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Seasonal Profiling of Bacterial Diversity in Balneological Mud Using MALDI-TOF MS: Public Health Implications from Burguç Mineral Spring (Türkiye)

Bahriye Çağla Çelik[1], İfakat Tülay Çağatay[2*]

[1]Institute of Natural and Applied Sciences, Akdeniz University, Antalya, Türkiye.
ORCID: https://orcid.org/0009-0006-0461-2508

[2]Department of Basic Sciences, Molecular Microbiology Laboratory, Faculty of Fisheries
Akdeniz University, Antalya, Türkiye.
ORCID ID: https://orcid.org/0000-0002-1868-8611

Abstract

Identification of bacterial communities in thermal mud is critical for evaluating microbiological safety in balneological applications, particularly in health tourism contexts where peloid is directly applied to the human body. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) provides a rapid, cost-effective approach for environmental microbial profiling. In this study, bacterial diversity of balneological mud was characterized across four seasons (April, July, October, December) at the Burguç mineral spring (Demre, Antalya, Türkiye) using MALDI-TOF MS. From 120 bacterial isolates yielding 200 colonies, 19 distinct species were identified. Species richness varied markedly by season, ranging from 2 species in summer to 10 in autumn. Alpha-diversity analysis confirmed autumn as the most diverse season (Shannon H′=2.27), while Pielou's evenness remained high across all seasons (J=0.97-0.99), indicating no single taxon dominated the recovered communities. Several taxa of potential clinical relevance were detected, including *Aeromonas* spp., *Stenotrophomonas maltophilia*, *Bacillus cereus*, and *Citrobacter freundii*. These findings support the need for systematic microbiological surveillance of mineral spring sites used in health tourism.

Keywords:
Balneological mud, Peloid, Health tourism, Microbial ecology, MALDI-TOF MS.

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Introduction

Mineral spring and balneological mud systems represent unique ecological niches that harbor diverse and largely unexplored microbial communities with significant implications for public health and health tourism. A considerable proportion of microorganisms constitute the human microbiota, contributing to host physiology and health. They are ubiquitously distributed across environmental matrices, including water, air, and soil, and exhibit diverse metabolic capabilities that enable adaptation to a wide range of ecological conditions (Ashfaq et al., 2022). Aquatic environments, in particular, host complex microbial communities comprising both saprophytic and pathogenic bacteria, whose distribution is strongly influenced by environmental, chemical, and physical factors (Costello & Chaudhary 2017). Geothermal resource availability, with more than a thousand mineral spring sources used for the treatment of rheumatic, dermatological, and musculoskeletal diseases through balneotherapy and peloidotherapy (Gianfaldoni et al.,

*Corresponding Author: İfakat Tülay Çağatay, E-mail: tulaycagatay@akdeniz.edu.tr

2017). Beyond their therapeutic value, these resources also constitute an important component of national health tourism.

In addition to their therapeutic value, mineral spring and balneological mud systems harbor rich and largely unexplored microbial diversity. These microorganisms have considerable potential for applications in biotechnology, pharmaceuticals, and cosmetics. However, such environments may also contain opportunistic bacteria of potential clinical relevance, which may warrant microbiological surveillance under direct-contact applications. In addition, the presence of chemical constituents such as sulfate, fluoride, and heavy metals may lead to adverse health effects, particularly under prolonged exposure (Quattrini et al., 2017; WHO, 2022). Therefore, comprehensive characterization of microbial communities in these environments is essential for both public health and sustainable resource management.

Microbial identification has traditionally relied on culture-based, microscopic, biochemical, serological, and histopathological methods. While these approaches provide foundational diagnostic information, they share key limitations, including the inability to detect non-culturable microorganisms, susceptibility to contamination, and reliance on phenotypic features that may vary among environmental strains (Alturkistani et al., 2015).

Molecular techniques have substantially advanced microbial characterization in complex environmental matrices. PCR-based methods (including qPCR, RT-PCR, multiplex, and nested PCR) enable rapid and highly sensitive detection of microbial genetic material (Green & Sambrook, 2019), while 16S rRNA gene-based approaches such as amplicon sequencing and metagenomics have revealed the true diversity of thermal environments, including taxa that escape culture-dependent detection (Hall et al., 2008).

Among these methods, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has emerged as a rapid, reliable and cost-effective technique for microbial identification. This approach analyses ribosomal protein profiles to generate unique spectral fingerprints, enabling accurate identification at the genus and species levels (Singhal et al., 2015; Piamsomboon et al., 2020). Compared with conventional and molecular techniques, MALDI-TOF MS offers significant advantages in speed, cost, and operational simplicity, and has been increasingly integrated into routine microbiological diagnostics (Popović et al., 2022). It has also been successfully applied to characterize bacterial communities in environmental matrices including water, sediment, and soil (Puk et al., 2023).

Previous studies have demonstrated the presence of diverse microbial communities in thermal environments, including potentially pathogenic genera such as *Pseudomonas*, *Bacillus*, *Acinetobacter* and *Aeromonas* (Ghilamicael et al., 2018; Pascale et al., 2020). More recent studies have confirmed the applicability of MALDI-TOF MS in both environmental and clinical microbiology, demonstrating its high reliability and diagnostic potential across diverse sample types (Gökdağ & Çağatay, 2025; Abbas et al., 2025). Recent environmental studies employing MALDI-TOF MS have reported the identification of bacterial taxa including *Pseudomonas*, *Aeromonas*, *Acinetobacter*, *Shewanella*, *Enterobacter hormaechei*, *Brevundimonas diminuta*, *Bacillus cereus*, *Stenotrophomonas maltophilia* and *Lysinibacillus* from water, sediment, and agricultural samples (Popović et al., 2022; Puk et al., 2023; Surányi et al., 2023), genera and species frequently reported in similar thermal and aquatic environments.

Despite these advances, studies specifically focusing on the comprehensive characterization of bacterial diversity in balneological mud systems using MALDI-TOF MS remain limited. The mud samples investigated in this study qualify as natural peloids in the balneological sense, defined as maturated fine-grained materials of geologic origin mixed with mineral water and biological metabolic products, regardless of water temperature (Gomes et al., 2013). The Burguç mineral spring, although classified as a hypothermal source, can still harbor thermotolerant and mesophilic bacterial communities of balneological and public health relevance. This sodium-chloride and magnesium-rich spring has been used therapeutically since antiquity. Therefore, the specific objectives of this study were: (i) to characterize the seasonal bacterial diversity of balneological mud from the Burguç mineral spring using MALDI-TOF MS across four seasons; (ii) to evaluate the seasonal dynamics of microbial community composition through alpha-diversity indices (Shannon, Simpson, Pielou's evenness, and Margalef); and (iii) to assess the public health and health tourism implications of the identified microbial community.

