Extremophilic microorganisms from natural and artificial environments: Bioprospecting and Biotechnological Applications

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‘Everything is everywhere, but, the environment selects’

Lourens G. M. Baas Becking

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Declaration

El Dr. Manuel Porcar Miralles, Investigador Contratado Doctor de la Universidad de Valencia, director del Laboratorio Biotecnología y Biología Sintética, y El Dr. Agustín Aranda Fernández, Científico Titular del CSIC y la Universidad de Valencia, director del Laboratorio Biotecnología de Levaduras Industriales,

INFORMAN QUE:

La presente memoria titulada 'Extremophilic microorganisms from natural and artificial environments: Bioprospecting and Biotechnological Applications' ha sido realizada bajo su supervisión por Leila Satari Faghihi, en el Instituto de Biología de Sistemas Integrativa, y que reúne los requisitos necesarios para ser defendida como tesis doctoral y optar al grado de Doctora por la Universidad de Valencia.

Y para que así conste a los efectos oportunos, se firma el presente en Paterna, 31 marzo de 2022.

Dr. Manuel Porcar Miralles

Dr. Agustín Aranda Fernández

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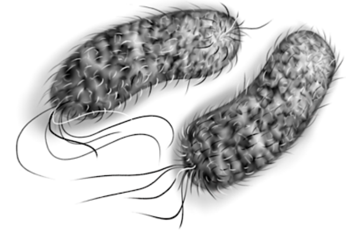
Introduction



Earth is home of a large biodiversity of microorganisms, which has recently predicted to be as high as 10^{12} or more microbial taxa inhabiting our planet (Lennon and Locey, 2020). For decades, natural environments have been explored to study their microbial communities (Gupta *et al.*, 2017), as well as the specific adaptation mechanisms of the species that compose them and their potential biotechnological applications. A clear example is the study of soil microorganisms able of producing molecules with plant growth promoting or antimicrobial activities (Chandra and Kumar, 2017; Ramakrishna *et al.*, 2019). Not only natural, but also artificial environments hold interest as sources of microbial diversity. These environments include solar salterns, wastewaters and, more recently, also nuclear waste (Christofi and Philp, 2018; Lee *et al.*, 2021; Sharma *et al.*, 2021). Some organisms can adapt to the new and constant changes that led in some cases to the development of local extreme conditions (Klempay *et al.*, 2021). Besides natural and semi-artificial environments, lots of artificial products were built in the last century due to modernization, most of them made of synthetic materials such as plastic polymers, glasses, and metals. Those products are generally very persistent in the environment and cannot be biodegradable by most microorganisms (Yang *et al.*, 2020). However, those microbial populations thrive in these kinds of waste have gotten more industrial attention during the last few years, as several studies have shown that specific microbial populations can colonize the surface of those products (Bahl *et al.*, 2021; Weaver *et al.*, 2021) and, in some cases, gradually modify and decompose those materials (Lekbach *et al.*, 2021; Weaver *et al.*, 2021), which opens up their potential in bioremediation, which is the biotechnological removal of pollutants. An interesting example is the biodegradation of plastics, composed mainly of synthetic polymers, in which many environmental microorganisms are able to degrade to a certain extent various types of plastics such as polypropiolactone (PPL), polycaprolactone (PCL), Polybutylene succinate (PBS), polyehtylene (PE), and polyvinyl chloride (PVC) (Bahl *et al.*, 2021).

Microbiome of the artificial products: Not only natural substrates are home of a myriad of microbial species. Man-made environments, including highly anthropized machines and locations such as elevators (Richardson *et al.*, 2019), the underground subways (Hernández *et al.*, 2020; Klimenko *et al.*, 2020), and small electronic devices (Hartmann *et al.*, 2004; Nepomuceno *et al.*, 2018) are also rich microbial niches. In this introduction, however, we will focus on man-made appliances, machines, and devices with their own associated microbiome, rather than large structures or “indoor” environments. That is, we will describe the microbial profile of “things”, which despite being in constant contact with humans or the human activity, do not necessarily share a microbiome characterized by human-associated microorganisms but, instead, represent micro-niches with their own selective pressures and

characteristic microbiomes. Here, we look briefly into the previous research on the natural microbiomes of artificial environments describing model examples such as photovoltaic panels, artificial devices with a standard design and operation procedure, in which the selective pressures they are under results in the development of a microbiome that does not significantly change within geographical or climatic variations (Tanner *et al.*, 2018; Moura *et al.*, 2021). Another example is the gasoline car tank lid (Vidal-Verdú *et al.*, accepted by npj Biofilms and Microbiomes) and pump microbiomes (Isola *et al.*, 2021) or the microbiome of automobile air-conditioning systems (Park Chulwoo *et al.*, 2019), which are colonized by very specialized microbial communities.

Microbial diversity of sun-exposed/UV irradiated artificial surfaces: Although almost all surfaces harbour microbial communities, their development can be influenced by several factors such as the surface composition, design, and geographical location (Elbourne *et al.*, 2019). Among all those, outdoor surfaces can be considered as microhabitats for the colonization of air-born and/or environmental microorganisms. Indeed, the microbial composition of artificial sun-exposed surfaces such as solar panels is especially interesting due to thermal fluctuations, ultraviolet (UV) irradiation, desiccation, and low availability of nutritional elements.

Photovoltaic panels: Solar panels are smooth, glass or glass-like, sun-exposed surfaces with a minimum water retention capacity. Microorganisms inhabiting them have developed specific adaptation mechanisms, for instance the production of adhesins for attachment, and the production of antioxidants such as pigments, enzymes, and proteins to survive irradiation, desiccation, low nutrient availability and heat. Therefore, only a small fraction of environmental air-born microorganisms can thrive on this surface. To date, several studies have shown that most culturable bacteria isolated from photovoltaic panels can produce a wide range of carotenoids to tolerate radiation. In addition, the presence of sphingolipids such as glycosphingolipids in some species (*Sphingomonas* spp.) was described as a facilitator of the bacterial attachment to polyamide and silica (Gutman *et al.*, 2014). Furthermore, protein analysis of the microbial communities inhabiting solar panels revealed metabolically active communities, characterized by the presence of proteins involved in biofilm formation and stress-response mechanisms (Dorado-Morales *et al.*, 2016). Regardless of the geographical location and climate differences in different locations of the world, there is a shared microbial community inhabiting the surface of solar panels dominated by *Hymenobacter* spp., *Sphingomonas* spp. and *Deinococcus* spp. (Dorado-Morales *et al.*, 2016; Porcar *et al.*, 2018; Tanner *et al.*, 2018), and to a lesser extent, *Methylobacterium* spp. (Moura *et al.*, 2021), *Roseomonas* spp. (Tanner, Molina-Menor, *et al.*, 2020) and *Novosphingobium* spp. (Dorado-Morales *et al.*, 2016). Members from the genus *Sphingomonas* are among the most abundant strains inhabiting sun-exposed surfaces such as solar panels (Tanner, Mancuso, *et al.*, 2020), and have a crucial role in the early steps of microbial colonization and biofilm formation (Romani *et al.*, 2019). Moreover, the antioxidant-related metabolisms of members of the genera *Hymenobacter* and *Methylobacterium* suggest that these microorganisms can have promising applications in the food and pharmaceutical industries (Moura *et al.*, 2021). The photovoltaic panel microbiome is very likely shaped from the strong selection pressure in the shape of UV irradiation; besides, other factors such as the limitation of nutrients, desiccation, and drastic temperature changes also play a major role in selecting for specific resident microbial taxa (Tanner *et al.*, 2020).

Spacecraft stations: Spacecraft facilities are considered extreme environments due to the desiccation, high temperature, and low-nutrient and -aeration conditions. For many years, the microbial sterilization of spacecrafts prior launching has been one of NASA indispensable tasks to avoid contaminating space vehicles with terrestrial microorganisms (Venkateswaran *et al.*, 2001). Previous research revealed that the most frequent taxa on the surface of space vehicles was *Bacillus* sp. based

on culture-based approaches and followed by less frequent genera such as *Kocuria* sp., *Staphylococcus* sp., and *Micrococcus* sp. Interestingly, most of those species exhibited intense growth at 60 °C and 10 % (w/v) NaCl (Venkateswaran *et al.*, 2001). This research showed that most of the culturable Gram-positive bacteria such as *Bacillus* sp. can tolerate desiccation by sporogenesis (Bressuire-Isoard *et al.*, 2018). On the other hand, through culture-independent RFLP analysis, the presence of many Gram-negative bacteria with approximately equal frequency to Gram-positive bacteria were confirmed (Venkateswaran *et al.*, 2001). With the emergence of NGS analysis, 16S rRNA gene sequencing of spacecraft facilities showed that samples from the International Space Station (ISS) were dominated by *Actinobacteria*, *Bacilli*, and *Clostridia*, while *Alphaproteobacteria* and *Gammaproteobacteria* were more frequent in the Jet Propulsion Laboratory (JPL) (Checinska *et al.*, 2015). The ISS environmental microbiome was also monitored to study the diversity of viable bacteria and fungi over a period of 14 months which revealed the temporal and spatial distribution of the ISS microbiome. Regardless of the routine microbial monitoring of the spacecraft assembly cleanrooms, where 1-10 % of viable microorganisms were cultured, approximately 46 % and 40 % of bacteria and fungi were cultured from other installations at the ISS. Among all, some human-associated microbiome, especially opportunistic pathogenic microorganisms, were identified. Beside the high abundance of human-associated bacteria from the family *Enterobacteriaceae,* species from the genera *Methylobacterium*, *Sphingomonas*, *Bacillus*, *Penicillium*, and *Aspergillus* were also identified on the ISS (Sielaff *et al.*, 2019). *Moraxallaceae* species present on the ISS facilities were also detected in spacecraft assembly cleanrooms (Bashir *et al.*, 2016; Sielaff *et al.*, 2019). Moreover, another microbial tracking experiment on the ISS revealed that some of the *Methylobacterium* species can survive unique selective pressures of the ISS such as microgravity. As *Methylobacterium* species are generally well known as plant symbionts, this finding opens up a possible biotechnological application of these bacteria for plant growth promotion in the space stations (Bijlani *et al.*, 2021). In summary, spacecraft stations harbour a fraction of environmental microorganisms, consisting of the genera *Bacillus*, *Methylobacterium*, *Sphingomonas*, which have several adaptation mechanisms to tolerate poly-extreme conditions of the spacecraft facilities.

Microbiome of the electronic interior devices: Artificial electronic devices such as smartphones or elevator buttons are inhabited by both human-associated microbiome and environmental microorganisms (Richardson *et al.*, 2019; Olsen *et al.*, 2021). Previous work has demonstrated that the microbial exchange between the human skin and a built environment can result in the formation of an independent microbial community after a while (Lax, Simon *et al.*, 2014). The stability of microbial communities is not only influenced by its size, but rather by the physical nature of the environment where they develop and abiotic factors such as temperature and nutrient availability (Bottos *et al.*, 2020). Although most built environments have received attention for being colonized by human-associated microorganisms that may have an impact on public health, some extremely environmental microorganisms co-habit on the surface of those artificial devices (Fujiyoshi *et al.*, 2017). On the other hand, electronic devices with particular selection pressures tend to display unique microbial communities.

Coffee-machine: A one-year study of the microbiome of ten coffee-machines based on the 16S rRNA gene amplicon analysis showed that their microbial profiles were similar in all the coffee-machines. Although there was a significant variation among samples, *Enterococcus* and *Pseudomonas* were the most frequent shared taxa, followed by other abundant genera such as *Stenotrophomonas, Sphingobacterium,* *Acinetobacter*, *Coprococcus, Paenibacillus* or *Agrobacterium,* which were also detected to a lesser extent. The colonisation process of the wasted coffee leach was also monitored during two months by Vilanova *et al.* (2015). The 16S rRNA gene

sequencing revealed that during the first two weeks, *Pantoea* sp., *Cloacomonas* sp. and *Brevundimonas* sp. were the most frequent taxa, whereas those taxa were substituted by species from genera *Pseudomonas, Acinetobacter* and *Sphingobium* later. Species of the genera *Pseudomonas* and *Enterococcus* dominated the bacterial community after two months. These results suggested that abiotic conditions such as drastic temperature and nutrient availability changes as well as the accumulation of caffeine may be the key factors shaping the final microbial community (Vilanova *et al.*, 2015).

Dishwasher: Recent research has studied the microbial colonization of dishwashers, another man-manufactured device subjected to extreme selective pressures (Zalar *et al.*, 2011; Zupančič *et al.*, 2018). Previous research revealed that dishwashers can be considered poly-extreme habitats for microorganisms due to thermal stress, high salt concentrations, presence of detergents, pH variations, and constant water pressure (Raghupathi *et al.*, 2018). The microbial communities that colonize these devices form biofilms not only on the surface of rubber seals, but also on the entire interior surfaces (Zupančič *et al.*, 2019). Although for sanitary reasons most efforts have focused on monitoring the presence of opportunistic microorganisms in dishwashers (Zalar *et al.*, 2011; Döğen *et al.*, 2013; Gümral *et al.*, 2016; Zupančič *et al.*, 2016), a small but important fraction of the dishwasher microbiome consists of environmental microorganisms such as *Gordonia* spp., *Micrococcus* spp., *Chryseobacterium* spp., *Exiguobacterium* spp. and *Meiothermu*s spp. (Raghupathi *et al.*, 2018). These microorganisms were previously reported as halotolerant, heavy metal tolerant, UV resistant and thermotolerant microorganisms (Kalyani *et al.*, 2016; Yolmeh *et al.*, 2017; Gurbanov *et al.*, 2019; Pandey, 2020). Moreover, besides opportunistic fungi, some human-associated and opportunistic bacteria such as *Staphylococcus* sp., *Streptococcus* sp., *Lactobacillus* sp., *Corynebacterium* sp., *Enterococcus* sp., *Acinetobacter* sp., *Escherichia*/*Shigella* sp., and *Pseudomonas* sp. have been vastly detected in the dishwasher biofilms (Raghupathi *et al.*, 2018; Zupančič *et al.*, 2019). Although fungi from the cluster *Ascomycota* followed by *Basidiomycota* tend to be the first colonizers of the rubber surfaces. The second microbial waves can be filtered by the first occupants, thus, this may explain the difference of the microbial composition of each dishwasher (Raghupathi *et al.*, 2018). Previous research showed that the fungal communities can also be affected by the high pressure of the pumping system water (Zupančič *et al.*, 2019). For the formation of biofilms, one essential factor is the microbial Extracellular Polymeric Substances (EPSs), responsible for assisting individual cell immobilization, accelerating cell to cell interactions, and finally, synergizing the formation of the microbial consortia (Flemming and Wingender, 2010). Dishwasher microorganisms such as *Gordonia* sp., *Micrococcus* sp., *Chryseobacterium* sp., *Exiguobacterium* sp., *Acinetobacter* sp. have been previously reported as EPSs producers (Kılıç and Dönmez, 2008; Fusconi *et al.*, 2010; Pandey, 2020; Hu *et al.*, 2022; Idris and Nadzir, 2022).

