in water distribution systems. We focused on temperature, since this is considered the most crucial one, chlorine, the presence of other pathogenic (*E. coli*, coliforms, *Pseudomonas*, *P. aeruginosa*) and non-pathogenic microorganisms (HPC), the type of operation of the hotel (annual or seasonal), other epidemiological factors (number of rooms, number of stars), and the type of sample collected (direct or following a two-minute flush). We also applied a holistic approach by implementing different mathematical analyses and models, to evaluate the potential significance and cross talk between the major risk factors.

We also performed individual analyses of the results on the presence of different *Legionella* spp., into separate for *L. pneumophila* sg 1, *L. pneumophila* (including all serogroups) and total *Legionella* (including any species or serogroup isolated). Moreover, we made our calculations based on both the LOD of our laboratory (50 cfu/L) and on the upper acceptable limit according to our National Legislation for *Legionella* (1000 cfu/L).

As regards temperature, *Legionella* species demonstrate the ability to survive in a wide spectrum of temperatures, ranging from lower than 20°C, maintaining their ability to multiply in slow rates in some of these temperatures, to as high as 70°C, although for short periods of time, while displaying differences in their preferred temperature ranges for growth and survival depending on the species and the serogroup. *Legionella*

TABLE 7 Analysis for the effect of chlorine against each of *L. pneumophila* sg 1 (L. p. sg 1) and Total *Legionella* (*Legionella* species).

Variable	Species	Cut-off	OR	p value
Linear	*Legionella*	1000	0.95	0.82
		50	1.06	0.65
	L. p. sg 1	1000	1.35	0.11
		50	1.46	0.00
Qualitative	*Legionella*	1000	0.16	0.00
		50	0.32	0.00
	L. p. sg 1	1000	0.46	0.32
		50	0.55	0.17
Qualitative	*Legionella*	1000	0.16	0.00
		50	0.30	0.00
	L. p. sg 1	1000	0.34	0.16
		50	0.58	0.21

Cut-offs of 1000 and 50 cfu/L were chosen based on the recommendation of the national legislation and the LOD of the laboratory, respectively.

pneumophila can be isolated from water temperatures ranging from 5.7 to 63°C, while proliferating at temperatures ranging between 25 and 45°C, peaking at 35 ± 2°C (Rasheduzzaman et al., 2020; Papagianeli et al., 2021). Even though the multiplication rate of *Legionella* increases in temperatures close to the optimal, the maintenance of a stable, albeit lower, population seems to be steadier in lower temperatures such as 25°C. This can be explained by the lower metabolic rates of the bacteria in these temperatures (Schwake et al., 2015). These low metabolic rates also seem to account for the low numbers of *Legionella* in biofilms forming in temperatures as low as 20°C, where the high number of *Acanthamoeba* theoretically provides a substrate for *Legionella* growth (Rogers et al., 1994).

As *Legionella* species exhibit limited survival in temperatures higher than 50°C, a constant high temperature can be used as a means of thermal disinfection. However, the current guidance for hot water temperature (50 to 55°C) seems inadequate as it is rejected by a number of studies which favor a range of temperatures from 55°C to 60°C (Rasheduzzaman et al., 2020; Papagianeli et al., 2021). While species such as *L. longbeachae*, a species found commonly in potting soil as well as in water systems are susceptible enough to a water temperature of 50°C, *L. pneumophila*, and especially the common serogroup 1, seems to be resistant to this temperature, whereas serogroup 7 seems to be resistant to temperatures as high as 55°C (Borella et al., 2005; Cervero-Arago et al., 2015). When it comes to temperatures higher than 55°C they were found to be significantly associated with decreased *Legionella* colonization coming in contrast with the positive correlation between water temperature <55°C and *Legionella* contamination (Rasheduzzaman et al., 2020). Above 60°C, all *L. pneumophila* serogroups exhibit similar behavior, surviving for several minutes (30 min 70°C). *Legionella* show higher tolerance for heat when associated with amoeba and biofilms, thus showing the need to examine other prevention factors such as disinfectants and nutrient availability (Cervero-Arago et al., 2015). Knezevic et al. recorded a statistically significant difference in the median temperature among the samples positive for Legionella and those negative for the bacterium, whether the water was warm or cold (Knezevic et al., 2022). The same study also concluded that *L. pneumophila* serogroup 1 showed a preference for higher temperatures compared to the rest serogroups.