Materials and Methods

Sample Collection and Bacterial Isolation

The Burguç mineral spring sampling station was located at 36.2497 °N, 29.9823 °E. Mud samples were collected from the Burguç mineral spring located in Demre, Antalya, Türkiye, using a four-season sampling design (April, July, October, and December) adopted to capture the full annual range of physicochemical and climatic variation at the site, which is known to influence microbial community structure in shallow sedimentary aquatic systems. In each sampling period, ten independent mud samples were aseptically

collected in sterile containers (n=40 in total across the four seasons) and transported to the laboratory within 3-4 hours to preserve microbial integrity. Each sample was processed in duplicate to ensure reproducibility and analytical reliability. Mud samples (1-10 g) were individually weighed and subjected to serial dilution ranging from 10^{-1} to 10^{-10} using a sterile diluent. Aliquots from dilutions yielding countable colony growth were selected for plating. Samples were inoculated onto Nutrient Broth, Nutrient Agar and Tryptic Soy Agar media (Amplichem, Germany). Non-selective general-purpose media were employed to allow growth of a broad range of heterotrophic bacterial groups. Incubation was carried out across a temperature range of 25-35°C to encompass mesophilic bacterial groups for 24-48 hours. Following incubation, distinct colonies were selected from countable plates based on morphological characteristics and subcultured to obtain pure isolates. The overall experimental workflow, from sample collection to species-level identification by MALDI-TOF MS, is summarized in Figure 1.

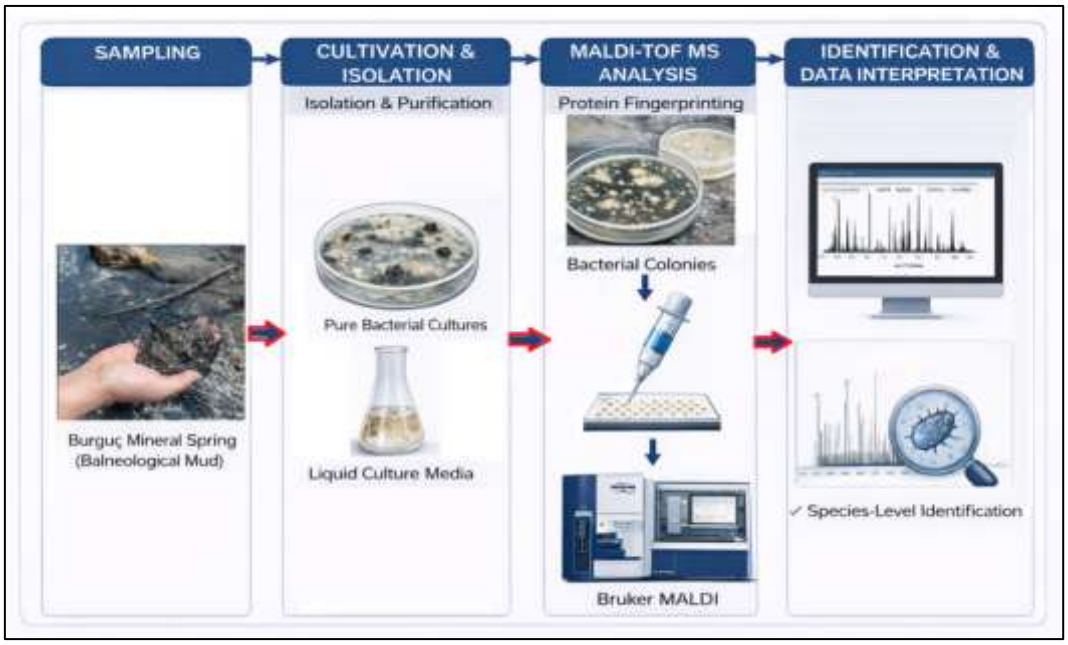

Figure 1. Workflow for the characterization of bacterial diversity in balneological mud from Burguç Mineral Spring by MALDI-TOF MS, including sample collection, bacterial isolation and cultivation, mass spectral profiling, and species-level identification via library matching.

Identification of Bacteria by MALDI-TOF MS

A total of 120 bacterial isolates were obtained from seasonal mud samples, yielding 200 colonies that were subjected to MALDI-TOF MS analysis, as some isolates were represented by more than one colony to ensure identification reliability. All MALDI-TOF MS analyses were conducted at the Health Research and Application Center of Akdeniz University, utilizing a Bruker MALDI Biotyper system (Bruker Daltonics, Germany), and acquired spectra were analysed using the integrated MALDI Biotyper software against the Bruker reference library (MBT Compass Library). Prior to each analytical run, mass calibration was performed using the Bacterial Test Standard (BTS, Bruker Daltonics) according to the manufacturer's specifications. For sample preparation, the on-target formic acid extraction protocol based on the direct smear approach described by Popovic et al., (2022) was followed. A sterile wooden applicator stick was used to transfer a single colony from the agar surface onto a 96-well stainless steel target plate, where it was spread into a uniform thin layer. To facilitate cell lysis and protein release, 1 µL of 70% formic acid (Kemika, Croatia) was applied to each spot. Following complete air-drying at ambient temperature, 1 µL of CHCA matrix solution (α-cyano-4-hydroxycinnamic acid; Bruker Daltonics, Germany) was deposited onto each spot and left to dry, enabling co-crystallization with the released proteins. The target plate was subsequently loaded into the instrument for spectral acquisition. Laser-induced desorption and ionization of biomolecules were performed during the analytical run, with mass spectra recorded across a range of 2,000-21,000 Da. To enhance spectral reliability, multiple laser shots were accumulated from various positions across each sample spot. Species and genus-level assignments were generated by matching acquired spectra against the Bruker reference library through the manufacturer's logarithmic scoring algorithm (0-3.00 scale). Score values were interpreted following the manufacturer's guidelines: scores ≥2.00 were considered reliable for species-level identification, whereas scores in the 1.70-2.00 range were accepted for genus-level identification, with the species-level result reported as the best match against the reference database. To

enhance reliability for borderline scores (1.70-2.00), each isolate was re-spotted in duplicate, and only consistent species-level matches between the two replicate spots were retained for the final dataset.

Diversity Analysis

Alpha-diversity indices were calculated using species-level colony-abundance data per sampling season. The following indices were computed: species richness (S), Shannon diversity index ($H' = -\Sigma p_i \ln p_i$), Simpson dominance ($D = \Sigma p_i^2$) and inverse Simpson (1/D), Pielou's evenness ($J = H'/\ln S$), and Margalef's richness index ($DMg = (S - 1)/\ln N$), where p_i is the proportional abundance of species i and N is the total colony count per season. All calculations were performed in Microsoft Excel using standard formulas, and results are reported in Table 2.