Washing machines: Another artificial device that has attracted the interest of microbial ecologists is washing machines, in which biofilm formation and microbial survival in the interior and exterior surfaces has been studied (Babič *et al.*, 2015; Nix *et al.*, 2015). Similar to the extreme selective conditions of the dishwashers, the washing machine microbiota tolerates a diverse range of temperatures, pHs, and detergent concentrations. Previous research revealed that the microbial community of a washing machine significantly depends on the site of sampling and the number of high temperature wash cycles per month (Jacksch *et al.*, 2020). For example, the composition of the microbial communities in the detergent drawer is different to that of the tub due to the enzymatic effect that concentrated detergents have on some microorganisms. Species from the genera *Pseudomonas*, *Acinetobacter*, and *Enhydrobacter* dominate the washing machine microbial communities regardless of the sampling site (Jacksch *et al.*, 2020). This study also revealed that human pathogens had low frequency in the washing machine microbiome.

Another work by Callewaert *et al*. (2015) corroborated that the presence of some pathogens such as *Leptospira* and *Legionella* is less than 1 % of the total microbial community (Callewaert *et al.*, 2015). The presence of members from the *Moraxellaceae* family was also detected, and previous studies demonstrated that bacteria such as *Moraxella* sp. cause the malodour of clothes after laundry (Kubota *et al.*, 2012; Bockmühl *et al.*, 2019).

Water heating systems: In the early 1970s, Brock and Boylen revealed that some environmental extremophiles can thrive in the extreme conditions of the water heating (WH) systems (Brock and Boylen, 1973). *Thermus aquaticus* was isolated from hot-water tanks after being found in natural hot springs. Interestingly, the *T. aquaticus* strains isolated from hot-water heaters did not show the pigmentation of the strains isolated from natural environments (Brock and Boylen, 1973), which these differences might explain the role of pigment _especially carotenoids_ production for quenching of singlet oxygen in intense sun-exposed irradiated environments (Ram *et al*., 2020). Later, Kjellerup *et al*. (2005) studied biofilm formation in WHs and demonstrated that the parameters playing a key role in the development of microbial biofilms were pH, conductivity and oxygen concentration (Kjellerup *et al.*, 2005). The result of FISH analysis revealed that *Betaproteobacteria* was the most abundant bacteria followed by sulfate-reducing bacteria (SRB), *Alphaproteobacteria*, and to a lesser extent, *Gammaproteobacteria*. At the genus level, the presence of *Acidovorax* sp., *Agrobacterium* sp., *Roseococcus* sp., *Flavobacterium* sp., and to a lesser extent, *Sphingomonas* sp. were monitored in the heating system biofilm by DGGE analysis (Kjellerup *et al.*, 2005).

Saunas: Saunas reach temperatures of approximately 75 to 80 °C. The human-associated bacteria cannot thrive in such a hot environment, however, some spore-forming microorganisms tolerate harsh and unfavourable conditions and can survive very high temperatures, even in the presence of detergent and disinfectants (Lee and Park, 2012). Previous research by Lee *et al*. showed that spore-forming bacteria such as *Bacillus* sp., *Virgibacillus* sp., and non-spore-forming thermophilic bacteria like *Tepidomonas* sp., *Pseudoxanthomonas* sp. as well as non-thermophilic bacteria belonging to the genera *Stenotrophomonas* and *Janthinobacterium* were detected in saunas by TGGE (Lee and Park, 2012). Kim *et al*. (2013) studied the bacterial diversity of two dry saunas that were operated at lower and higher temperatures (64 °C and 76 °C, respectively). Members of the genera *Moraxella* and *Acinetobacter* were detected in the low temperature sauna, whereas strains belonging the genera *Aquaspirillum*, *Chromobacterium*, *Aquabacterium*, *Gulbenkiania*, *Pelomonas*, and *Aquitalea* dominated the high temperature sauna. Thermophilic strains such as *Bacillus megaterium* and *Deinococcus geothermalis* were also found in both low and high temperature saunas (Kim *et al.*, 2013). In another report, Tanner *et al*. (2017) reported a similar taxonomic distribution in another sauna sample characterised by a high abundance of *Alpha*-, *Beta*- and *Gammaproteobacteria*, to lesser extent, *Bacteroidetes*, *Actinobacteria* and *Acidobacteria*. Scanning Electron Microscopy (SEM) of the sample showed a dense microbial biofilm within a smooth matrix of EPSs. By culture-dependent techniques, a moderate number of bacteria with thermophilic lipolytic activity were isolated in this study that shed light on the biotechnological potential of the extremophiles selected by man-made interior devices (Tanner *et al.*, 2017).

Air conditioning cooling systems: Air conditioning (AC) systems are another type of indoor devices that harbour microbial communities (Schmidt *et al.*, 2012; Bakker *et al.*, 2018). In an AC system, the warm and humid air passes through the ACs cooling coil and blows out cold air. Besides this process, the condensed water generated as output accumulates on the coil surfaces and is a habitat for microorganisms (Acerbi *et al.*, 2017). Research by Acerbi *et al*. (2017) showed that the microbial populations from the air and the condensed coil water were significantly different, although *Agaricomycetes* was the most abundant taxa in both.

Table 1. The presence of microorganisms in different man-made devices with particular selective pressures such as thermal stress, pH variations, Desiccation, UV-irradiation etc.

Artificial devices	Poly-extreme Condition of the Device	Frequent Microorganisms	References
Solar panels	UV-irradiation Desiccation Low nutrition	**Bacteria:** *Hymenobacter* sp. *Sphingomonas* sp. *Deinococcus* sp. *Methylobacterium* sp. *Roseomonas* sp. *Novosphingobium* sp.	(Dorado-Morales *et al*., 2016) (Porcar *et al*., 2018) (Tanner *et al*., 2018, 2020) (Moura *et al*., 2021)
Spacecraft facilities	Desiccation High temperature Low nutrient Low aeration Microgravity	**Fungi:** *Penicillium* sp. *Aspergillus* sp. **Bacteria:** *Bacillus* sp. *Kocuria* sp. *Staphylococcus* sp. *Micrococcus* sp. *Methylobacterium* sp. *Sphingomonas* sp. ***Moraxallaceae* family**	(Venkateswaran *et al*., 2001) (Bashir *et al*., 2016) (Sielaff *et al*., 2019) (Bijlani *et al*., 2021)
Coffee-machines	Thermal stress Water pressure Particular nutrient availability	**Bacteria:** *Enterococcus* sp. *Pseudomonas* sp. *Stenotrophomonas* sp. *Sphingobacterium* sp. *Acinetobacter* sp. *Coprococcus* sp. *Paenibacillus* sp. *Agrobacterium* sp. *Sphingobium* sp.	(Vilanova *et al*., 2015)
Dishwashers	Thermal stress High salt concentrations Presence of detergents pH variations water pressure	**Fungi:** *Ascomycota* *Basidiomycota* **Bacteria:** *Gordonia* sp. *Micrococcus* sp. *Chryseobacterium* sp. *Exiguobacterium* sp. *Meiothermus* sp. *Staphylococcus* sp. *Streptococcus* sp. *Lactobacillus* sp. *Corynebacterium* sp. *Enterococcus* sp. *Acinetobacter* sp. *Escherichia/Shigella* sp. *Pseudomonas* sp.	(Raghupathi *et al*., 2018) (Zupančič *et al*., 2019)
Washing machines	Thermal stress Presence of detergents pH variations Constant water pressure	**Bacteria:** *Pseudomonas* sp. *Enhydrobacter* sp. *Leptospira* sp. *Sphingomonas* sp. *Legionella* sp.	(Kubota *et al*., 2012) (Callewaert *et al*., 2015) (Bockmühl *et al*., 2019) (Jacksch *et al*., 2020)

		***Moraxellaceae* family:** *Acinetobacter* sp.	
Water heating systems	High temperature High humidity High pH conductivity and oxygen concentration	**Bacteria:** *Thermus* sp. *Acidovorax* sp. *Agrobacterium* sp. *Roseococcus* sp. *Flavobacterium* sp. *Sphingomonas* sp. *Brochothrix* sp. *Buchnera* sp. *Polynucleobacter* sp. *Ralstonia* sp. *Thermicanus* sp. *Parascardovia* sp. *Micrococcus* sp. *Rothia* sp. *Brachybacterium* sp. *Methylobacterium* sp. *Sejonia* sp. ***Moraxellaceae* family**	(Brock and Boylen, 1973) (Kjellerup *et al*., 2005) (Savage *et al*., 2016)
Saunas	High temperature Low nutrient	**Bacteria:** *Bacillus* sp. *Virgibacillus* sp. *Tepidomonas* sp., *Pseudoxanthomonas* sp. *Stenotrophomonas* sp. *Janthinobacterium* sp. *Aquaspirillum* sp. *Chromobacterium* sp. *Aquabacterium* sp. *Gulbenkiania* sp. *Pelomonas* sp. *Aquitalea* sp. *Deinococcus* sp. ***Moraxellaceae* family**	(Lee and Park, 2012) (Kim *et al*., 2013) (Tanner *et al*., 2017)
Air conditioning cooling systems	Low temperature Low nutrient	**Fungi:** *Malassezia* sp. *Cladosporium* sp. *Leotiomycetes* sp. **Bacteria:** *Agaricomycetes* *Pseudomonas* sp. *Sphingomonas* sp. *Propionibacterium* sp. *Methylobacterium* sp. *Enhydrobacter* sp. ***Moraxellaceae* family:** *Perlucidibaca* sp. *Acinetobacter sp.*	(Acerbi *et al*., 2017) (Bakker *et al*., 2020)

The results showed that the air samples were more diverse than the condensed water samples. *Moraxellaceae* (such as *Perlucidibaca*, *Acinetobacter*) and *Enhydrobacter* followed by *Pseudomonas* spp. were the less abundant bacteria after *Agaricomycetes* in the air samples. Meanwhile, the population of *Sphingomonas* spp. grew over time and dominated the microbiome at the late phase of the research in the water samples (Acerbi *et al.*, 2017). Another study on the microbial diversity of forty large-scale commercial ACs showed that *Methylobacteriaceae* and *Propionibacterium* spp., and, to a lesser extent, *Acetobacteraceae,* and *Sphingomonas* spp. dominated the ACs bacteriome (Bakker *et al.*, 2020). The analysis of the fungal communities within those forty commercial ACs showed no specific pattern about the distribution of fungal taxa, although the presence of *Malassezia* spp., *Cladosporium* spp., and *Leotiomycetes* was confirmed with different relative abundances (Bakker *et al.*, 2020). Interestingly, previous works also demonstrated that *Methylobacterium* sp. and *Propionibacterium* sp. can grow in oligotrophic environments such as the surface of the cooling coils in air-handling systems and air conditioning systems of automobiles by biofilm formation (Hugenholtz, 1994; Simmons *et al.*, 1999). The presence of *Malassezia* spp. is not surprising either, as this common skin-commensal yeast has been widely reported in indoor environments (Hanson *et al.*, 2016). Another study by Wilson *et al.* (2007) also reported that spore-forming *Cladosporium* sp. dominated the microbial communities of various parts of air-handling systems (Wilson *et al.*, 2007).

Although microorganisms can colonize artificial surfaces, the microbial community is not only limited to the human-associated microbiome but also consists of a considerable fraction of the environmental microorganisms which can thrive on those multi-stress niches. Table 1 shows the summary of the mentioned artificial environments, their microbiota, and extreme selective pressures of those devices.

The relation between the niche and the microbial profile: The selective pressure that environmental factors play on microbial populations was first described by the Baas-Becking hypothesis; "*Everything is everywhere, but, the environments selects*" (Becking, 1934). The Baas-Becking hypothesis suggests that the biotic and abiotic factors of an environment, rather than its geographical location, are key in the establishment of the specific taxa present in a microbial niche. This is not limited to natural environments. In the previous parts of this introduction, we described the presence of different microbial communities on/in different man-made items. Those artificial environments have particular conditions such as UV-irradiation, desiccation, low nutritional resources, extreme temperatures, water pressure, and pH variations. The microbial communities that thrive under these conditions should have specific adaptation mechanisms, as the proliferation under those conditions is not generally the best for the majority of microorganisms.

On the other hand, the primary colonizers of any surface are those microorganisms, able not only to attach and colonize the surface but also to modify the surface's chemical properties enabling the subsequent colonization by other microorganisms. The primary colonizers may also shape the availability of nutrients, oxygen, or pH, "choosing" that way who is coming next.

Regardless of the physicochemical properties of the sampling sites, *Sphingomonas* spp. was detected in most artificial and natural environments; from high UV irradiated surfaces such as solar panels (Tanner, Mancuso, *et al.*, 2020) to oligotrophic water distribution systems (Zhang *et al.*, 2018). Members of this genus are oligotrophic and can thrive under nutrient deprivation on the surface of different plumbing materials of the distribution water systems (Gulati and Ghosh, 2017). *Sphingomonas* spp. are also considered key players in biofilm formation (Acerbi *et al.*, 2017).