Based on the results of our study, according to the generalized linear model both hot and cold-water supply seemed to affect the presence of *Legionella* when tested in separate at the cut-off proposed by the national legislation (1000 cfu/L). When applying a univariate approach, the exposure to temperature (under 25°C for cold water and over 47°C for hot water) also showed a high association with *Legionella*.

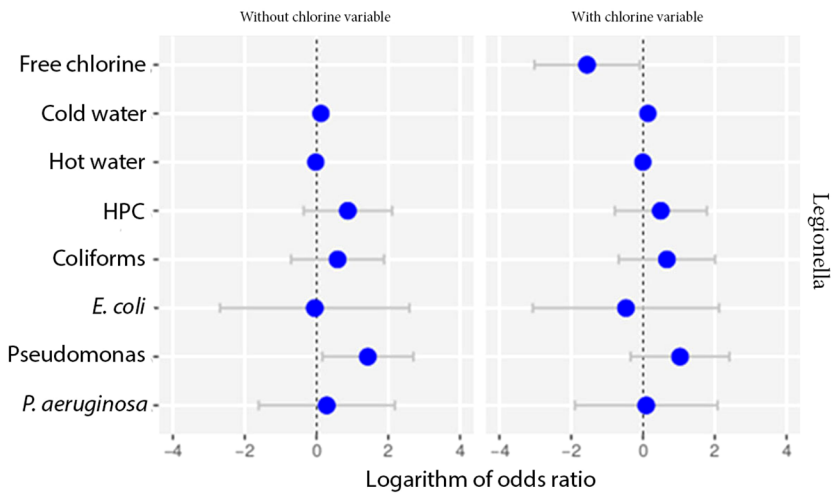

FIGURE 6
Odds ratio analysis testing the effect of chlorine against other parameters using multivariate models.