Results

Water-quality parameters of the sampling station were monitored across the four sampling seasons and showed minimal variation throughout the study period; the annual mean values were as follows: temperature 17.9 °C, salinity 12.35 ‰, dissolved oxygen 6.0 mg L^{-1}, pH 7.8, conductivity 20.7 mS cm^{-1}, and total dissolved solids 10.4 g L^{-1}. These values are consistent with the previously reported character of the spring as a hypothermal sodium-chloride and magnesium-rich mineralized water (total mineralization 9 382.2 mg L^{-1}) and were used to contextualize the microbial community findings.

A total of 120 isolation plates (30 per season) were obtained from balneological mud samples of the Burguç mineral spring collected across four seasons. Based on morphological characteristics, 1-5 representative colonies were selected from each plate, yielding a total of 200 bacterial colonies subjected to MALDI-TOF MS identification. All 200 colonies analyzed yielded successful species-level identifications. Of these, 19 distinct bacterial species were identified across all sampling periods, with certain taxa appearing in multiple seasons. Isolates with identification scores ≥2.00 were reliably assigned at the species level, while those with scores in the 1.70-2.00 range were also confirmed at the species level based on the best match against the reference database, demonstrating the overall high identification performance of MALDI-TOF MS for environmental isolates.

The identified bacterial community exhibited distinct seasonal variation across sampling periods. In spring, six bacterial species were identified, including *Brevundimonas diminuta*, *Enterobacter hormaechei*, *Citrobacter freundii*, *Pseudomonas monteilii*, *Stenotrophomonas maltophilia* and *P. putida*. Summer samples exhibited comparatively lower diversity, with only two species identified, namely *Comamonas aquatica* and *P. putida*. The reduced species richness observed in summer may reflect the influence of seasonal physicochemical conditions, such as elevated temperature and altered mineral concentrations, which may limit the growth of certain bacterial groups. In contrast, autumn samples exhibited the highest diversity, with ten bacterial species detected, including *Shewanella putrefaciens*, *P. monteilii*, *P. putida*, *Lysinibacillus sphaericus*, *Aeromonas salmonicida*, *A. veronii*, *Acinetobacter johnsonii*, *Bacillus pumilus*, *P. azotoformans* and *B. cereus*. Winter samples also revealed relatively high diversity, with nine bacterial species identified, including *P. monteilii*, *P. putida*, *A. enteropelogenes* (syn. *A. trota)*, *S. putrefaciens*, *A. caviae*, *Kosakonia cowanii*, *P. azotoformans*, *P. veronii* and *A. johnsonii*.

Certain taxa demonstrated consistent ecological persistence across multiple seasons. *P. putida* was the only species detected in all four seasons, indicating strong adaptability to the varying physicochemical conditions of the Burguç mineral spring mud system. *P. monteilii* was identified in spring, autumn, and winter, while *S. putrefaciens*, *A. johnsonii* and *P. azotoformans* were detected in both autumn and winter samples, suggesting seasonal adaptation patterns associated with cooler environmental conditions.

MALDI-TOF MS identification scores ranged from 1.720 to 2.420 across the 27 representative isolates listed in Table 1. Of these, 22 isolates yielded identification scores ≥2.00, indicating reliable species-level identification, while 5 isolates showed scores between 1.70 and 2.00, corresponding to genus-level identification. The highest identification score was recorded for *Pseudomonas monteilii* (score: 2.420, winter) and *Acinetobacter johnsonii* (score: 2.420, winter), while the lowest score was observed for *P. azotoformans* (score: 1.720, autumn). Identification scores and corresponding spectral (m/z) ranges of isolates across seasons are presented in Table 1. Representative MALDI-TOF MS spectra of selected isolates reflecting seasonal diversity are presented in Figure 2. Alpha-diversity indices calculated from the seasonal colony-abundance data are summarized in Table 2, and the seasonal pattern of bacterial diversity is visualized in Figure 3.

Table 1. MALDI-TOF MS identification scores, colony counts, and corresponding spectral (m/z) ranges of bacterial isolates obtained from the balneological mud of the Burguç mineral spring across four seasons.

Season	Isolat	Species	Colon	MALDI-TOF MS	m/z Range

	e No.		y No.	Score	
Spring	1	*Brevundimonas diminuta*	5	2.137	2552.865–18558.858
	2	*Enterobacter hormaechei*	7	2.104	3123.667–9513.689
	3	*Citrobacter freundii*	6	2.351	2615.360–11742.566
	4	*Pseudomonas monteilii*	9	2.015	2564.808–10611.249
	5	*Stenotrophomonas maltophilia*	5	1.786	2775.091–18744.678
	6	*P. putida*	11	2.193	3748.144–11366.136
Summer	7	*P. putida*	12	2.199	2566.509–13112.805
	8	*Comamonas aquatica*	10	2.297	2918.682–12807.996
Autumn	9	*Shewanella putrefaciens*	5	2.140	2512.920–9560.200
	10	*P. monteilii*	7	2.410	2570.740–7581.890
	11	*P. putida*	9	1.880	2563.900–9798.980
	12	*Lysinibacillus sphaericus*	8	2.340	2698.940–8624.630
	13	*Aeromonas salmonicida*	10	2.400	2526.010–9399.550
	14	*A. veronii*	11	2.260	2526.220–6302.780
	15	*Acinetobacter johnsonii*	6	1.790	2589.170–7380.800
	16	*Bacillus pumilus*	6	1.730	2643.270–7409.640
	17	*P. azotoformans*	6	1.720	2562.640–7232.010
	18	*Bacillus cereus*	6	2.400	2168.530–7366.650
Winter	19	*P. monteilii*	7	2.420	2568.97–7211.85
	20	*P. putida*	9	2.240	2569.30–7617.93
	21	*A. enteropelogenes**	5	2.300	2525.05–9379.49
	22	*S. putrefaciens*	5	2.060	2510.79–7330.40
	23	*A. caviae*	10	2.200	2524.97–7329.31
	24	*Kosakonia cowanii*	6	2.030	2103.69–7155.81
	25	*P. azotoformans*	4	2.140	2217.14–7233.90
	26	*P. veronii*	9	2.160	2217.33–7233.92
	27	*A. johnsonii*	6	2.420	2587.37–9216.07

**A. enteropelogenes (*syn. *A. trota)*

Table 2. Alpha-diversity indices of bacterial communities recovered from the balneological mud of the Burguç mineral spring across four seasons. N, total number of colonies; S, species richness; H′, Shannon diversity index; D, Simpson dominance index; 1/D, inverse Simpson index; J, Pielou's evenness; DMg, Margalef's richness index.