Moraxellaceae is also detected in almost all water-dependent devices such as washing machines, WHs, and ACs.The members of this family are common in extreme environments but also on the human skin (Zupančič *et al.*,

2018; Wilpiszeski *et al.*, 2019; Jacksch *et al.*, 2020). Interestingly, *Acinetobacter* sp. are the most abundant species within this family found in the coffee-machines and dishwashers. Members of this genus can tolerate thermal stress (Campos *et al.*, 2019), high pressure (Dutta *et al.*, 2019), and nutrient deprivation (Fan *et al.*, 2021).

Intriguingly, members of the genus *Methylobacterium* are found in both desiccated and humid environments. Solar panels and spacecraft facilities as well as WHs and ACs are nutrient-depleted environments, in which oligotrophic microorganisms such as *Methylobacterium* spp. can attach to different surfaces, form a biofilm, and survive to thermal stress (Szwetkowski and Falkinham, 2020).

Members of the genus *Bacillus* were isolated from the space station and saunas. Both environments share some parameters such as high temperature, desiccation, and low nutrients availability. One of the most common strategies to tolerate those unfavourable conditions is through sporogenesis (Bressuire-Isoard *et al.*, 2018). Therefore, sporogenesis is a survival mechanism to thrive as vegetative dormant cells under extreme conditions (Milanesi *et al.*, 2015).

In table 2, we summarised the shared microbial genera with the potential to colonize and stabilize in the poly-extreme conditions of the mentioned man-made devices. The widespread presence of the genera *Sphingomonas*, *Methylobacterium*, *Pseudomonas*, and *Acinetobacter*, to a lesser extent, *Bacillus*, and *Micrococcus* in artificial environments reveals that these microorganisms harbour adaptation mechanisms to thrive and proliferate in such multi-stress conditions.

In the present thesis, we have tried to expand the knowledge on the microbiome of artificial environments poorly explored to date. From those, the wasted chewing gum can be considered as both artificial and/or semi-artificial environment due to the properties of its main ingredient, gum base (Konar *et al.*, 2016). Gum base is non-nutritive water-insoluble elastic parts of the chewing gum, allowing chewing gum to be chewed for a long time without any structural modifications (Hartel and Hartel, 2014). The plastic-like property of the gum base results in a long-lasting environmental pollutant after being discarded in the environment (Dominguez-moñino *et al.*, 2014). Although previous research revealed that the oral microbiome can be entrapped during the mastication in the matrix of chewing gum (Wessel *et al.*, 2015), there is a lack of information on the biodegradability of this product once discarded.

We would like to shed light on whether the wasted chewing gum is biodegradable and whether the oral microbiome is responsible to decompose this artificial product. Moreover, we planned to study microbial successions -if any- taking place after the gum is disposed and what is distribution of the wasted chewing gum microbiome in different locations of the world.

Table salt is another semi-artificial environment which is a micro-habitat for some halophilic archaea (Henriet *et al.*, 2014; Gibtan *et al.*, 2017). One of the most important saline sites, as a source for table salts production, is solar saltern which is a man-made pond-like environment constructed to harvest high-quality sodium chloride (NaCl) from seawater (Burns *et al.*, 2007; Oren and Meng, 2019).

The microbial communities of solar salterns have been previously studied, and both halophilic archaea and bacteria have been identified in the solar salterns (Lee *et al.*, 2021). However, there is a lack of knowledge about the bacterial density and diversity of the table salts. In the present thesis, we study the microbial community of the commercially available table salts from different origins to shed light on the bacterial diversity of those semi-artificial environments. Beyond the taxonomic characterisation, we wanted to understand if the table salts from different locations can present the same microbial profiles or there are any differences correlated to geographical origins of the salts.

Table 2. Shared microorganisms among the artificial devices described in this chapter.

Microorganisms	Artificial device	Poly-extreme conditions
***Deinococcus* sp.**	Solar panels Sauna	UV-irradiation, Desiccation, Low nutrition High temperature, Desiccation, Low nutrient
***Sphingomonas* sp.**	Solar panels Spacecraft facilities Washing machines Water heating systems Air conditioning systems	UV-irradiation, Desiccation, Low nutrition Desiccation, High temperature, Low nutrient and oxygen concentration, Microgravity Thermal stress, Presence of detergents, pH variations, Constant water pressure High temperature and humidity, High pH, conductivity, and oxygen concentration Low temperature and nutrient, High humidity
***Methylobacterium* sp.**	Solar panels Spacecraft facilities Water heating systems Air conditioning systems	UV-irradiation, Desiccation, Low nutrition Desiccation, High temperature, Low nutrient and oxygen concentration, Microgravity High temperature and humidity, High pH, conductivity, and oxygen concentration Low temperature and nutrient, High humidity
***Pseudomonas* sp.**	Coffee-machine Dishwasher Washing machines Air conditioning systems	Thermal stress, Constant water pressure, Low nutrient Thermal stress, High salt concentrations, Presence of detergents, pH variations, Constant water pressure Thermal stress, Presence of detergents, pH variations, Constant water pressure Low temperature and nutrient, High humidity
***Bacillus* sp.**	Spacecraft facilities Sauna	Desiccation, High temperature, Low nutrient and oxygen concentration, Microgravity High temperature, Desiccation, Low nutrient
***Acinetobacter* sp.**	Coffee-machine Dishwasher Washing machines Air conditioning systems	Thermal stress, Constant water pressure, Low nutrient Thermal stress, High salt concentrations, Presence of detergents, pH variations, Constant water pressure Thermal stress, Presence of detergents, pH variations, Constant water pressure Low temperature and nutrient, High humidity
***Enhydrobacter* sp.**	Washing machines Air conditioning systems	Thermal stress, Presence of detergents, pH variations, Constant water pressure Low temperature and nutrient, High humidity
***Micrococcus* sp.**	Spacecraft facilities Dishwasher Water heating systems	Desiccation, High temperature, Low nutrient and oxygen concentration, Microgravity Thermal stress, High salt concentrations, Presence of detergents, pH variations, Constant water pressure High temperature and humidity, High pH, conductivity, and oxygen concentration

We have also tackled in the present thesis the study a of a biofilm which was detected in a laboratory device: a standard ice machine. Ice machines have a particular operating system (low temperature and constant water pressure, besides low nutrition availability), which can be considered as a harsh micro-niche for the growth and colonization of microbial cells. However, the biofilm clogging a pipe drain of the ice machine suggested that microorganisms could get adapted to this unfavourable poly-extreme environment and colonize this artificial device. We were interested in studying the microbial diversity of this high resistant biofilm by using both culture-based and NGS techniques. Moreover, we discuss the possible abiotic and biotic factors which accelerate the biofilm formation.

In this thesis, we study the polyphasic characterization of a new species isolated from the inner sediments of marine waste, an aluminium can, to explore their potential role for biotechnology. In recent years, the identification and characterization of novel microorganisms with a wide range of biotechnological applications have allowed us to better understand the role of microorganisms in our daily life especially regarding health promotion and lifestyle quality. In this dissertation, we study the new strain of the genus *Sagittula*, of which only two species have previously been reported.

Finally, in the present doctoral thesis, we discuss the possible potential of some isolated/detected microorganisms from those semi-artificial and artificial environments which might hold potential for biotechnological applications.

Objectives

In the present thesis, we have tried to uncover the microbial communities of some artificial and semi-artificial environments which are under constant particular selective pressures such as UV irradiation, desiccation, salinity, nutrient deprivation, low temperature and oxygen availability. All in all, the objectives of the present thesis can be summarized as:

• Description of the microbial communities associated with unexplored semi-artificial and artificial products such as the wasted chewing gum and the laboratory ice machine by using NGS and culturomics analysis (Chapter I and III).

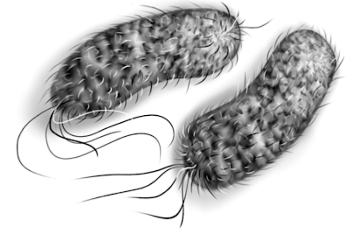
• Study the succession of the microbial community for colonization of semi-artificial and/or artificial products such as the wasted chewing gum (Chapter I).

• Identification of microbial species associated with semi-artificial and artificial food-grade products such as chewing gum, table salts and fully man-made device; ice machine, and uncovering their potential for biotechnological applications (Chapter I, II and III).

• Isolation and characterization of individual microorganisms from those environments by using different media and methods to determine their biological activity (Chapter I, II and III).

• Characterization of a new species of the genus *Sagittula* which is previously isolated from the marine waste (an aluminium can) by multi-omics phenotypic approaches (Chapter IV).

Chapter I – The wasted chewing gum bacteriome



Abstract

Here we show the bacteriome of wasted chewing gums from five different countries and the microbial successions on wasted gums during three months of outdoors exposure. In addition, a collection of bacterial strains from wasted gums was set, and the biodegradation capability of different gum ingredients by the isolates was tested. Our results reveal that the oral microbiota present in gums after being chewed, characterised by the presence of species such as *Streptococcus* spp. or *Corynebacterium* spp., substituted in a few weeks to an environmental bacteriome characterised by the presence of *Acinetobacter* spp., *Sphingomonas* spp. and *Pseudomonas* spp. Wasted chewing gums collected worldwide contain a typical sub-aerial biofilm bacteriome, characterised by species such as *Sphingomonas* spp., *Kocuria* spp., *Deinococcus* spp. and *Blastococcus* spp. Our findings have implications for a wide range of disciplines, including forensics, contagious disease control, or bioremediation of wasted chewing gum residues.

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Introduction

Chewing gums may have been used for thousands of years, since wood tar from the Mesolithic and Neolithic periods have been found with tooth impressions, which suggests a role in teeth cleaning as well as its usage as early adhesives (Aveling *et al.*, 1999; Van Gijn *et al.*, 2006). The first modern chewing gum was introduced in the market in the late 19th (Imfeld, 1999) and chewing gums are today vastly consumed worldwide: it is estimated that Iran and Saudi Arabia are the countries with the highest chewing gum consumption, where 80 % of the population are regular chewing gum consumers (Nieburg *et al.*, 2012). Moreover, global online surveys on gum intake conducted in Europe and United States displayed similar chewing gum patterns among them, where more than 60 % of adolescents and adults had chewed gums in the last 6 months before the survey and the mean intakes ranged from 1 to 4 pieces of chewing gum per day (Hearty *et al.*, 2014; Martyn *et al.*, 2019). Significantly, Hearty *et al.* (2014) reported the lowest chewing gum intake (46 %) in the United Kingdom (Hearty *et al.*, 2014). Finally, the value of chewing gum trade has been estimated as more than 30 billion U.S. dollars in 2019 (Loose *et al.*, 2020).

Chewing gums are generally composed of two phases: the water-insoluble phase (gum base) and the water-soluble phase, which can be made of sugar (sugar chewing gums) or sugar alcohols such as polyols (sugar-free chewing gums). Some chewing gums present a solid coat, which is involved in flavour release as well as in chewing gum protection to physicochemical agents, that can be specified as a third phase (Konar *et al.*, 2016). The main component of any chewing gum is the gum base (20-30 %), that is not edible, nor digestible, but allows chewing, during which added flavours and sweeteners are released. Indeed, chewing gum can be chewed for a long period without any structural modifications because of the water-insoluble property of the gum base (Hartel *et al.*, 2018). Gum base can be produced from either natural polymers, such as latex or waxes, or synthetic polymers, particularly polyvinyl acetate (15-45 %) _a key ingredient in chewing gum formulation_ and synthetic elastomers (10-30 %) including co-polymers of butadiene-styrene, isobutylene-isoprene as well as, polyethylene, polyisobutylene and polyisoprene (Konar *et al.*, 2016). Hence, this inert part of the formula constitutes the support for the water-soluble components which consist of: (i) sweeteners, whether sugar or sugar alcohols that constitute the 60 % of the chewing gum; (ii) humectants, such as glycerine; (iii) antioxidants, supplemented to avoid oxidation of other ingredients; (iv) colours, flavours and organic acids, added to define an specific taste of the chewing gum; and (v) optionally, “active” ingredients such as nicotine in chewing gums as an alternative to smoking (Konar *et al.*, 2016; Hartel *et al.*, 2018). The particular amounts of these components in the chewing gum formula are a well-kept secret of each confectionery industry. As hinted before, sweeteners comprise more than half of the chewing gum composition. Sucrose, dextrose, and glucose syrup are the most frequently used in sugar-containing gums. However, most of chewing gums present in the European market are sweetened with polyols (sugar alcohols) such as xylitol, sorbitol, mannitol, maltitol and isomalt (Burt *et al.*, 2006; Zumbél *et al.*, 2001) as well as artificial sweeteners such as aspartame (Zamzam et al., 2019), all of which being labelled as sugar-free chewing gums. The effect of the sugar-free chewing gums in the control of dental disease, salivary pH, and the oral microbiome has been reported (Burt *et al.*, 2006; Rafeek *et al.*, 2019; Takeuchi *et al.*, 2018; Wessel *et al.*, 2016).