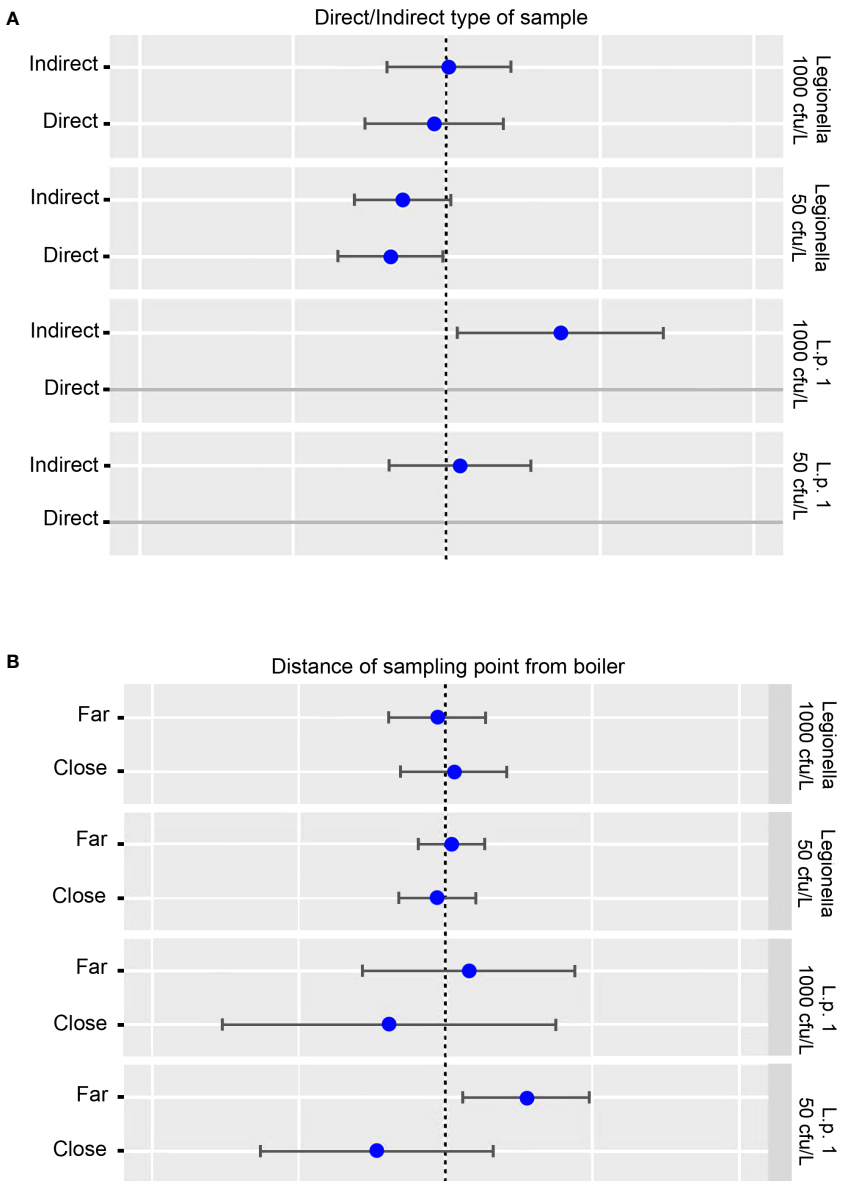

FIGURE 7
(A) Potential effect of direct and indirect sampling on *L. pneumophila* sg 1 (L. p. sg 1) and Total *Legionella* (*Legionella* species) presence. Cut-offs of 1000 and 50 cfu/L were chosen based on the recommendation of the national legislation and the LOD of the laboratory, respectively. **(B)** Potential effect of proximity of the sampling point to the boiler on *L. pneumophila* sg 1 (L. p. sg 1) and Total *Legionella* (*Legionella* species) presence. Cut-offs of 1000 and 50 cfu/L were chosen based on the recommendation of the national legislation and the LOD of the laboratory, respectively.

Moreover, according to the logistic regression and the binomial model created, exposure to different water temperatures seemed to be the covariate with the greater association with *Legionella*. In particular, cold water seemed to have positive linear relationship with *Legionella* while hot water seems to have negative linear relationship with the bacterium. Similar results were obtained when Count and Hurdle models were also applied.

In general, it seems that it is agreed that a hot water temperature of <50°C is a risk factor for *Legionella* contamination, but there still seems to exist a controversy when it comes to the temperature range between 50°C and 60°C. There are studies that have suggested that contamination at temperatures between 50°C and 60°C or >60°C don't differ significantly and accept the guideline of >55°C (Volker and Kistemann, 2015), while others have pointed out the survival of a colonization of water distribution systems even at >60°C and hold temperatures as high as 57°C as a risk factor if water chlorination/hygiene is inadequate. Thus, these studies strictly suggest a temperature of over 60°C unless these other parameters can be regularly and closely monitored (Domenech-Sanchez et al., 2022).

Of interest, temperature also affects both the physico-chemical parameters which include chlorine decay and the absorption of