Season	N	S	H′	D	1/D	J	D_Mg
Spring	43	6	1.75	0.18	5.49	0.97	1.33
Summer	22	2	0.69	0.50	1.98	0.99	0.32
Autumn	74	10	2.27	0.11	9.38	0.99	2.09
Winter	61	9	2.15	0.12	8.29	0.98	1.95

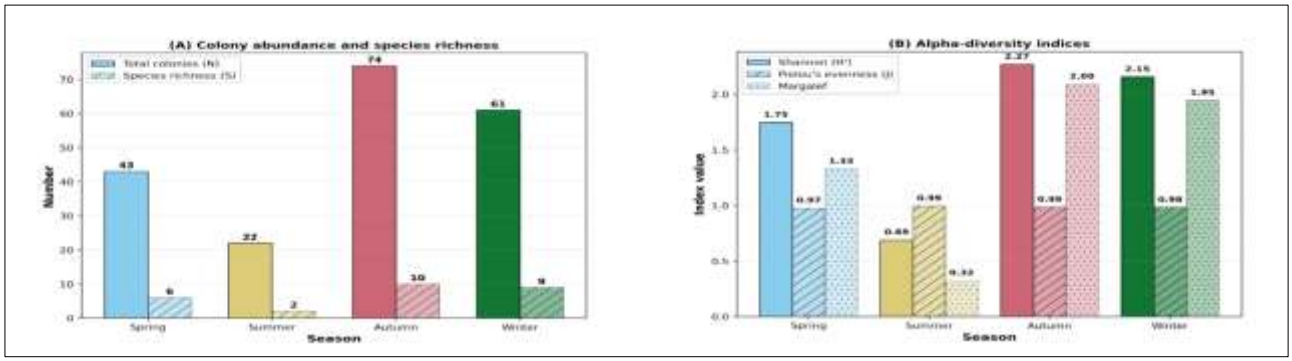

Figure 3. Seasonal bacterial diversity in the balneological mud of the Burguç mineral spring. (A) Total colony abundance (N) and species richness (S) per season; (B) alpha-diversity indices: Shannon (H′), Pielou's evenness (J), and Margalef's richness index. The figure illustrates the autumn maximum and summer minimum in microbial diversity.

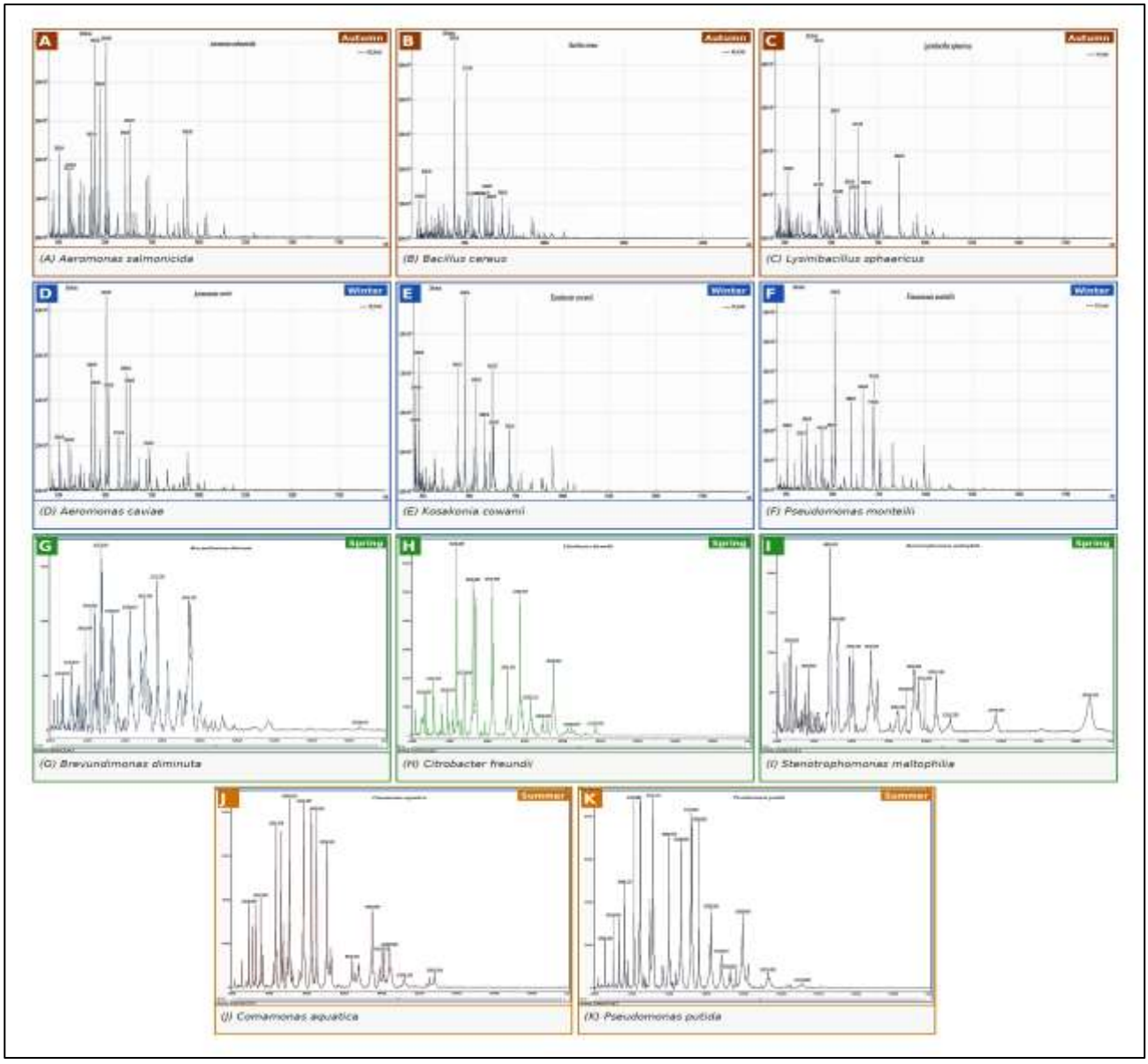

Figure 2. Representative MALDI-TOF MS spectra of bacterial isolates identified from the balneological mud of the Burguç mineral spring across four seasons. (A) *Aeromonas salmonicida*, Autumn; (B) *Bacillus cereus*, Autumn; (C) *Lysinibacillus sphaericus*, Autumn; (D) *Aeromonas caviae*, Winter; (E) *Kosakonia cowanii*, Winter; (F) *Pseudomonas monteilii*, Winter; (G) *Brevundimonas diminuta*, Spring; (H) *Citrobacter freundii*, Spring; (I) *Stenotrophomonas maltophilia*, Spring; (J) *Comamonas aquatica*, Summer; (K) *Pseudomonas putida*, Summer.