Wasted chewing gums are often improperly discarded and end up as long-lasting residues on both indoor and outdoor pavements and surfaces. Local councils spend millions of euros cleaning up gum residues from the pavement. For instance, it is estimated that in the UK the annual cost of cleaning up wasted gums form streets is almost 70 million euros (Rudgard, 2018). Besides, there is a big concern regarding chewing gum residues stuck to historic buildings or art works, which contribute negatively to its conservation (Dominguez-Moñino *et al.*, 2014). Moreover, the popularity of chewing gums as well as the widespread presence of those long-lasting

residues have allowed using wasted chewed gums for human genetic analysis in criminology (Bond *et al*., 2008) and archaeology (Kashuba *et al*., 2019; Jensen *et al*., 2019). It has to be stressed, though, that beyond its contents in the consumer's DNA, used chewed gum can contain an important fraction of the oral microbiome (Wessel *et al*., 2015), toxins (Saberi *et al*., 2018), and some opportunistic pathogens such as *Streptococcus* spp. and *Actinomyces* spp. (Wessel, 2016; Forssten *et al*., 2010), which remain trapped in the sticky residue, and whose survival over time has received only limited attention to date (Dominguez-Moñino *et al*., 2014; Wessel *et al*., 2015). Wasted chewing gums are considered as environmental pollutants, mainly for aesthetic reasons, and their removal from pavements can be economically expensive, and time-consuming (Emsley, 2004). To date, most studies aiming at improving wasted chewing gums cleaning have mainly focused on the production of less adhesive, water-soluble, and degradable chewing gums (Mehta *et al*., 2017; Saberi *et al*., 2018).

The present work describes a complete characterization on the bacterial contents of wasted chewed gum, by using culture-dependent and -independent techniques. We have studied the microbial content of wasted chewing gums sampled in different locations worldwide as well as the distribution of bacteria depending on the depth (surface, intermediate and bottom layers of the residue) and conducted a dynamic study to shed light on the microbial succession that takes place in the chewing gum during the first weeks after its disposal on an outdoor surface. On the other hand, we have screened the biodegradation capability of the gum ingredients of a collection of bacterial strains we isolated from chewing gum residues. Our results have implications in fields such as criminology, contagious disease control, waste management and bioremediation.

Material and Methods

Chewing gum samples: Wasted chewing gums were collected directly from outdoor pavements in the vicinity of the Scientific Park of the University of Valencia as well from other locations worldwide. In particular, ten wasted gum samples were gathered from five countries (Fig. 1). All samples were removed from the pavement with a sterile scraper, and transported to the lab, where they were kept frozen at − 80 °C until required. In one case, a sample from Valencia (Spain), of approximately 3 mm thick, was sliced in situ in three different layers of roughly 1 mm each (upper, sun-irradiated, dark part; an intermediate part and the bottom fraction of the wasted gum, in contact with the pavement). The resulting three sub-samples were processed independently.

For the assays, two commercial sugar-free chewing gums were used, Orbit and Trident chewing gums. Both chewing gums were ground with a sterilized coffee grinder, and they were added to a minimal medium as the sole carbon source. Gum powder I and II were prepared from the same source (Orbit gum pieces). The only difference between those two was that in the gum powder II, the white cortex layer was entirely removed before preparation in order to study whether bacteria only utilize the cortex layer, or they are able to degrade the whole compositions. Gum powder III was prepared from Trident brand.

Media: M9 salt medium (ATCC 2511) was prepared (gl^{-1}): Na_2HPO_4 12.8, KH_2PO_4 3.0, NaCl; 0.5, NH_4Cl; 1.0, pH adjusted to 7.2. After autoclaving, the following filter-sterilized solutions were added to the medium; 2 ml of 1 M $MgSO_4$ solution, 0.1 ml of 1 M $CaCl_2$ solution, and 20 ml of a Glucose Solution (20 % w/v). Glucose as a source of carbon was replaced, when necessary, by possible compositions of the chewing gum. M9 salt medium was modified and enriched with 2 % (w/v) gum powder I as a selective medium. In addition to this medium, two rich media, TSA (composition in g l^{-1}: Tryptone 15.0, Soya Peptone 5.0, NaCl 5.0, Agar 15.0) and LB (composition in gl^{-1}: Tryptone 10.0, NaCl 10.0, Yeast Extract 5.0, Agar 15.0), were also used.

In order to study the biodegradation of the chewing gum compositions by isolates, the modified M9 salt medium (without glucose) was enriched with 2 % (w/v) carbon sources including Sucrose, Sorbitol, Mannitol, Xylitol, Aspartame, Gum powder I, II, III, and 2 % (v/v) glycerol. Gum powders were used as other

possible complementary ingredients of chewing gum.

Culture-dependent approaches: Chewed gums were taken off with a sharp scraper from three locations in an open area from the Scientific Park of the University of Valencia, Spain. Two samples were incubated in liquid M9 salt solution (without glucose) supplemented with 2 % (w/v) chewing Gum Powder I, at room temperature orbital shaking (150 rpm) for 24 and 48 h, respectively. The third sample was directly plated on the same solid medium after resuspension. Several serial dilutions were prepared and cultured on the selective media. Plates were incubated at room temperature for a week under aerophilic, anaerobic and microaerophilic conditions. Isolated colonies were selected based on their shape and colour and they were re-streaked on fresh media. Pure isolates were resuspended in 20 % glycerol (v/v) and cryo-preserved at − 80 °C.

Polymerase chain reaction was performed with universal primers 8F (5′- AGA GTT TGA TCC TGG CTC AG -3′) and 1492R (5′- CGG TTA CCT TGT TAC GAC TT -3′) to amplify 16S rRNA gene. A loopful of bacterial cells was resuspended in 100 µl MilliQ water, pre-incubated at 100 °C for 10 min, and 1 µl of each bacterial suspension was used as DNA template. Thermal cycler program was set as the following procedure: Initial step of incubation at 95 °C for five min followed by PCR amplification (30 cycles of 30 s at 95 °C, 30 s at 54 °C, 30 s at 72 °C), and final step at 72 °C for 10 min. PCR products were monitored by 1 % agarose gel electrophoresis to confirm the amplification of the 16S rRNA gene fragment amplicon. Next, dsDNA was purified from the PCR products and resuspended in 10 µl MilliQ water. 16S rRNA Sanger sequencing was performed by tagging with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), at the Sequencing Service (SCSIE) of the University of Valencia. All sequences were edited and compared with EzBioCloud online database (https://www.ezbiocloud.net/).

Carbon source use: To study the ability of the bacterial strains isolated from wasted chewing gum to degrade different components, chewing gum sweeteners and glycerol were added as carbon sources to the minimal medium (described in media section). Moreover, three different media containing Gum Powder I, II, III separately were prepared to observe its biodegradation. For each isolated strain, a dilution of 0.2 optical density (OD_{600}) was prepared with fresh liquid M9 medium. Then 2 µl of the dilutions were spread on the three different media, in three replicates. The plates were incubated at room temperature under aerobic condition for five days. Also, the minimal M9 medium without any carbon source was used as the control medium.

Ecological succession and bacterial colonisation: A study of the microbial colonisation of wasted chewing gum under controlled conditions was carried out. The study protocol was approved by the board of directors of The Institute for Integrative Systems Biology (I2SysBio) and was conducted under the guidelines of Helsinki declaration of 2013. Informed consent of the volunteer was obtained prior the study. A healthy volunteer (36 years old female) chewed two chewing gum pieces every day for 30 min. The first chewed gum was stored in − 80 °C as a control of the oral microbiome. Twelve chewed gums processed this way were placed on the sidewalk of the Scientific Park (University of Valencia, Spain) on an outdoor, sun-oriented location. The experiment was conducted in mid-June, and wasted gums were processed as described for 12 consecutive days. Then, they were picked up in intervals of one week during a total period of twelve weeks. Total DNA was extracted from each sample and 16S rRNA metagenomic analysis was carried out.

16S rRNA sequencing: The following procedures for DNA extraction and 16S rRNA sequencing were performed in all the experiments. All chewing gum residues were frozen after sampling and stored in − 80 °C at least for overnight. Frozen samples were ground to fine powder and added to 1 ml PBS buffer. Mixtures of wasted gum and PBS buffer were frozen at − 20 °C overnight. Then, 10 glass plating beads (3 mm diameter) were added to the tubes and mixed with a vortex for 3 min. Samples were let at room temperature

for 2 min, and then 500 µl of the supernatant was transferred to a 2 ml micro-tube. DNA extraction was then carried out as described by Latorre *et al*. (Latorre *et al*., 1987), and the extracted DNA was quantified through Qubit dsDNA HS Assay kit (Qubit 2.0 Fluorometer, Q32866).

The conserved regions V3 and V4 (459 bp) of the 16S rRNA gene were then amplified using forward and reverse primers: 5′- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG 3′ and 5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C -3′, respectively (Klindworth *et al*., 2013). Amplification was carried out using the KAPA HiFi HotStart ReadyMix PCR kit (KK2602) and the following PCR cycle: initial denaturation at 95 °C for 3 min; 25 cycles of amplification (30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C); and 5 min of extension at 72 °C. Amplicons were mixed with Illumina sequencing adaptors and dual-index barcodes (Nextera XT index kit v2, FC-131-2001). Libraries were normalized and merged before the sequencing. Then, the pool containing indexed amplicons was loaded onto the MiSeq reagent cartridge v3 (MS-102-3003), spiked with 10 % PhiX control to enhance the quality of the sequencing. Finally, paired-end sequencing (2 × 300 bp) was carried out on the Illumina MiSeq sequencing system. Illumina outcomes were analysed via Qiime2 software (Bolyen *et al*., 2019; Liu *et al*., 2020). The Demux plugin was used to assess the quality of reads, and the Qiime2-integrated Dada2 pipeline was employed for trimming and joining the sequences, removing chimeras, and detecting sequence variants (> 99.9 % of similarity). The classify-Sklearn module (feature-classifier plugin) was applied for assessing the taxonomy of each sequence variant, using the SILVA (v. 132) database as reference.

Data was subsequently analysed by different R packages. Rarefaction curves were constructed with the iNEXT and ggiNEXT functions (iNEXT) (Hsieh *et al*., 2016). Alpha diversity plots were generated by employing the plot_richness function (Phyloseq) (McMurdie *et al*., 2013) based on the richness, Shannon and Simpson diversity indexes. PCoA were created with the plot_ordination function (phyloseq) using Bray–*Curtis* dissimilarities as distance method. Finally, heatmap was constructed by the heatmap.2 function (gplots).

Results

Bacterial communities in wasted chewing gums analysed through NGS: The bacterial diversity of eight chewing gum samples collected in five different countries was analysed through NGS as described in the Materials and Methods section and the taxonomic profiles are shown in Fig. 1. Two chewing gum samples collected in Spain, France, and Singapore were analysed, while from Greece and Turkey one chewing gum sample was analysed. The bacterial profile deduced by the analysis of the pool of 16S rRNA genes reveals relatively similar bacterial profiles, but yet with differences in some genera. Interestingly, one of the samples from Singapore displayed a very high biodiversity with a total of 427 identified taxa, which results in a low relative abundance of the most common genera, being approximately the 15 % in this sample. However, the number of samples per country being too small to draw conclusions at the geographic level, these results have to be considered as a first approach on the chewing gum bacteriome. Chewing gum samples from France (Paris) and Turkey (Istanbul) proved also rather diverse representing the most common genera the 49 % and 42 % of total abundance, respectively.

Although the eight samples exhibited an important variation among locations, several genera were found in all the analysed samples (*Kocuria*, *Sphingomonas*, *Deinococcus*, *Blastococcus*, *Skermanella*, *Hymenobacter*, *Modestobacter*, *Paracoccus, Roseomonas*, *Rubellimicrobium*, *Methylobacterium*, and Uncultured *Sphingomonas*). Their relative abundance changed significantly among samples. The genus *Kocuria* with a 54.18 % frequency and *Sphingomonas* with a 38.9 %, were the most abundant genera in one of the samples from Singapore and France, whereas in other samples, such as Greece, the most frequent genera were *Deinococcus* with a 25.2 % frequency, followed by *Kocuria*,

Pseudokineococcus and *Modestobacter* with relative abundances from 10 to 16 %. In samples from Valencia, other frequent genera were *Blastococcus*, *Nesterenkonia* and *Hymenobacter* with a 23.2 %, 27.8 % and 14.4 % relative abundance, respectively. Finally, the genus *Skermanella* was the most abundant one in the chewing gum from Turkey (20.9 %). *Arthrobacter* spp. was identified in all samples except the two samples from Singapore. Similarly, *Pseudomonas* spp. was found in all samples except in the one from Greece. Interestingly, in the two Mediterranean locations, three genera constituted approximately 25 % of the biodiversity. These genera were: *Blastococcus* and *Nesterenkonia* from Valencia, Spain; and *Deinococcus* from Spetses Island, Greece. The presence of genus *Nesterenkonia*was found exclusively in the two samples from Spain. In addition, the genus *Curtobacterium*was found mainly in Turkey and in a lower abundance in France (Paris).

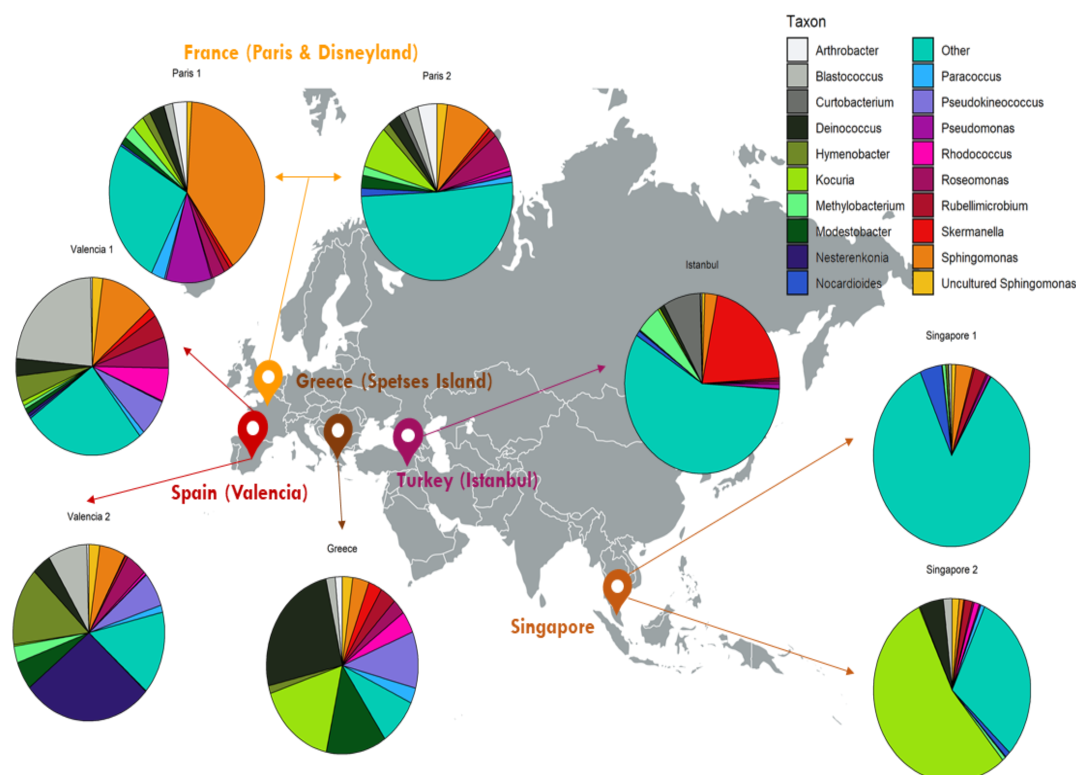

Figure 1. The taxonomic profiles of wasted chewing gum samples collected from outdoor locations in five countries. The most abundant taxa from each location are shown in pie charts.