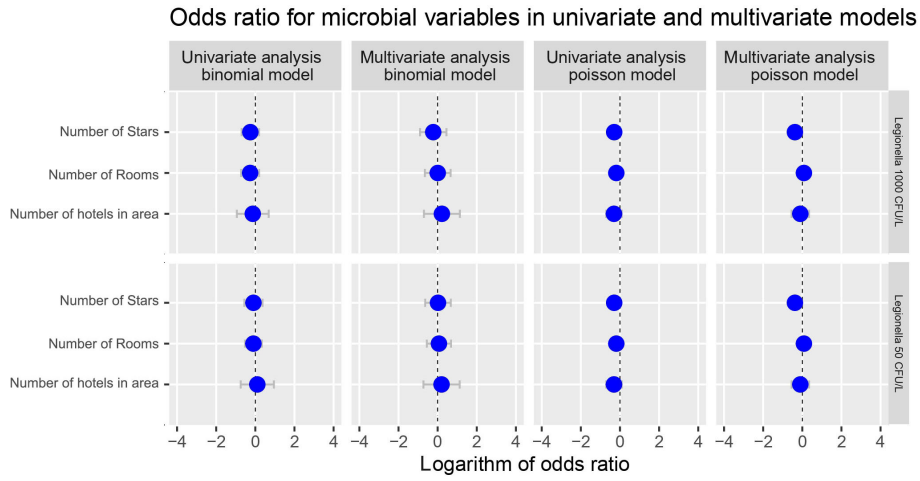

FIGURE 8
Potential effect of number of Stars and number of rooms of the hotel and the number of hotels situated at the area of sampling on Total *Legionella* (*Legionella* species) presence. Cut-offs of 1000 and 50 cfu/L were chosen based on the recommendation of the national legislation and the LOD of the laboratory, respectively.

chemicals and determine the formation of biofilms as well as the dynamics of formed biofilms allowing *Legionella* to detach and spread through the water system (Calero Preciado et al., 2021).

As regards disinfection, chlorination of hot water systems is one of the two major preventive measures against *Legionella* contamination, alongside thermal disinfection, and it can be used either as a secondary method of disinfection or as an installation disinfection method (hyper-chlorination). It is recommended by the WHO that drinking water has a minimum chlorination of 0.2 mg/L or 0.5 mg/L, depending on the risk of said water source for contamination (Cervero-Arago et al., 2015). The chlorine levels of the water seem to correlate negatively with *L. pneumophila* growth except for serogroup 1 which seems to be more resistant to the disinfectant. Knezevic et al. reported a simultaneous presence of chlorine along with Legionella, however the presence of the bacterium was reported at low to very low levels of the disinfectant (Knezevic et al., 2022).

According to our results and regardless of the models used, only total *Legionella*, dis-regarding the cut-off value (50 or 1000 cfu/L, revealed a statistically significant value and only when chlorine was treated as a qualitive parameter.

Other studies have also pointed out the negative correlation between chlorine concentration and *Legionella* growth however, they note that inside *Acanthamoeba* vesicles, where *Legionella* grow parasitically especially in biofilms, there is an increase in chlorine resistance. However, increasing chlorination is not a viable option as it would cause odor and taste problems to the water while also posing a health threat itself (Falkinham et al., 2015).

Apart from the physico-chemical factors, we also investigated the potential influence of other pathogenic microorganisms on the presence of *Legionella*. The pathogens whose presence and dynamics are currently studied to assess the water quality, mainly include Enterococci, *E. coli* and other coliforms. However, these pathogens' presence or absence fails to determine the growth of opportunistic pathogens (OPs), which are usually not of Enteric or Fecal origin (Felfoldi et al., 2010; Mapili et al., 2020). It has also been suggested that the presence of certain other opportunistic pathogen species or other *Legionella* species in biofilms promote *Legionella* growth through increased resistance to temperature and disinfectants by horizontal gene transfer (Girolamini et al., 2022). This however is still controversial as studies suggest both an association and a lack thereof between the growth of *Legionella* and other OPs such as *P. aeruginosa* (Masaka et al., 2021; Atnafu et al., 2022).

According to the generalized linear model that we created, *E. coli*, coliforms and *P. aeruginosa* seemed to have a positive correlation with *L. pneumophila* sg 1, *L. pneumophila* and total *Legionella*. When testing our results by univariate analysis, *Pseudomonas* species and total coliforms showed a statistically significant impact on the presence of *Legionella* regardless of the

TABLE 8 Odds ratio for Poisson model considering different aspects of the areas from which the samples were collected.

Coefficient Analysis	OR		p-value	
	Univariate	Multivariate	Univariate	Multivariate
No of hotels in the region tested	0.77	1.06	0.03	0.76
No of hotels sampled in the region tested	0.91	0.90	0.00	0.03

species. It seems that increased numbers of *E. coli*, coliforms and *P. aeruginosa* may be present with increased numbers of *Legionella*. It is not clear whether the parallel presence of *E. coli* and coliforms is due to adequate presence of nutrients or the presence of biofilm that can provide a rescue room for these bacteria. To assess this issue the study of biofilm presence and perhaps of amoebae should have been necessary.