Discussion

In the present study, bacterial diversity in balneological mud samples from the Burguç mineral spring was systematically characterized using culture-based methods combined with MALDI-TOF MS analysis. A total of 19 bacterial species were identified, collectively representing a diverse and ecologically significant microbial community. The range of taxa recovered points to the microbial complexity of balneological mud environments and to the capacity of such systems to harbor functionally diverse bacterial populations.

Balneological mud systems constitute dynamic natural ecosystems shaped by physicochemical parameters such as temperature, pH, mineral composition, dissolved oxygen, and organic matter content. The Burguç mineral spring, characterized by its hypothermal nature and enrichment in magnesium, iron, sulfur, and phosphorus, provides a chemically distinctive substrate that supports a complex and functionally diverse microbial assemblage. These physicochemical conditions create selective ecological niches that favor certain bacterial taxa over others, and their interplay is likely a key determinant of the seasonal compositional patterns observed in this study.

Seasonal variation in bacterial diversity was evident throughout the sampling period, with autumn samples yielding the highest species richness (10 spp.) and summer samples the lowest (2 spp.). This pattern likely reflects the influence of seasonal fluctuations in environmental parameters such as temperature, precipitation,

and organic matter input, which are known to shape microbial community structure in sediment-associated systems (Costello & Chaudhary, 2017). The reduced diversity observed during summer may reflect summer reductions in moisture availability and organic matter input, which could restrict the growth of less tolerant bacterial groups. In contrast, the higher species richness detected in autumn may coincide with more moderate physicochemical conditions and increased nutrient availability, creating a more favorable environment for a broader range of bacterial taxa. Similar seasonal dynamics have been reported in thermally influenced aquatic and sedimentary systems, where transitional seasons tend to support greater microbial diversity (Ghilamicael et al., 2018).

The identified isolates were predominantly affiliated with the phyla Proteobacteria and Firmicutes, with a clear dominance of Gram-negative taxa, consistent with previous reports from natural and thermal aquatic systems (Ghilamicael et al., 2018). This dominance likely reflects metabolic flexibility, efficient nutrient utilization, and biofilm-forming capacity in sediment-associated environments.

Among the identified taxa, members of the genus *Pseudomonas* (*P. putida, P. monteilii, P. azotoformans* and *P. veronii*) were repeatedly detected across multiple seasons, indicating strong ecological persistence and adaptability. *Pseudomonas* spp. are well-documented for their metabolic versatility and adaptability across diverse aquatic systems (Duman et al., 2021; Chauhan et al., 2023). Among these, *P. monteilii*, originally isolated from clinical specimens (Elomari et al., 1997), has been increasingly detected in environmental matrices, confirming its dual ecological and clinical relevance. *P. putida*, despite being considered primarily environmental, has been documented in nosocomial settings (Fernández et al., 2015). The continuous detection of *P. putida* throughout all seasons suggests that this species constitutes a stable and dominant component of the Burguç microbiota. This observation is consistent with previous studies reporting the ability of *P. putida* to degrade a wide range of organic pollutants and adapt to environmental stress conditions (Nikel & de Lorenzo, 2018).

The detection of *Aeromonas* spp. (*A. caviae, A. salmonicida, A. veronii* and *A. enteropelogenes* (syn. *A. trota*) is of particular ecological and clinical importance. These bacteria are common inhabitants of aquatic environments and are recognized as opportunistic taxa affecting both fish and humans. The presence of *A. salmonicida*, a well-known fish pathogen, suggests that the spring environment may be directly influenced by aquatic fauna, including the potential presence of infected or carrier fish within the water system. This provides a plausible ecological explanation for the occurrence of fish-associated bacteria in the studied environment. In line with previous reports, *Aeromonas* spp. have been associated with gastrointestinal and soft tissue infections in humans (Chen et al., 2024). In particular, *A. veronii* was first characterized as a clinically relevant species capable of causing diarrhea and extraintestinal infections (Hickman-Brenner et al., 1987), while *A. caviae* and *A. enteropelogenes* (syn. *A. trota)* have similarly been implicated in human disease. The reliable identification of these species in environmental samples using MALDI-TOF MS, as also demonstrated by Benagli et al., (2012) for *Aeromonas* strains from clinical and environmental sources. Therefore, their detection also points to the potential need for microbiological monitoring of untreated natural water sources, particularly in environments used for therapeutic or recreational purposes; however, the present data, based on presence/absence detection without virulence-factor screening or quantitative load assessment, do not allow for direct quantification of public health risk. In addition to these dominant groups, several environmentally relevant bacteria were identified, including *Shewanella putrefaciens*, *Acinetobacter johnsonii*, *Comamonas aquatica*, *Lysinibacillus sphaericus*, *Bacillus pumilus*, *B. cereus*, *Brevundimonas diminuta*, *Citrobacter freundii* and *Enterobacter hormaechei*. These taxa represent functionally diverse groups with distinct ecological roles and varying levels of clinical significance. *S. putrefaciens* plays a key role in metal reduction processes in sediment-associated environments (Fredrickson et al., 2008) and has previously been reported as a rare but clinically significant bacterium capable of causing bacteremia and wound infections, particularly in immunocompromised individuals (Brink et al., 1995). Its environmental persistence and clinical relevance in aquatic systems have also been described in recent genomic and ecological studies (Müller et al., 2023). *A. johnsonii*, a member of the clinically important *Acinetobacter* genus widely recognized for its nosocomial significance and antimicrobial resistance potential (Bergogne-Bérézin and Towner, 1996), was detected in both autumn and winter samples, suggesting seasonal persistence in the balneological mud environment. *C. aquatica*, a Gram-negative betaproteobacterium commonly found in aquatic and soil habitats, has been increasingly recognized as an emerging opportunistic taxon of clinical relevance, with cases of bacteremia and septic shock in susceptible individuals. *B. diminuta*, detected in spring samples, is a non-fermenting Gram-negative rod widely distributed in water and soil environments (Willems et al., 2006) and considered an emerging opportunistic bacteria, particularly associated with nosocomial infections in immunocompromised patients (Ryan & Pembroke, 2018). *B. cereus*, while forming part of the environmentally ubiquitous spore-forming Firmicutes, is of notable concern due to its well documented toxin producing capacity and its association with food borne illness and opportunistic infections (Bottone, 2010), while *B. pumilus* and *L. sphaericus* represent additional spore-