In one case, a single gum sample was collected and divided in situ in three different fractions that were processed independently. The sample, located in one of the outdoor car parks of the Scientific Park of the University of Valencia, was collected in three consecutive sections, corresponding to the upper, medium, and bottom layers of the residue. As shown in Fig. 2, the taxonomic profiles of the three sub-samples proved almost identical in composition. The surface layer, which is the most exposed to environmental conditions, presented a higher abundance of chloroplasts. The main genus identified in the three samples was *Curtobacterium* with a relative abundance between 32-47 %, being more frequent in the intermediate layer. Other, less frequent genera were *Sphingomonas*, which was the second most abundant genus and represented more than 16 % of the biodiversity; *Hymenobacter* with an 8 %; and *Kineococcus,* with a 4-6 %. Finally, *Massilia*, an uncultured *Sphingomonadaceae*, *Methylobacterium*, *Aureimonas* and members of the *Rhizobium* clade (*Allorhizobium, Neorhizobium, Pararhizobium* and *Rhizobium*) had similar frequencies, of 1.5-5 %. The remaining genera presented similar abundances and constituted approximately 9 % of the total biodiversity in each layer.

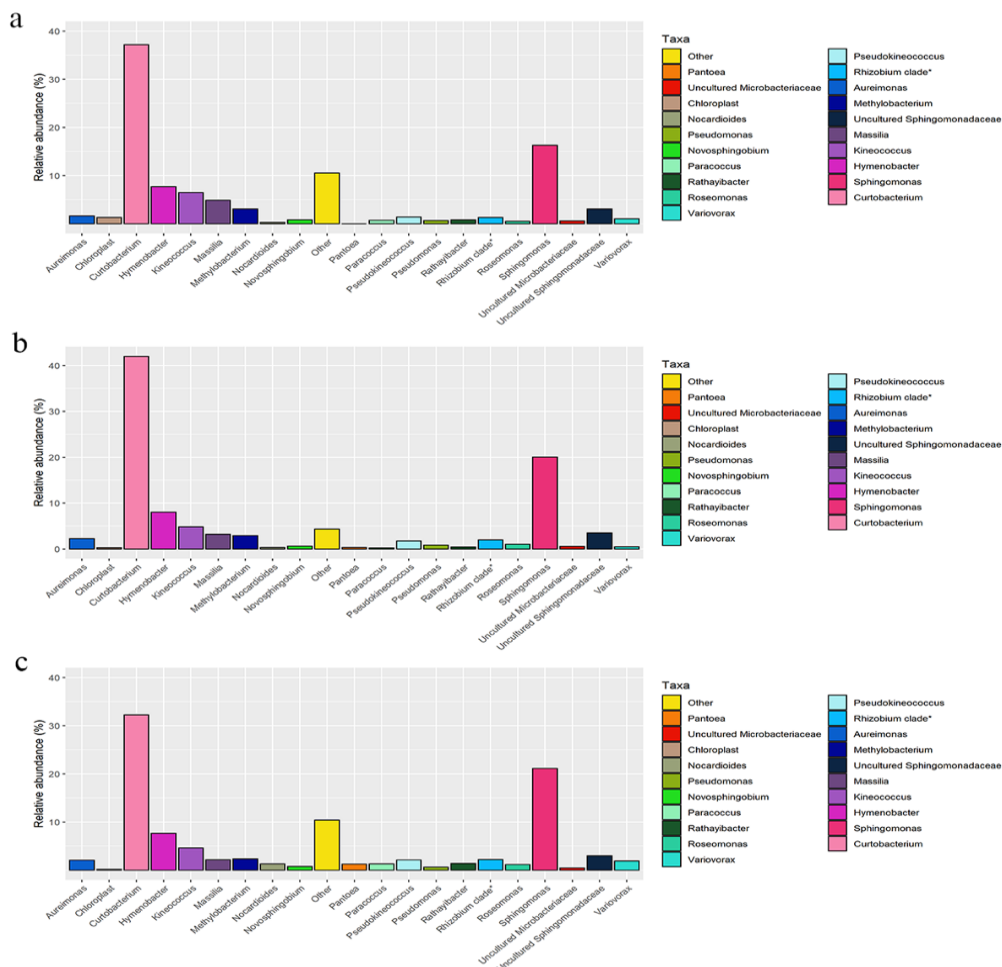

Figure 2. Comparison between the bacterial communities of three layers from a chewed gum residue. (**a**) Upper layer, (**b**) medium layer, and (**c**) bottom layer (described in M&M) showing small variation in biodiversity.

Colonisation process of wasted chewing gums: A specific experiment was carried out to shed light on the microbial successions taking place in wasted chewing gum once discarded. Thirteen gums were chewed and placed on an outdoor pavement over a period of up to 12 weeks and high throughput 16S rRNA gene sequencing was carried out to follow the dynamic changes in their bacterial contents. The shape of rarefaction curves at OTU level revealed a good coverage of the actual bacterial diversity (Supplementary Fig. 1). The high throughput sequencing and analysis of the 16S rRNA gene amplicons from all the samples revealed shifts in bacterial diversity in time (Fig. 3a), reaching the highest alpha diversity values after 6-8 weeks. The most abundant genera (Fig. 3b) included the genus *Streptococcus,* with a relative abundance of more than 25 % in the control sample (which had its total DNA immediately extracted after being chewed). The relative frequency of this genus slowly decreased in time and reached its lowest abundance by the ninth week. Other frequent genera found in the control sample were the oral microbiome members *Rothia*, *Haemophilus*, *Corynebacterium*, *Veillonella*, *Actinomyces* and, to a lesser extent, *Granulicatella* and *Gemella*. All these genera remained detectable in the analysed samples throughout the whole experiment, but they clearly decreased in time, although with different temporal patterns. Interestingly, the relative abundance of non-oral, environmental genera, such as *Rubellimicrobium,* *Sphingomonas*, *Acinetobacter*, and *Pseudomonas* increased with the time the gums were kept outdoors, reaching maximum values of 12 %, 19 %, 23 % and 16 % respectively, after sixth, eighth, eleventh, and twelfth weeks each.

A Principal Coordinate Analysis (PCoA) proved that the bacterial communities in wasted chewing gums during a period of 12 weeks clustered in three groups (Fig. 3c). These groups corresponded to samples collected after 1-4; 5-8; and 9-12 weeks, thus indicating a clear association between taxonomic profile similarities and time of outdoor exposition.

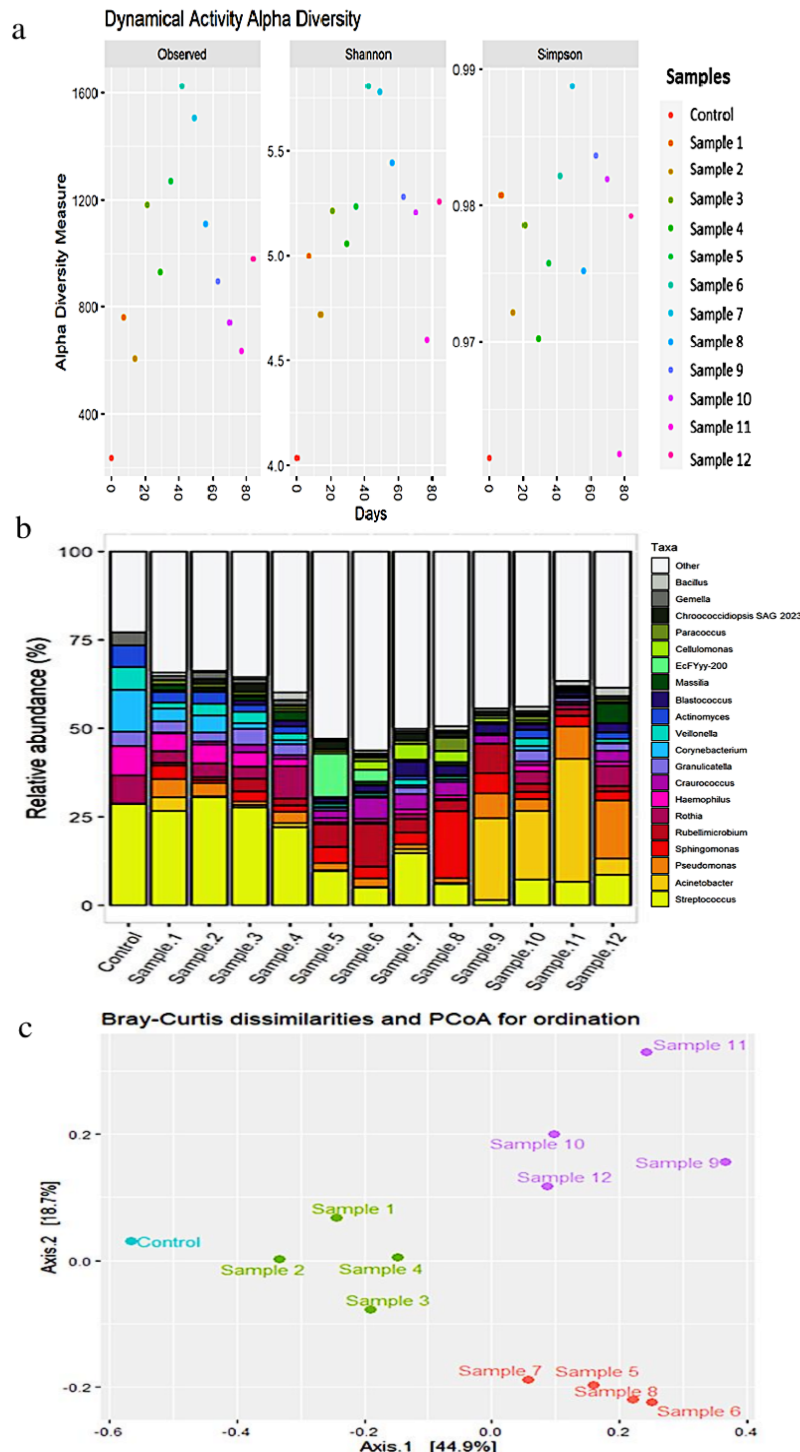

Figure 3. Dynamic experiment showing the variation of the chewed gum microbial communities in a twelve weeks period. (**a**) Alpha diversity shows the richness of the samples based on the Richness, Shannon, and Simpson index, (**b**) Clustered-Bar chart represents the modification of the oral bacterial profile over time, according to 16S rRNA gene monitoring. (**c**) Beta diversity (PCoA) based on Bray–Curtis illustrates correlations among the bacterial genera in different samples. Distances to the linear statistical correlation and the colours indicate the similarity of the microbial communities affected by environmental factors or removing time during the three-month period of the experiment.

In order to identify the fate of the most common genera in the control sample in time, their relative abundance as well as that of the most frequent genera at the end (12th week) of the experiment were compared, as shown in Fig. 4. The four most abundant genera in the control sample were *Streptococcus*, *Corynebacterium*, *Haemophilus*, and *Rothia*, which constituted 56 % of biodiversity of the sample (Fig. 4a). On the other hand, sample 12 included 9 main genera (51 % of the reads) corresponding to environmental bacteria. In fact, and as Fig. 4 shows, the presence of the most abundant taxa in the control sample decreased significantly and was substituted by a soil-related profile in which the most frequent genera were *Acinetobacter*, *Sphingomonas* and *Pseudomonas*. The ecological succession of seven selected environmental genera (*Sphingomonas*, *Rubellimicrobium*, *Craurococcus*, *Granulicatella*, *Deinococcus*, *Hymenobacter*, and *Kocuria*) were monitored over time (Supplementary Fig. 2).

All these genera, except *Granulicatella,* with a 4 % abundance at the start, were not present in the control sample or only in a very low quantity, as the case of *Sphingomonas* and *Rubellimicrobium,* which showed an upward trend over time, peaking in the intermediate stages and stabilizing towards the end of the monitoring.

Culture-dependent experiments Strain collection from wasted chewing gums:

Different chewing gum samples from the vicinity of our laboratory (Valencia, Spain) were ground and spread on different microbiological media. A total of 21 bacterial colonies, were selected, isolated, and identified by 16S rRNA gene sequencing. The isolates selected from aerobically-incubated Petri dishes belonged to the following genera; *Curtobacterium* ($S_{1\text{-}1}$), *Pantoea* ($S_{1\text{-}2}$), *Microbacterium* ($S_{1\text{-}3}$ and $S_{1\text{-}6}$), *Pseudomonas* ($S_{1\text{-}4}$), *Paenibacillus* ($S_{1\text{-}5}$), *Arthrobacter* ($S_{2\text{-}1}$, $S_{2\text{-}3}$, $S_{2\text{-}6}$), *Serinicoccus* ($S_{2\text{-}2}$, $S_{2\text{-}5}$), *Sphingomonas* ($S_{2\text{-}4}$), *Aureimonas*($S_{2\text{-}7}$), *Bacillus* ($S_{3\text{-}1}$), *Agrococcus* ($S_{3\text{-}17}$), *Williamisia* ($S_{3\text{-}18}$) (Supplementary Table 1). Five additional bacterial isolates (Supplementary Table 2) identified as members of the genera *Arthrobacter*, *Cellulosimicrobium*, *Sphingomonas*, *Terribacillus*, *Bacillus* were isolated under microaerophilic conditions.