Our multivariate analysis showed that *P. aeruginosa* seems to prefer lower temperatures than *Legionella* for its survival and multiplication; a similar observation was recorded following PCA analysis. On the contrary, hot water exerts a negative effect on all pathogens tested when chlorine and temperature were not considered as variables. Free chlorine seems to exert a similar effect on all pathogens.

It has been suggested that *Legionella* could be used as an alternate indicator of water quality. On the other hand, the need for specific culture media, the different sensitivity to certain factors such as disinfectants between *Legionella* and other opportunistic pathogens and Fecal bacteria and the existence of viable but not culturable *Legionella* limit the pathogen's potential usage as a sole indicator of water quality and it is suggested that it should be used in conjugation with other "typical" pathogens (Zhang et al., 2021b).

As far as HPC is concerned, it is considered an early indicator of microbial growth within a water distribution system since it signalizes the availability of organic nutrients, formation of biofilms and the presence of amoebae (Stojek and Dutkiewicz, 2011; Wingender and Flemming, 2011). HPC is used to monitor and assess the quality of water, as its increase is indicative of a post treatment biological activity in a hot water system, essentially signifying the existence of biofilms or the nutritional conditions needed for their formation (Bargellini et al., 2011; Volker et al., 2016). Formation of biofilms allows for *Legionella* to grow both intracellularly, as parasites to protozoan hosts, and extracellularly, being supported by various cyanobacterial species (Rogers et al., 1994). Specifically, *Legionella* grows intracellularly in vesicles contained in various *Acanthamoeba* spp. and extracellularly by harvesting carbon and energy sources excreted by other bacteria (Declerck et al., 2009; Stojek and Dutkiewicz, 2011). Biofilms also provide protection from disinfectants and temperature while serving as a substrate for exchange of genetic material and play a pivotal role in the maintenance of *Legionella* through the water system as parts of them get detached as a result of many factors such as temperature (Whiley et al., 2019; Calero Preciado et al., 2021; Girolamini et al., 2022).

While it stands to reason that *Legionella* occurrence could correlate with HPC, studies have shown both the existence and the lack between HPC and *Legionella* occurrence and it should be noted that the studies which do suggest a correlation point out that their results are limited by other factors such as temperature, with >50°C not showing any correlation between HPC and *Legionella* occurrence (Serrano-Suarez et al., 2013; Whiley et al., 2019). Some other studies have shown a significant correlation between *Legionella* contamination and HPC at specific temperatures, for example at 30°C, while others have only associated the two parameters in certain temperatures and only for very high HPC values (>100 CFU/ml) revealing the need for HPC and biofilm to be examined with more parameters such as water stagnation (Bargellini et al., 2011; Volker et al., 2016). A recent study (Knezevic et al., 2022) reported that HPC was positively associated with *Legionella* only when HPC was recorded following incubation on blood agar plates at 36°C for four days. At less days of incubation or when yeast extract was used as a medium, no significant correlation was reported. These studies perhaps indicate that HPC may not be a rigid parameter that can be used to monitor the presence of Legionella.

Stagnation of water occurs when there is an inadequate water flow rate, often due to a lack of a circulation pump or the existence of a physical dead leg in the hot water system. The stagnation zones allow bacteria to create biofilms providing both the time necessary for their formation and by making it difficult to maintain high temperatures and disinfectant concentrations (Volker et al., 2016). Although we did not study the role of water stagnation in our study, there have been studies that agree upon the correlation of water stagnation and *Legionella* contamination. Some authors attribute it to the dissipation of chlorine, going as far as to point out the correlation of winter and spring, the seasons with the lowest water usage and thus the highest water stagnation, with *Legionella* growth (Al-Bahry et al., 2011; Zhang et al., 2021b).

In our study we chose to test for the effect of number of stars and rooms upon the presence of *Legionella* in the water samples collected. The choice of these two factors was made as an indirect way to test for the effect of the complexity of a water distribution system assuming that the higher the number of stars and rooms the bigger the building and the more complex the water distribution system. However, we failed to show any correlation between the presence of *Legionella* compared to the number of stars and rooms of the hotel tested irrespective of the model, or the analysis chosen.