forming taxa that exhibit broad resistance to environmental stress conditions (Nicholson et al., 2000). The detection of *C. freundii*, which has been reported to produce cytotoxins and to form aggregative biofilms and has been associated with bloodstream infections in clinical settings (Heljanko et al., 2023), along with *E. hormaechei*, both of which are commonly associated with fecal contamination, suggests possible external biological inputs into the balneological mud system, consistent with reports indicating their use as environmental contamination indicators (WHO, 2022). Finally, *Kosakonia cowanii*, first described as a novel genus reclassified from *Enterobacter* (Brady et al., 2013) and previously isolated from infant formula (Yang et al., 2018) and reported as a rare cause of clinical infection (Berinson et al., 2020), was identified in winter samples. While primarily regarded as an environmental and plant-associated bacterium (Merlino et al., 2025), its detection in the present study reveals the ecological breadth of microbial communities in mineral spring mud systems and warrants further attention given its emerging clinical relevance. Likewise, *S. maltophilia*, identified in spring samples, is a ubiquitous Gram-negative environmental bacterium that has emerged as an important multidrug-resistant nosocomial concern, commonly isolated from water bodies, soil and plant rhizospheres. Its intrinsic resistance to multiple antibiotic classes and its capacity to form biofilms in water-associated environments make its presence in therapeutic settings of particular concern (Brooke, 2021). The detection of *S. maltophilia* adds to the public health relevance of microbiological surveillance in environments used for therapeutic or recreational purposes.

The taxa identified in this study, including *Pseudomonas*, *Aeromonas*, *Stenotrophomonas*, *Acinetobacter*, *Brevundimonas*, *Shewanella*, *Bacillus* and *Enterobacter*, have also been detected and identified by MALDI-TOF MS in various aquatic and environmental matrices in previous studies, supporting the present findings. Popovic et al., (2022) demonstrated the effective use of MALDI-TOF MS for identifying *Aeromonas, Acinetobacter, Pseudomonas* and *Shewanella* from riverine water and sediment samples, reporting identification score distributions comparable to those obtained here. Puk et al., (2023) applied MALDI-TOF MS to agricultural water samples and successfully identified *P. azotoformans*, *P. veronii*, *A. salmonicida*, *Acinetobacter*, *B. diminuta*, *B. cereus*, *S. maltophilia* and *Lysinibacillus* among environmental isolates, genera and species that overlap substantially with the microbial community documented. Surányi et al., (2023) also confirmed that MALDI-TOF MS reliably identified *P. veronii*, *E. hormaechei*, *Brevundimonas* and *Acinetobacter* from irrigation water samples, with identification performance comparable to 16S rRNA gene sequencing. Together, these studies support the use of MALDI-TOF MS as a high-throughput identification tool for environmentally derived bacterial isolates and confirm the ecological significance of the microbial taxa reported here.

The present results demonstrate that MALDI-TOF MS is a rapid, cost effective, and reliable tool for the identification of environmental bacterial isolates. While this technique is widely used in clinical microbiology, its application in environmental microbiology remains relatively limited. Consistent with previous studies, MALDI-TOF MS enabled accurate identification of diverse bacterial taxa based on ribosomal protein profiles (Pascale et al., 2020). The use of MALDI-TOF MS for identification of environmental bacterial isolates from aquatic environment has been validated in previous studies (Benagli et al., 2012; Duman et al., 2021), supporting its applicability of the method in nonclinical contexts. The obtained identification scores (1.72-2.42) fall within accepted ranges for genus and species level identification (Puk et al., 2018; Surányi et al., 2023). Variations in score values may be associated with environmental strain diversity and limitations of the reference database. The use of formic acid extraction further improved identification performance, particularly for Gram-positive bacteria (Singhal et al., 2015), which confirms its potential as a practical and scalable tool for routine environmental monitoring. The spectral (m/z) ranges obtained in this study (2103.690-18744.678 m/z) are consistent with previously reported MALDI-TOF MS profiles, typically ranging between 2,000-21,000 Da (Çağatay, 2024). This agreement further confirms the accuracy of spectral data and the overall identification results.

Overall, the results indicate that the Burguç mineral spring harbours a diverse microbial community comprising environmental bacteria together with several taxa of potential clinical relevance. This ecological complexity supports the value of routine microbiological surveillance in systems actively used for therapeutic and recreational purposes, while also emphasizing that detection alone does not equate to active health risk.

Several limitations of this study should be acknowledged. First, the present study relies on culture-dependent isolation followed by MALDI-TOF MS identification using general-purpose, non-selective media, which inherently excludes viable but non-culturable (VBNC) microorganisms, slow-growing taxa, and strict anaerobes; culture-independent molecular approaches such as 16S rRNA amplicon sequencing or shotgun metagenomics would be expected to detect a substantially broader microbial spectrum and are recommended for future characterization of this site. Second, identification relied on a single platform (MALDI-TOF MS) without complementary 16S rRNA gene sequencing for cross-validation, and the diversity indices reported

here are based on 27 representative isolates selected from the successful MALDI identifications, which constrains finer-scale community-structure analyses.

Conclusion

This study provides the first culture-based microbiological characterization of bacterial diversity in the balneological mud of the Burguç mineral spring, a hypothermal mineral water system of growing relevance to health tourism. Using MALDI-TOF MS, 19 bacterial species were identified across four seasons, supported by quantitative alpha-diversity analysis (Shannon, Simpson, Pielou's evenness, and Margalef indices). The recovered community showed pronounced seasonal variation, with autumn yielding the highest diversity and summer the lowest, while *P. putida* was detected across all seasons, suggesting a stable core microbiome adapted to the physicochemical conditions of the spring.

Several taxa of potential clinical relevance, including *Aeromonas* spp., *S. maltophilia*, *B. cereus*, *C. freundii*, and *E. hormaechei*, were detected, supporting the value of routine microbiological surveillance in mineral spring environments used for therapeutic or recreational purposes. However, presence alone does not establish active public health risk, and quantitative virulence and antimicrobial-resistance assessments would be required for formal risk evaluation.

The novel contribution of this work lies in providing the first quantitative seasonal alpha-diversity baseline for the Burguç site, establishing a reference for future environmental and health-tourism monitoring. Two priority directions are recommended for follow-up studies: (i) integration of 16S rRNA amplicon sequencing or shotgun metagenomics to capture viable but non-culturable taxa, and (ii) coupling of microbiological monitoring with antimicrobial-resistance and virulence-factor profiling to enable risk-based assessment in health-tourism contexts.