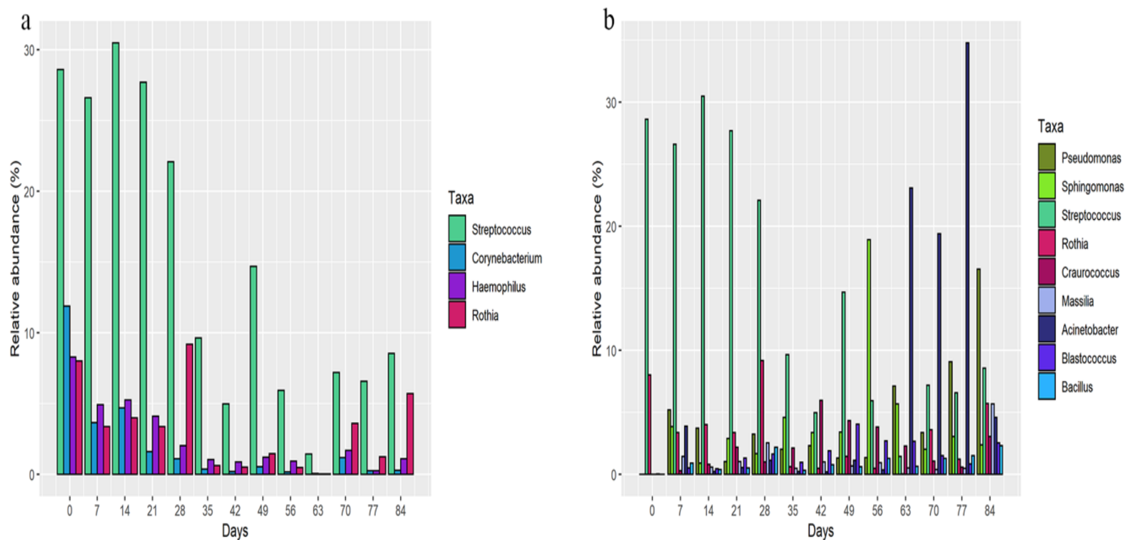

Figure 4. Comparison between the most frequent genera in (**a**) a control gum directly analysed after being chewed and (**b**) a sample taken after 12 weeks placed outdoors.

Characterization of the degradation of chewing gum components: In order to identify possible degrading activities from the chewing gum isolates, a screening was carried out by growing those strains on minimal M9 medium supplemented with different possible components of chewing gum (Fig. 5a). In general, all the isolates grew better in the media supplemented with complete ground chewing gum. The *Bacillus altitudinis* strain was the only isolate that could grow well in minimal medium alone. *Pantoea vagans* showed the

highest growth when sucrose, mannitol and glycerol were added to the medium as the only carbon source while it grew poorly in the presence of xylitol and sorbitol. *Paenibacillus illinoisensis* showed a wide spectrum of degradation of chewing gum components except xylitol and sorbitol. In fact, as shown in Fig. 5a, xylitol showed a significant inhibiting effect on the growth of the isolates. Finally, one strain, S_{2-4}, with 98.54 % similarity to *Sphingomonas insulae*, could not grow well on the solid media after isolation. The morphology of some strains was different depending on the type of chewing gum powder that was used as a carbon source for the selective media (Fig. 5b).

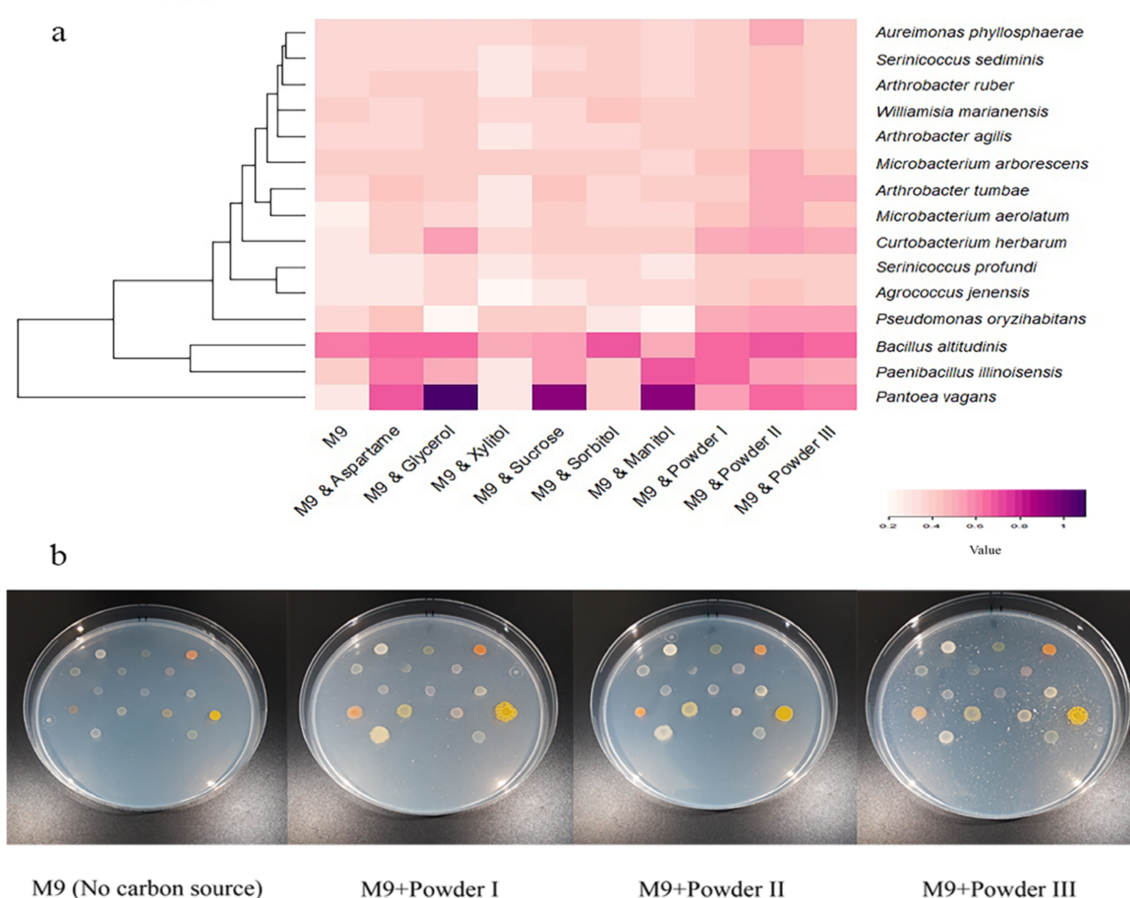

Figure 5. Degradation of various chewing gum ingredients by strains isolated from wasted chewing gums. (**a**) Heat map representing the ability to grow on different compounds of gum pieces as carbon sources. The phylogenetic tree and taxonomy of the chewed gum isolates based on 16S rRNA gene are shown in the left and right Y-axis, respectively. (**b**) The strains growth on modified M9 salt media enriched by chewing gum powder I, II, III, compared with the same medium without any carbon source as a control (left).

Discussion

In this work, we describe the complete characterisation of the wasted chewing gum bacteriome. We report here that wasted chewing gums display a moderate diversity of bacterial population with variations among the samples analysed. Additionally, we show in a specifically designed assay that the oral community-based microbial pool is largely substituted, in a matter of few weeks, by an environmental bacteria-rich microbial community after a process of ecological successions.

The microbial community of the wasted gum samples consists of bacteria from phyla *Proteobacteria, Actinobacteria, Deinococcus-Thermus,* and *Bacteroides*. The most abundant families were *Sphingomonadaceae*, *Micrococcaceae*, *Geodermatophilaceae*, and *Deinococcaceae* and, at the genus level, *Sphingomonas*, *Kocuria*, *Blastococcus*, *Deinococcus*, and *Skermanella*; other frequent

genera were *Nesterenkonia* and *Hymenobacter*. All those taxa have previously been reported as microbial communities associated with natural environments such as the phylloplane (Ren *et al*., 2020), flowers (Shade *et al*., 2013) or soil ecosystems (Alteio *et al*., 2020). The taxonomic profile we have detected is similar to abundant phyla on the surface of rocks in Maritime Antarctic glacier forefields (Garrido-Benavent *et al*., 2020) and also the bacteriome of other outdoor surfaces such as photovoltaic, solar panels. In fact, solar panels are characterised by a desert-like bacteriome rich in *Sphingomonas*, *Deinococcus*, or *Hymenobacter* (Dorado-Morales *et al*., 2016). It is obvious that rocks or plant surfaces, solar panels and sun-exposed wasted chewing gums share similar ecological pressures in terms of irradiation, low water availability, thermal variations, or oxidative stress.

Streptococcus, *Rothia*, *Haemophilus*, *Granulicatella*, *Corynebacterium*, *Veillonella*, *Actinomyces*, and *Gemella* were the most frequent genera in the control, chewed sample. These taxa are typical inhabitants of the mouth cavity, which bacteriome is composed of approximately 700 species from 185 genera (Yang *et al*., 2018; Deo *et al*., 2019). Our results reveal that the pool of oral microbiome bacteria present initially in the chewed samples is substituted after a colonisation process by environmental bacteria. However, some oral genera, specifically *Streptococcus*, was also detected at relatively high frequencies weeks after being used, as deduced by the colonisation experiment, we conducted. Interestingly, *Streptococcus* was detected with very low relative abundance in most of the older-wasted chewing gum samples we studied, and not detected in samples of Greece and Singapore, suggesting that the stability in time of *Streptococcus* in the wasted chewing gum may not be more than a few weeks. Furthermore, other oral bacteria *Corynebacterium*, *Haemophilus*, *Veillonella* and *Gemella* were the most abundant genera in the control sample and these genera remained in the samples through the whole experiment, but their populations clearly decreased over time. The presence of *Corynebacterium* immediately decreased during the first month of outdoors incubation; however, it was observed with low intensity between 5-12 weeks. Furthermore, the lowest relative abundances from genera *Haemophilus*, *Veillonella* and *Gemella* were detected in sample 12. While, in comparison, the genus *Streptococcus* was the most frequent in this sample. These results suggest that wasted chewing gums constitute carriers of oral bacteria, some of which could be pathogens, even several weeks after being discarded. The biodiversity of the chewed gum microbial community increased after a few weeks outdoors, and the analysed samples after 6 or 9 weeks were more diverse and richer in genera such as *Craurococcus* and *Sphingomonas*, which were found in gum samples until the end of the period analysed. Other genera, such as *Cellulomonas* and *Rubellimicrobium* were observed in the gums of the same age as well, but they did not persist in time. Finally, the last samples analysed were rich in *Actinobacteria*, *Blastococcus* and other environmental bacteria. A possible explanation for this rearrangement may be that the transient taxa have degraded polymeric substrates remaining in the chewed gum to short carbon chains, and these simple carbon sources could then be hydrolysed by environmental bacteria. Additionally, the changes in abiotic factors such as pH, temperature, oxygen levels or water contents may play a role as selection forces driving community successions.

It is interesting to compare the bacterial profile of the chewing gums exposed outdoors for several weeks in the controlled experiment we performed with that of the “old” wasted chewing gums we sampled and analysed from different locations worldwide. *Pseudomonas*, *Sphingomonas*, *Rubellimicrobium*, and *Blastococcus* were present as the most abundant genera in almost all samples collected from different countries as well as the last sample of dynamic experiment (sample 12), which indicates that these taxa are fast, common colonisers of the wasted gums. Moreover, other environmental bacteria such as *Kocuria*, *Modestobacter*, *Deinococcus*, and *Roseomonas* were observed in all samples collected worldwide; however, they only constituted a small percentage of the microbial community in the dynamic samples labelled as

"others" (data not shown). This strongly suggests that these taxa correspond to "second wave", of slower but yet also cosmopolitan, microbial colonisers of the gum substrate.

Wasted gum being compact masses, the access of water and oxygen to the central part of the residues could be at least partially prevented, and it could thus be hypothesized that microbial communities would be different depending on their physical distance to the external environment. As a first approach to find out whether there was a variation in the bacterial composition across the depth of the waste, a single sample was divided into three successive layers that were analysed independently. Surprisingly, no significant differences were observed in the microbial communities of the different chewed gum layers of this sample. This result is striking, since even if the physico-chemical properties of a chewing gum may not change across its depth, UV radiation and water activity would be expected to be strong selective factors shaping a specific bacterial composition. *Sphingomonas* was detected in all three layers of the analysed chewed gum, as well as *Hymenobacter* and *Deinococcus,* all of which have been reported from extreme environments under strong desiccation and radiation conditions (Tanner *et al*., 2018; Maeng *et al*., 2020). Due to the limitation of the analysis of a single sample, further research including more samples is needed to address the colonisation of the interior of chewing gums.