Other studies have revealed that the age and height of a building are positively correlated with *Legionella* contamination of the water distribution system, possibly because they make it difficult for proper water temperatures to be maintained throughout the entirety of its extent, especially at higher floors. An age of 20 or more years and a number of floors over 10 are also considered risk factors (Masaka et al., 2021; Atnafu et al., 2022; Girolamini et al., 2022). On the contrary, a newer study failed to exhibit a correlation with *Legionella* growth with the age of a water distribution system pipes (Wingender and Flemming, 2004). In general, however, it seems that lower floors of taller buildings are not protected, as *Legionella* spreads to them from the higher floors (Borella et al., 2005; Gamage et al., 2022). Furthermore, the outlet distance has also been associated with *Legionella* contamination, as the outlets further away from temperature decrease, biofilm formation and stagnation of water (Volker et al., 2016).

According to the analysis that we performed, the proximity of the sample point to the boiler seemed to exert an effect (positive or negative based on the distance from the boiler) to *L. pneumophila* sg 1 but not total *Legionella* regardless of the cut-off used.

Although we did not test for the effect of nutrients, *Legionella* species have very low requirements to survive and grow. The presence of copper seems to limit the bacterium's growth. The hardness and the pH of the water seem to influence *Legionella*'s

growth, both directly and indirectly, as a low pH leads to corrosion of pipe walls which allows for biofilms to develop (Borella et al., 2005; Volker et al., 2016).

Other studies agree that the release of nutrients in the water distribution system, possibly by like rubber coated valves favor the formation of biofilms and some of these nutrients such as ammonia have been correlated with *Legionella* contamination (Wingender and Flemming, 2004; Zhang et al., 2021b).

We also tried to test for the effect of direct or indirect sampling. The results that we revealed were ambiguous since no relationship was calculated at a cut-off of 1000 cfu/L for *Legionella* and of 50 cfu/L for *L. pneumophila* sg 1 while a negative effect was calculated at a cut-off of 50 cfu/L for *Legionella* and a positive effect at a cut-off of 1000 cfu/L. Certainly, more studies need to be carried out to study the potential effect of flushing on the detection of *Legionella*.

Lastly, we did not reveal any correlation between the presence of *Legionella* compared to the number of hotels situated at the region of sampling when applying complicated analysis, while when we focused strictly on the number of hotels tested in the area from which the samples were collected, a statistically significant p-value was calculated regardless the type of analysis followed. Again, more studies need to be carried out to reveal any potential effects lying behind.

The findings of the current study further support the belief that simple recording of temperature, chlorine, Legionella concentrations, etc. is no longer enough to minimize the risk of colonization of water distribution systems. A more holistic approach is need to properly survey Legionella presence since the latter seems to be affected by far more factors than just temperature and chlorine (or any other disinfectant). There is a need to take under consideration and routinely test for the presence of opportunistic and other bacteria since their presence may either act as indicator of Legionella presence or may enhance the presence of the bacterium. Other epidemiological factors should also be considered when trying to set up a guidance for the risk factors that affect Legionella. Water stagnation and age/type of pipelines should be included into the risk factors considered. There have been several cases of hotel units that try to get rid or even minimize the presence of the bacterium in their water distribution systems without any success. Simple hyper-chlorination and/or heat shock do not always prove enough to minimize the risk. To keep the system under control and to maintain an equilibrium of the bacterium concentration in the water distribution system, there is a need to remove all these factors that enhance the survival and proliferation of Legionella.

The results of this study add up to the existing knowledge recorded from past studies on the area and could be used as a guide to proper hotel management in implementing effective water treatment strategies to reduce Legionella risk in water distribution systems.