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References

1. Abbas, R., Haris, M., Rahman, S. Shahid, S.A., Gul, T., Farooq, A., Ismail, A., Alotaibi, M.O., Gaber El-Saber Batiha, G., Ali, M. (2025). The hidden bacterial world of natural springs: Insight through MALDI-TOF mass spectrometry. *MicrobiologyOpen*, *14*(5), e70021. https://doi.org/10.1002/mbo3.70021
2. Alturkistani, H. A., Tashkandi, F. M., & Mohammedsaleh, Z. M. (2015). Histological stains: a literature review and case study. *Global Journal of Health Science*, *8*(3), 72-79. https://doi.org/10.5539/gjhs.v8n3p72
3. Ashfaq, M. Y., Da'na, D. A., & Al-Ghouti, M. A. (2022). Application of MALDI-TOF MS for identification of environmental bacteria: a review. *Journal of Environmental Management*, *305*, 114359. https://doi.org/10.1016/j.jenvman.2021.114359
4. Benagli, C., Demarta, A., Caminada, A. Ziegler, D., Petrini, O., & Tonolla, M. (2012). A rapid MALDI-TOF MS identification database at genospecies level for clinical and environmental *Aeromonas* strains. *PLoS ONE*, *7*(10), e48441. https://doi.org/10.1371/journal.pone.0048441
5. Bergogne-Bérézin, E., & Towner, K. J. (1996). *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clinical Microbiology Reviews*, *9*(2), 148-165. https://doi.org/10.1128/CMR.9.2.148

6. Berinson, B., Bellon, E., Christner, M., Both, A., Aepfelbacher, M., & Rohde, H. (2020). Identification of *Kosakonia cowanii* as a rare cause of acute cholecystitis: Case report and review of the literature. *BMC infectious diseases*, *20*(1), 366. https://doi.org/10.1186/s12879-020-05084-6
7. Bottone, E. J. (2010). *Bacillus cereus*, a volatile human pathogen. *Clinical Microbiology Reviews*, *23*(2), 382-398. https://doi.org/10.1128/CMR.00073-09
8. Brady, C., Cleenwerck, I., Venter, S., Coutinho, T., & De Vos, P. (2013). Taxonomic evaluation of the genus Enterobacter based on multilocus sequence analysis (MLSA): proposal to reclassify *E. nimipressuralis* and *E. amnigenus* into Lelliottia gen. nov. as *Lelliottia nimipressuralis* comb. nov. and *Lelliottia amnigena* comb. nov., respectively, *E. gergoviae* and *E. pyrinus* into *Pluralibacter* gen. nov. as *Pluralibacter gergoviae* comb. nov. and *Pluralibacter pyrinus* comb. nov., respectively, *E. cowanii, E. radicincitans, E. oryzae* and *E. arachidis* into *Kosakonia* gen. nov. as *Systematic and applied microbiology*, *36*(5), 309-319. https://doi.org/10.1016/j.syapm.2013.03.005
9. Brink, A. J., van Straten, A., & van Rensburg, A. J. (1995). *Shewanella putrefaciens* bacteremia. *Clinical Infectious Diseases*, *20*(5), 1327-1332. https://doi.org/10.1093/clinids/20.5.1327
10. Brooke, J. S. (2021). Advances in the microbiology of *Stenotrophomonas maltophilia*. *Clinical Microbiology Reviews*, *34*(3), 10-1128. https://doi.org/10.1128/cmr.00030-19
11. Chauhan, M., Kimothi, A., Sharma, A., & Pandey, A. (2023). Cold adapted *Pseudomonas*: ecology to biotechnology. *Frontiers in Microbiology*, *14,* 1218708. https://doi.org/10.3389/fmicb.2023.1218708
12. Chen, Y. H., Chen, P. L., Ko, W. C., & Lamy, B. (2024). Human infections caused by *Aeromonas* species: clinical features, pathogenic mechanisms and antimicrobial resistance. *Frontiers in Cellular and Infection Microbiology*, 14, 1298457. https://doi.org/10.3389/fcimb.2024.1298457
13. Costello, M. J., & Chaudhary, C. (2017). Marine biodiversity, biogeography, deep-sea gradients and conservation. *Current Biology*, *27*(11), 511-R527. https://doi.org/10.1016/j.cub.2017.04.060
14. Çağatay, İ. T. (2024). Use of proteomic-based MALDI-TOF mass spectra for identification of bacterial pathogens in aquaculture: a review. *Aquaculture International*, *32*(6), 7835-7871. https://doi.org/10.1007/s10499-024-01448-2
15. Duman, M., Mulet, M., Altun, S., Saticioglu, I. B., Ozdemir, B., Ajmi, N., Lalucat, J., & Garcia-Valdes, E. (2021). The diversity of *Pseudomonas* species isolated from fish farms in Turkey. *Aquaculture*, *535,* 736369. https://doi.org/10.1016/j.aquaculture.2021.736369
16. Elomari, M., Coroler, L., Verhille, S., Izard, D., & Leclerc, H. (1997). *Pseudomonas monteilii* sp. nov., isolated from clinical specimens. *International Journal of Systematic Bacteriology*, *47*(3), 846-852. https://doi.org/10.1099/00207713-47-3-846
17. Fernández, M., Porcel, M., de la Torre, J. Molina-Henares, M.A., Daddaoua, A., Llamas, M.A., Roca, A., Carriel V., Duque, E. (2015). Analysis of the pathogenic potential of nosocomial *Pseudomonas putida* strains. *Frontiers in Microbiology*, 6, 871. https://doi.org/10.3389/fmicb.2015.00871
18. Fredrickson, J. K., Romine, M. F., Beliaev, A. S. et al. (2008). Towards environmental systems biology of *Shewanella*. *Nature Reviews Microbiology*, *6,* 592-603. https://doi.org/10.1038/nrmicro1947
19. Ghilamicael, A. M., Boga, H. I., Anami, S. E., Mehari, T., & Budambula, N. L. (2018). Potential human pathogenic bacteria in five hot springs in Eritrea revealed by next generation sequencing. *PLoS ONE*, *13*(3), e0194554. https://doi.org/10.1371/journal.pone.0194554
20. Gianfaldoni, S. et al. (2017). History of the baths and thermal medicine. *Dermatologic Therapy*, 7, 1-11. https://doi.org/10.1007/s13555-017-0187-4
21. Gökdağ, K., & Çağatay, İ. T. (2025). Identification of *Vibrio parahaemolyticus* found on plastics via matrix-assisted laser desorption/ionization time of flight mass spectrometry. *Journal of Biometric Studies*, *5*(1), 36-44. https://doi.org/10.5281/jbs.2025
22. Gomes, C., Carretero, M. I., Pozo, M., Maraver, F., Cantista, P., Armijo, F., Legido, J. L., Teixeira, F., Rautureau, M., & Delgado, R. (2013). Peloids and pelotherapy: historical evolution, classification and glossary. *Applied Clay Science*, *75-76*, 28-38. https://doi.org/10.1016/j.clay.2013.02.008
23. Green, M. R., & Sambrook, J. (2019). PCR amplification of DNA. *Cold Spring Harbor Protocols*, *7*, pdb.prot095125. https://doi.org/10.1101/pdb.prot095125
24. Hall, J. R., Mitchell, K. R., Jackson-Weaver, O., Kooser, A. S., Cron, B. R., Crossey, L. J., & Takacs-Vesbach, C. D. (2008). Molecular characterization of the diversity and distribution of a thermal spring microbial community by using rRNA and metabolic genes. *Applied and Environmental Microbiology*, *74*, 4910-4922. https://doi.org/10.1128/AEM.00233-08
25. Heljanko, T., Rantala, M., Kantele, A., & Lyytikäinen, O. (2023). Trends in bloodstream infections caused by *Citrobacter* species in a tertiary care hospital. *Infection*, *51*(2), 361-369. https://doi.org/10.1007/s15010-022-01876-3