Aspartame, mannitol, and glycerol were hydrolysed by several strains that we isolated from wasted chewing gums and identified as belonging to the species *Pantoea vagans*, *Paenibacillus illinoisensis*, and *Curtobacterium herbarum*. Aspartame was also remarkably degraded by *Pseudomonas oryzihabitans*, *Microbacterium arborescens*, and *Arthrobacter agilis*. The biodegradation of several artificial sweeteners including aspartame was recently studied (Tran *et al*., 2014; Gatidou *et al*., 2020), but the particular microbial key-players involved in the biodegradation process have been poorly studied. Degradation of mannitol and glycerol by *Pantoea vagans* and *Paenibacillus illinoisensis* has also previously been reported (Dai *et al*., 2019; Nascimento *et al*., 2020) Kuranishi *et al*. proved that *Pantoea* species can use mannitol as a substrate, by cultivating this genus on a designed semi-selective medium enriched with this compound (Kuranishi *et al*., 2019). Another sweetener, sorbitol, was used as carbon source by one of our strains belonging to the genus *Aureimonas*. This genus has previously been found to hydrolyse a variety of carbon sources, such as carbohydrates, polyols, and organic acids (Ávila *et al*., 2020). Our *Curtobacterium* strain was able to degrade almost all the tested chewing gum ingredients. Recently, Chase *et al*. reported that *Curtobacterium* spp. is able to degrade a wide range of carbohydrates, especially structural polysaccharides (Chase *et al*., 2016). It is thus tempting to hypothesize that bioaugmentation of some of the strains we characterized, specially *Curtobacterium herbarum,* may be used as a bioremediation strategy to contribute to remove chewing gum residues from contaminated pavements.

On the other hand, one of the main compositions of gum base are natural or artificial rubbers (Edwards, 2003). Previous research reported that these polymers could be degradable by bacteria; nevertheless, the biodegradation of rubbers is a long-term process (Shah *et al*., 2013) since polymeric chains are cross linked by sulphur binding (Joseph *et al*., 2015). In this study, we have detected, either by culture-dependent or culture-independent techniques some taxa that have been previously described as rubber degraders. Tatangelo *et al*. showed that *Rhodococcus* can oxidize sulphur to sulphate, and desulphurization can facilitate the rubbers biodegradation (Tatangelo *et al*., 2019). We also detected *Bacillus* spp. in both culture-dependent and -independent experiments, which has been described as a natural and synthetic rubber degrader (Shah *et al*., 2012, 2013). Interestingly, the population of *Bacillus* in the control sample of the dynamic experiment was very low; however, the presence of these bacteria gradually increased after several weeks of outdoors incubation. We also identified *Sphingomonas* spp. as one of the most frequent genera in culture-independent experiments and it was also isolated by culture-

dependent methods, which could theoretically have a role in the biodegradation of polycyclic aromatic chains present in the gum base (Edwards, 2003; Leys *et al.*, 2004). Finally, *Corynebacterium* spp., have been reported as an opportunistic oral bacterium (Sedghizadeh *et al.*, 2017) as well as a natural rubber degrader that needs direct contact with the surface of rubber particles (Shah *et al.*, 2013). In this research, this genus was detected as a component of the oral microbiome in the control, and it remained during 12 weeks in a low-stable frequency that suggests it could play a role in long-term chewing gum biodegradation.

This study is the first report revealing from a holistic approach the bacterial composition of wasted chewing gum. Taken together, our results suggest that bacteria can play a role in the natural biodegradation of the chewing gum and may also be a source of strains with other biodegradation properties. The relative stability of the oral microbiome in a sun-irradiated outdoor space even after several weeks of outdoor and solar exposition raises concerns on the possible role of wasted chewing gums as long-term carriers of pathogenic microorganisms. On the other hand, the fact that the oral microbiome is partially maintained after many days suggest that, besides human DNA analysis, NGS aiming at determining the oral microbiome remaining in a chewing gum could hold potential for legal and forensic applications.

Data availability: Raw reads are available at NCBI's Sequence Read Archive (SRA) (Bioproject Accession PRJNA641111).

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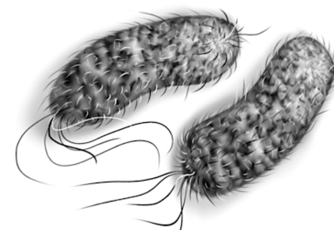
Chapter II – Beyond archaea: the table salt bacteriome



Abstract

Commercial table salt is a condiment with food preservative properties. Salt is also a source of halophilic bacteria and archaea. In the present research, the diversity of halotolerant and halophilic microorganisms was studied in six commercial table salts by culture-dependent and culture-independent techniques. Three table salts from marine origins were studied: Atlantic Ocean, Mediterranean (Ibiza Island), and Odiel marshes (supermarket marine salt). Other salts supplemented with mineral and nutritional ingredients were also used: Himalayan pink, Hawaiian black, and one with dried vegetables known as Viking salt. The results of 16S rRNA gene sequencing reveal that the salts from marine origins display a similar archaeal taxonomy, but with significant variations among genera. Archaeal taxa *Halorubrum Halobacterium*, *Hallobellus*, *Natronomonas*, *Haloplanus*, *Halonotius*, *Halomarina*, and *Haloarcula* were prevalent in those three marine salts. Furthermore, the most abundant archaeal genera present in all salts were *Natronomonas*, *Halolamina*, *Halonotius*, *Halapricum*, *Halobacterium*, *Haloarcula*, and uncultured *Halobacterales*. *Sulfitobacter* sp. was the most frequent bacteria, represented almost in all salts. Other genera such as *Bacillus*, *Enterococcus*, and *Flavobacterium* were the most frequent taxa in the Viking, Himalayan pink, and black salts, respectively. Interestingly, the genus *Salinibacter* was detected only in marine-originated salts. A collection of 76 halotolerant and halophilic bacterial and haloarchaeal species was set by culturing on different media with a broad range of salinity and nutrient composition. Comparing the results of 16S rRNA gene metataxonomic and culturomics revealed that culturable bacteria *Acinetobacter*, *Aquibacillus*, *Bacillus*, *Brevundimonas*, *Fictibacillus*, *Gracilibacillus*, *Halobacillus*, *Micrococcus*, *Oceanobacillus*, *Salibacterium*, *Salinibacter*, *Terribacillus*, *Thalassobacillus*, and also Archaea *Haloarcula*, *Halobacterium*, and *Halorubrum* were identified at least in one sample by both methods. Our results show that salts from marine origins are dominated by Archaea, whereas salts from other sources or salt supplemented with ingredients are dominated by bacteria.

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Introduction

Sodium chloride, table salt, is widely used in the food industry as a taste, texture, and flavor enhancer (Henney *et al.*, 2010a), stabilizer and food preservative (Albarracín *et al.*, 2011). Most processed products contain sodium chloride as a preservative, which strongly affects osmotic pressure (Albarracín *et al.*, 2011). In the process of salting, sodium and chloride can interact with water molecules and reduce the water activity of the food (Tim, 2002). Therefore, salinity can prevent microbial spoilage (Henney *et al.*, 2010b). Additionally, salinity decreases oxygen solubility, thus controlling aerobic growth. Moreover, high amounts of sodium and chloride can interfere with the enzymatic activity of some microorganisms (Man, 2007). Finally, in the presence of salt, microbial cells spend more energy by pumping sodium out from their cells to cope with the harsh effect of the osmotic shock (Henney *et al.*, 2010b).

Saline environments such as solar ponds, saline lakes, brine springs, rock salts, and seawater are sources of commercial table salt (Antonites, 2020). Most commercial salts are refined and finely ground before distribution. Saline environments can also be the origin of salt-loving microorganisms, halophiles (Oren, 2015). Therefore, those microbes might be entrapped in the fluid inclusions during the crystallization process and remain viable after extraction and packaging (Baati *et al.*, 2010).

In saline habitats, archaea and halophilic bacteria are the predominant microbial communities. Those extremophiles are classified into several subgroups according to their optimum salt requirements (DasSarma and DasSarma, 2017). Most of the halophilic microorganisms belong to the slight (0.2-0.85 M) and moderate (0.85-3.4 M) halophiles. However, some extreme halophiles have been detected in hypersaline environments where the salt concentration lies between 3.4 and 5.1 M (DasSarma and DasSarma, 2017). Moreover, some studies described another subgroup for halophiles; those can thrive under the borderline range of salinity (1.5-4.0 M salt) (Ghosh *et al.*, 2019). Also, some halotolerant bacteria can tolerate low salinity and even non-saline environments. Halophiles have developed different adaptations to cope with the saline environment (Harding *et al.*, 2016). In the "salt in" strategy (Koller, 2019), the presence of K^+ and, less frequently, Na^+ is central to balance the intracellular osmotic pressure with that of the environment (Roberts, 2005). Additionally, some halophiles balance their intracellular osmotic pressure by accumulating organic compatible solutes in the cytoplasm (Roberts, 2005). Besides, most of the extreme halophiles belong to the domain of Archaea, whose optimum salt requirements are usually more than 25 % w/v dissolved salts (Bowers and Wiegel, 2011). Among those, Haloarchaea have a higher content of acidic amino acids, and a lesser amount of hydrophobic amino acids in their enzymes and proteins, respectively (Oren, 2002; Leigh *et al.*, 2011).

Table salt or very salty food are sources of halophiles especially halophilic bacteria, a part of which may originate from the original location from which salt was extracted. Considering this, processed food can be seen as a habitat for halophiles provided that salt levels are high (Lee, 2013). Indeed, halophiles are found in fermented seafood (Das *et al.*, 2020), cheeses (Kothe *et al.*, 2020), sauces (Sagdic *et al.*, 2017; Ohshima *et al.*, 2019), green table olives (Randazzo *et al.*, 2017) and pickles (Stoll *et al.*, 2020). Some halophilic and halotolerant microorganisms can be established in the human gut microbiome, and the gut halophilic microbiota is considered to be linked to some chronic diseases (Seck *et al.*, 2019). In patients with obesity, Type 2 diabetes, celiac disease or inflammatory bowel disease (IBD), an increased frequency of these microorganisms has been detected in samples from the intestine, colon and stool, compared to healthy individuals (Seck *et al.*, 2019; Biswas and Rahaman, 2020).

There are very few reports on the microbiome of table salt, and they tend to focus on the archaeal taxa (Henriet *et al.*, 2014; Gibtan *et al.*, 2017). The present work describes a complete characterization of the bacteriome of six table salts by using culture-dependent and

-independent techniques. We have studied the microbial contents of different commercial table salts, from regular ones to some with added spices or minerals, and from salts of marine origin to some originating from salt mines and identified the distribution of both archaea and bacteria isolates and sequences among the studied salts.

Materials and Methods

Table salt samples: Seven commercial table salts were analyzed in this study. Salts were purchased from local supermarkets (el Corte Inglés and Mercadona, Valencia, Spain) and stored at room temperature in a dark and dry place. These salts can be grouped in salts from marine origin (Atlantic salt, Ibiza salt and supermarket marine salt); larger granulated salts supplemented with other ingredients (two Black salts and Viking salt), and salt from inland salt mines (Himalayan pink salt). Visual characters of food-grade salts such as color, size of their particles, origins and other features are shown in Supplementary Table and Fig. 1. In order to have the homogenized microbial diversity all over the packages, salt particles were thoroughly mixed before sampling.

Solutions and media: The following media and solutions were used to isolate different halotolerant and halophilic microorganisms based on their salt requirements. SMM and saline R2A are supplemented with approximately 100 g^{-1} total salt, while their nutritional compositions are different. SM15 contains almost 170 g^{-1} total salt. Modified DBCM2 and DBCM2 have 25 and 250 g^{-1} total salt, respectively.

Sea salt solution (g^{-1}): NaC1, 81; $MgC1_2$, 7.0; $MgSO_4$, 9.6; $CaCl_2$, 0.36; KCI, 2.0; $NaHCO_3$, 0.06; NaBr, 0.026. Sea salt solution contains almost 10 % (w/v) total salts (Rodriguez-Valera *et al.*, 1981).

Sea Water (SW) solution (g^{-1}): NaC1, 240; $MgC1_2 \cdot 6H_2O$, 30.0; $MgSO_4$, 35.0; KCI, 7.0; $NaHCO_3$, 0.2; NaBr, 0.8, 1 M Tris.Cl, pH 7.5. SW solution contains approximately 30 % (w/v) total salts (Dyall-Smith, 2009).

SMM medium (g^{-1})**:** NaCl, 46.8; $MgCl_2.6H_2O$, 19.5; $MgSO_4.7H_2O$, 30.5; $CaCl_2$, 0.5; KCl, 3.0; $NaHCO_3$, 0.1; NaBr, 0.35; Casein digest, 5.0 and Sodium pyruvate, 1.1, Agar;15.

Saline R2A medium (g^{-1}): Peptone, 1; Yeast extract, 0.5; Dextrose, 0.5; Soluble starch, 0.5; K_2HPO_4, 0.3; $MgSO_4$, 0.05; Sodium pyruvate; 0.3 which is supplemented with 10 % sea salts solution (Rodriguez-Valera *et al.*, 1981).

SM15 medium(g^{-1})**:** NaCl, 117; $MgCl_2.6H_2O$, 19.5; $MgSO_4.7H_2O$, 30.5; $CaCl_2$, 0.5; KCl, 3.0; $NaHCO_3$, 0.1; NaBr, 0.35; Casein digest, 5.0; Yeast extract, 2.5; Sodium pyruvate, 1.1; and Glucose, 1.0, Agar; 18 (León *et al.*, 2014).

DBCM2 medium: DBCM2 medium was prepared by mixing 833 ml 30 % SW solution with 167 ml low nutritional solution with the following compositions: Sodium pyruvate 0.11 g, Glucose; 0.0025 g, Peptone; 0.0125 g, yeast extract; 0.0125 g, and Agar; 20.0 g (Henriet *et al.*, 2014).

Modified DBCM2 medium: This medium was prepared by mixing 833 ml 0.1X SW solution with 167 ml of the same low nutritional solution and Agar. This medium is optimized for the growth of halotolerant bacteria.

The media were selected based on the similarity of their minerals to the seawater and the pH of all was adjusted to 7.5±0.05 with 1 M KOH or 1M HCl.