Limitations of the study

Certainly, the results of the current study may be limited by the specific geographic focus on Crete; further studies in diverse locations may provide more generalized insights on the effect of the above-mentioned factors on Legionella. Moreover, although we tried to implement as many statistical approaches as possible and study as many parameters as possible, it would be inevitable to include all of them. Under this term, we need to point out that part of the study was performed during the SARS-CoV-2 pandemic where several hotels either remained closed or started their operation with great delay during the summers. These prolonged closures may have affected the microbiological quality of the water distribution systems and may have made it more difficult to clean the pipelines from any microorganisms, biofilms, etc. Therefore, if the same study was carried out before the pandemic different results could have been recorded. Moreover, we did not test for the potential effect of stagnation of water or the protective effect that amoebae may confer to *Legionella*. We did not also study the presence of viable but not culturable forms of *Legionella* or the potential effect that these forms of the bacteria may have had upon our results. Although we tried to investigate the effect of several epidemiological factors associated with the hotel (for example the number of rooms or stars), we did not study the effect of seasonality upon our results. We did not also test for the presence of chemical agents, such as Mg, Mn, Fe, etc., that may affect the presence of Legionella in the water distribution systems. Of course, in case we needed to do so, we would have to repeat all our analyses for each season separately. Application of different models did not always give us back similar results. Under this concept, processing of our data with neural networking or artificial intelligence may had provided us with different outcomes. In general, more studies could or should focus on these aspects as well.

Conclusions

Plumbing drinking water systems show great short- and long-term variability in terms of the presence and concentration of *Legionella*. Regular testing by classical culture methods cannot always reliably reveal contamination risks. There is a need for a longitudinal sampling approach, better risk assessment and better specification of the number of samples that need to be collected to be representative of the water distribution system. We need to consider the entire drinking water plumbing system as an ecological system and each outlet as an ecological niche. The analysis of single parameters specific to individual sampling points cannot reliably predict the possibility of *Legionella* contamination. The application of empirical modeling using logistic regression can provide a valuable contribution to a better risk assessment for hydraulic drinking water systems than conventional culture-based detection methods. Moreover, the use of different models may provide different results. It is crucial to assess all these parameters prior to sampling since this can help: in risk assessment, in the prioritization of risk areas, in identifying suitable sampling points, in determining control measures within a building and in the development of a water safety plan for a specific building.

The results of the current study provide further knowledge on the study of *Legionella* presence in water distribution systems. Several factors may affect its presence and multiplication and certainly more complex approaches are needed to control the bacterium.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

DC designed the experiment, did the laboratory testing and wrote the manuscript. VS wrote the manuscript and did the laboratory testing. AN wrote the manuscript. M-OD did the statistical analysis. TL did the statistical analysis. NT did the laboratory testing. RM did the laboratory testing. CP collected samples. AP did the supervision. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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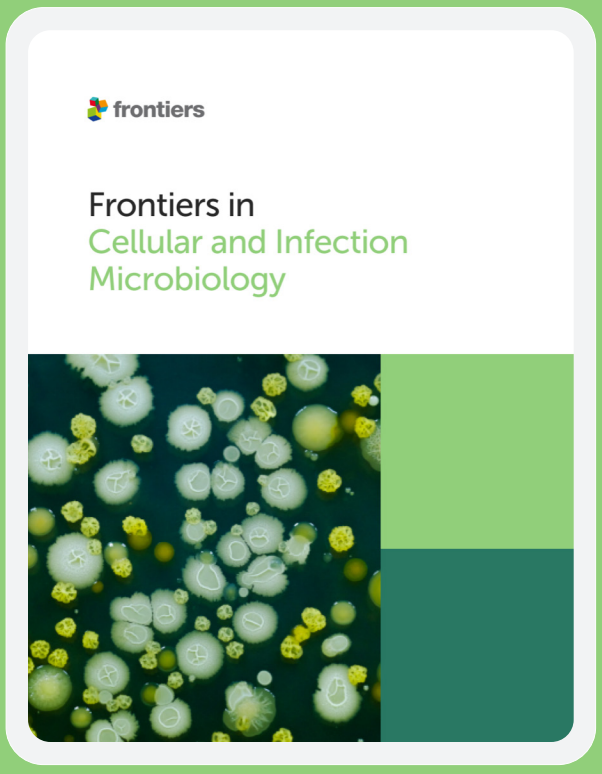

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