26. Hickman-Brenner, F. W., MacDonald, K. L., Steigerwalt, A. G., Fanning, G. R., Brenner, D. J., & Farmer 3rd, J. J. (1987). *Aeromonas veronii*, a new ornithine decarboxylase-positive species that may cause diarrhea. *Journal of Clinical Microbiology*, *25*(5), 900-906. https://doi.org/10.1128/jcm.25.5.900-906.1987
27. Merlino, G., Rossi, F., Balestrazzi, A., & Biondi, E. (2025). Environmental and plant-associated lifestyles of *Kosakonia cowanii*. *Environmental Microbiology Reports*, *17*(2), e1035. https://doi.org/10.1111/1758-2229.13035
28. Müller, A. L., Kjeldsen, K. U., Rattei, T., & Loy, A. (2023). Environmental adaptation and pathogenic potential of the genus *Shewanella*. *Environmental Microbiology*, *25*(2), 512-526. https://doi.org/10.1111/1462-2920.16313
29. Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., & Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology Reviews*, *64*(3), 548-572. https://doi.org/10.1128/MMBR.64.3.548-572.2000
30. Nikel, P. I., & de Lorenzo, V. (2018). *Pseudomonas putida* as a functional chassis for industrial biocatalysis: from native biochemistry to trans-metabolism. *Metabolic Engineering*, *50,* 142-155. https://doi.org/10.1016/j.ymben.2018.05.005
31. Pascale, M. R., Mazzotta, M., Salaris, S., Girolaminin, L., Grotola, A., Simone, M.L.,Cordovana, m., Bisognin,f., Cristino, S. (2020). Evaluation of MALDI-TOF mass spectrometry in diagnostic and environmental surveillance of *Legionella* species: a comparison with culture and mip-gene sequencing. *Frontiers in Microbiology*, *11,* 589369. https://doi.org/10.3389/fmicb.2020.589369
32. Piamsomboon, P., Pornsukarom, S., & Srisuk, N. (2020). Identification of bacteria by MALDI-TOF mass spectrometry: principles and applications. *Journal of Microbiological Methods*, *173,* 105944. https://doi.org/10.1016/j.mimet.2020.105944
33. Popović, N. T., Kazazić, S. P., Strunjak-Perović, I., & Čož-Rakovac, R. (2022). Identification of environmental aquatic bacteria by mass spectrometry supported by biochemical differentiation. *PLoS ONE*, *17*(6), e0269423. https://doi.org/10.1371/journal.pone.0269423
34. Puk, K., Banach, T., Wawrzyniak, A., Adaszek, Ł., Zietek, J., Winiarczyk, S., & Guz, L. (2018). Detection of *Mycobacterium marinum*, *M. peregrinum*, *M. fortuitum* and *M. abscessus* in aquarium fish. *Journal of Fish Diseases*, *41*(1), 153–156. https://doi.org/10.1111/jfd.12686
35. Surányi, B. B., Taczman-Brückner, A., Mohácsi-Farkas, C., & Engelhardt, T. (2023). Rapid identification of bacteria from agricultural environment using MALDI-TOF MS. *Acta Alimentaria*, *52*(1), 113-125. https://doi.org/10.1556/066.2022.00202
36. Quattrini, S., Pampaloni, B., & Brandi, M. L. (2017). Natural mineral waters: chemical characteristics and health effects. *Clinical Cases in Mineral and Bone Metabolism*, *14*(2), 173-180. https://doi.org/10.11138/ccmbm/2017.14.1.173
37. Ryan, M. P., & Pembroke, J. T. (2018). *Brevundimonas* spp: emerging global opportunistic pathogens. *Virulence*, *9*(1), 480-493. https://doi.org/10.1080/21505594.2017.1419116
38. Singhal, N., Kumar, M., Kanaujia, P. K., & Virdi, J. S. (2015). MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Frontiers in Microbiology*, *6,* 791. https://doi.org/10.3389/fmicb.2015.00791
39. Surányi, B. B., Zwirzitz, B., Mohácsi-Farkas, C., Engelhardt, T., & Domig, K. J. (2023). Comparing the efficacy of MALDI-TOF MS and sequencing-based identification techniques (Sanger and NGS) to monitor the microbial community of irrigation water. *Microorganisms*, *11*(2), 287. https://doi.org/10.3390/microorganisms11020287
40. Vogel, B. F., Venkateswaran, K., Satomi, M., & Gram, L. (1997). Identification of *Shewanella putrefaciens* from spoilage of marine fish. *Applied and Environmental Microbiology*, *63*(9), 3708-3714. https://doi.org/10.1128/aem.63.10.3708-3714.1997
41. WHO (World Health Organization). (2022). *Fluoride in Drinking-Water: Background Document for Development of WHO Guidelines for Drinking-Water Quality*. World Health Organization. Geneva.
42. Willems, A., Gillis, M., & De Vos, P. (2006). Phenotypic and phylogenetic characterization of *Brevundimonas* species. *International Journal of Systematic and Evolutionary Microbiology*, 56, 1481-1491. https://doi.org/10.1099/ijs.0.63817-0
43. Yang, J. E., Kim, J. O., Park, Y. J., & Chang, H. C. (2018). Occurrence and characterization of *Kosakonia cowanii* isolated from powdered infant formula. *Food Microbiology*, *75,* 1-7. https://doi.org/10.1016/j.fm.2018.02.012