Moreover, two following Modified Growth Media (MGMs) were specially used to isolates haloarchaea. Modified Growth medium supplemented with 23 % and 25 % SW solutions were prepared by mixing 767 ml and 833 ml 30 % SW solution with 200 ml and 134 ml Pure water, respectively. Each medium is supplemented with (g^{-1}): Peptone (Oxide), 5.0; Yeast extract, 1.0; and Agar, 15.0. The pH of the MGM media was adjusted to 7.5 with 1 M Tris.Cl pH7.5 and its final volume adjusted to 1000 ml. The only difference between these two media is the total salt concentrations. MGM with 23 % salt is an appropriate medium to recover various halobacteria. However, genus *Halobacterium* can be retrieved on 25 % MGM (Dyall-Smith, 2009).

Culture-dependent approach: For culture-based analysis, 10 g of each salt was added to 40 ml sterile Milli-Q H_2O in a 50 ml Falcon tube [final concentration of the saline solution: 25 % (w/v)]. Salt was dissolved by vortexing and then the saline solution (except saline solution from Viking salt) was centrifuged for 45 min 6000 rpm at 4 °C, and the pellets were resuspended in 2 ml phosphate-buffered saline (PBS) buffer (1x, pH 7.4). Serial dilutions were prepared by resuspending 10 µl of the bacterial suspension in the PBS buffer (diluted 1:10) and plated on SMM, Saline R2A, SM15, DBCM2, modified DBCM2, MGMs. The plates were sealed with parafilm and incubated at 30 °C (for 23 % and 25 % MGM media, at 37 °C) for at least ten weeks. Other saline solutions such as PBS buffer and sea salt solution [10 % (w/v) total salts] were also used for isolation of bacteria and archaea from table salts.

In the case of saline solution from Viking salt, it was centrifuged at 500 rpm for 10 min at 4 °C, and its bulky insoluble particles were removed. Then, the supernatant was transferred to another 50 ml Falcon tube and the extraction was continued as described above.

Individual colonies were selected based on the pigmentation and morphology of the colonies, and they were re-streaked on fresh media. Finally, glycerol stocks were prepared from the fresh biomass from the plates in 20 % glycerol (v/v) supplemented with a saline medium and stored at –80 °C.

Universal 16S rRNA gene primers 8F (5’-AGA GTT TGA TCC TGG CTC AG -3’), 1492R (5’- CGG TTA CCT TGT TAC GAC TT -3’) for bacteria; and A616D (5’- CGK TTG ATC CTG CCG GA -3’), and P1525R (5’-WAG GAG GTR ATC CAD CC -3’) for Archaea were used for PCR. Qiagen master mix 2X was used for all reactions. A loopful of a fresh culture growing on solid medium was resuspended in 100 µl of Milli-Q H_2O. The suspension was pre-incubated at 100 °C for 10 min and 1 µl of the suspension used as the DNA template. PCR for bacteria and archaea was performed based on the following program: Initial step of incubation at 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C (30 s), annealing at 50 °C (30 s) and an extension step at 72 °C (1 min 30 s). The last stage was the final extension step at 72 °C for 10 min. The amplification of the targeted gene fragment was monitored by 1 % agarose gel electrophoresis. Then, dsDNA was purified from the PCR products and resuspended in 10 µl MilliQ H_2O. Sanger sequencing was performed by tagging with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), at the Sequencing Service (SCSIE) of the University of Valencia. All sequences were edited and compared with the EzBioCloud online database (https://www.ezbiocloud.net) for bacteria and Archaea.

Culture-independent approach: DNeasy PowerSoil Pro Kit (Qiagen, Germany) was used for DNA extraction from food-grade salt samples. In preparation steps, 20 g of each sample was completely dissolved in 100 ml sterile Milli-Q H_2O (20 % w/v) and centrifuged for 45 min, at 6000 rpm and 4 °C to collect microbial cells. The pellets were resuspended in 100 µl solution C1, transferred into two-ml bead tubes and the extraction carried out according to the manufacturer instructions. As a negative control, 100 mL sterile Milli-Q H_2O (salt-free) were centrifuged, and DNA extraction was carried out in the same way that DNA extraction for the saline samples. The quantity of the extracted DNA was analyzed through the Qubit dsDNA HS Assay kit (Qubit 2.0 Fluorometer, Q32866).

For 16S rRNA gene sequencing analysis, the V3-V4 and V4-V5 regions were targeted for Bacteria and Archaea, respectively. Primers 341F (5’- CCT AYG GGR BGC ASC AG -3’) and 806R (5’- GGA CTA CNN GGG TAT CTA AT -3’) were used to amplify the bacterial 16S rRNA gene, while primers Arch519F (5’-CAG CCG CCG TAA -3’) and Arch915 (5’-CGT GCT CCC CCG CCA ATT CCT -3’) were used for the archaeal 16S rRNA gene (Klindworth *et al.*, 2013). All PCR reactions were carried out with Phusion High-Fidelity PCR Master Mix (New England Biolabs). PCR products were mixed at equal density ratios. The pool was then purified with the Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated with NEBNext UltraTM DNA Library Prep Kit for Illumina

and quantified via Qubit and q-PCR. Finally, the NovoSeq 6000 Sequencing System (2 × 300 bp) was employed for sequencing the samples at Novogene (Cambridge, UK). Raw Illumina reads were analyzed via Qiime2 software (v. 2020.8) (Bolyen *et al*., 2019). The Demux plugin was used to assess the quality of reads, and the Qiime2-integrated DADA2 pipeline was employed for trimming and joining the sequences, removing chimeras, and detecting amplicon sequence variants (ASVs; > 99.9 % of similarity). The classify-Sklearn module (feature-classifier plugin) was applied to assess the taxonomy of each sequence variant, using the SILVA (v. 138) database as a reference.

Data were subsequently analyzed by using different R packages. Rarefaction curves were constructed with the iNEXT and ggiNEXT functions (iNEXT) (Hsieh *et al*., 2016). PCoA was created with the plot_ordination function (phyloseq) (McMurdie and Holmes, 2013) using Bray-Curtis dissimilarities as a distance method. Taxonomic barplots were created with the following R libraries: ggplot2, forcats and tidyr.

Results

Culture-dependent approach: A strain collection was set by culturing table salt samples on a range of saline media, with a broad range of salinity and different nutritional and trace elements, and after incubation at 30 and 37 ºC for more than two months (see material and methods section). This allowed us to isolate 79 different strains from 28 bacterial and archaeal taxa (Supplementary table 2). Comparing the culturomics results with the previous studies, of those, 27 isolates belonged to halotolerant and slightly halophilic bacteria; 45 strains to moderately halophilic bacteria; and one genus corresponded to extremely halophilic bacteria; whereas six archaeal strains were isolated and identified. Bacteria were mostly isolated during the first four weeks of incubation, while the archaeal colonies were in general more frequent within the small colonies that were only noticeable after five weeks of incubation. Interestingly, all strains were isolated from table salts by dissolving salt samples in sterile Milli-Q H_2O. Using other saline solutions was not efficient enough to recover this vast microbial biodiversity. However, most bacteria were isolated by this method, especially slightly and moderately halophilic bacteria were spore-forming strains. Surprisingly, no colonies from the Himalayan black salt were observed in any media, while only three genera could be isolated from the Hawaiian black salt on saline R2A media with 10 % (w/v) total salt concentration. Most of the bacterial isolates from Viking salt were isolated on the media with less than 17 % (w/v) total salt. Regarding bacterial isolates from media with very high salt concentrations, *Acinetobacter* sp. was the only genus isolated on the MGM with 23 % (w/v) NaCl. From Ibiza salt, a few extremely halophilic bacteria were isolated on the DBCM2 medium. Genus *Bacillus* was the most frequent taxon isolated on four of the media.

The microbial diversity of the commercial table salts by using the various media is shown in Fig. 1. Surprisingly, no *Bacillus* sp. was identified among the colonies growing on the media with high salt concentrations (DBCM2 and MGMs), while *Salibacterium* sp. and *Pontibacillus* sp. were mostly isolated on media with more than 20 % salinity. More interestingly, *Thalassobacillus* sp. was identified in isolates from a broad range of salinity (2.5–25 % (w/v) salt concentrations). Genus *Lentibacillus* was only recovered from the moderate and hyper-saline media (SM15, DBCM2 and MGMs). By using MGM supplemented with 23 % (w/v) salt, we were able to recover verity of halobacteria such as *Virgibacillus*, *Oceanobacillus*, *Halobacillus*, and *Acinetobacter,* while none of those genera recovered on 25 % MGM. *Salinibacter* sp. was isolated only from the DBCM2 medium, while genera *Aquibacillus* and *Terribacillus* were isolated from saline R2A and SM15 media, respectively. Some potential pathogens, *Cytobacillus* sp. and *Peribacillus* sp., were also isolated from the media with a 2.5 % (w/v) total salt concentration. Finally, human-associated bacteria such as *Fictibacillus* and *Dermacoccus* were found on SMM and saline R2A media, respectively.

The genus *Bacillus*, with more than 50 isolates, was the most abundant genera in all

table salt samples and was particularly frequent among the Viking salt isolates (Fig. 2).

Genera *Halobacillus* and *Lentibacillus* were also frequent genera with 14 isolates, and they were isolated from three different commercial salts. However, both genera were the most frequent taxa recovered from the Himalayan pink salt sample with 8 and 12 isolates, respectively. *Thalassobacillus* sp. was another abundant genus with 13 isolated bacteria in our collection. This genus was identified from four commercial salts and was not isolated from Black salt nor Viking salt. Genera *Salibacterium* and *Metabacillus* were isolated from Himalayan pink salt and Ibiza salt with 9 and 7 culturable isolates, respectively. *Lentibacillus* sp. and *Oceanobacillus* sp. were also recoverd from Ibiza salt and Himalayan pink salt. At the genus level, *Aquibacillus* sp. and *Gracilibacillus* sp. were only isolated from supermarket salt and Himalayan pink salt, respectively, while genus *Terribacillus* was only found in the Viking salt.

The highest bacterial diversity was observed within the strains isolated from the Himalayan pink salt, followed by Atlantic and Ibiza salts. Although the microbial compositions of these salts were different, the shared microbial profile among these three samples were *Bacillus* sp., *Cytobacillus* sp. and *Thalassobacillus* sp. Moreover, some genera; *Pontibacillus* sp., *Acinetobacter* sp. and *Piscibacillus* sp., were only isolated from the Atlantic, Ibiza and Himalayan pink salt, respectively. Interestingly, haloarchaea *Halorubrum* sp. and extremely halophilic bacteria *Salinibacter* sp. were only isolated from the Ibiza salt beside other bacteria; *Acinetobacter*, *Alkalihalobacillus*, *Bacillus*, *Cytobacillus*, *Halorubrum*, *Lentibacillus*, *Metabacillus*, *Oceanobacillus*, *Peribacillus* and *Thalassobacillus*. Moreover, seven genera were recovered from the supermarket marine salt: *Alkalihalobacillus*, *Aquibacillus*, *Bacillus*, *Brevundimonas*, *Metabacillus*, *Thalassobacillus*, and *Virgibacillus*, whereas Black salt displayed the lowest microbial diversity of culturable strains from genera *Bacillus*, *Halobacillus*, and *Staphylococcus*.

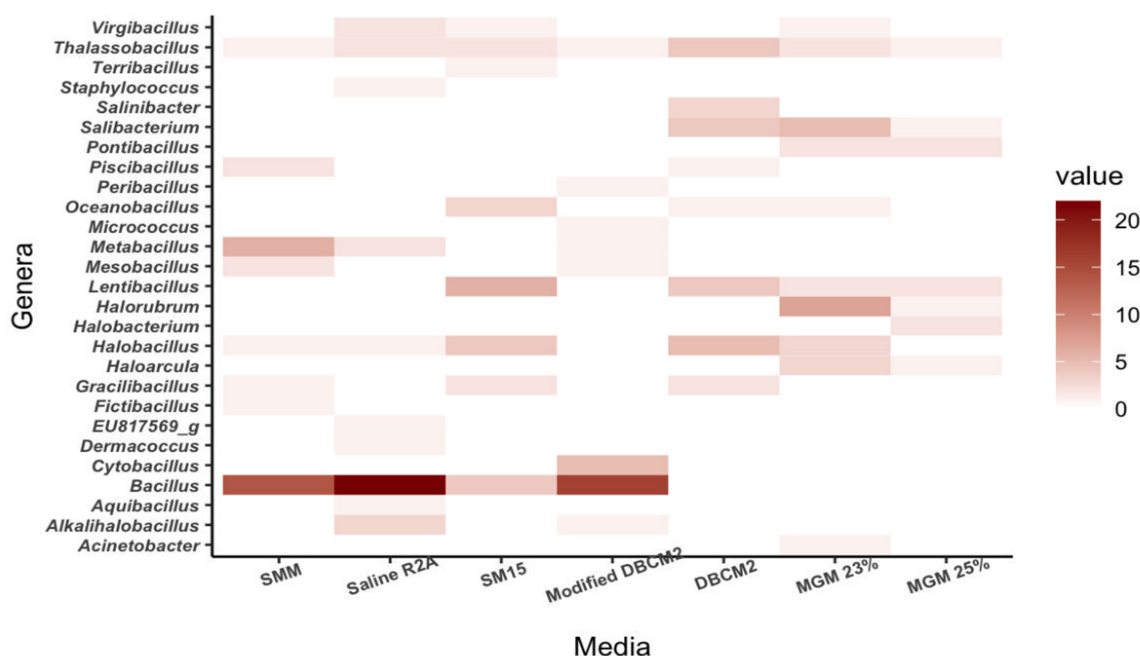

Figure 1. Bacterial and archaeal genera from table salts recovered on culture media with different salt concentrations and nutritional compositions. SMM and Saline R2A are supplemented with 10 % w/v total salt, while their nutritional compositions are different. SM15 contains 17 % w/v total salt. Modified DBCM2 and DBCM2 have 2.5 % w/v total salt, 25 % w/v total salt, respectively. Also, MGM was especially used for isolation of archaea supplemented with 23 % and 25 % w/v total salt. The color code indicates the number of microorganisms isolated on each medium.

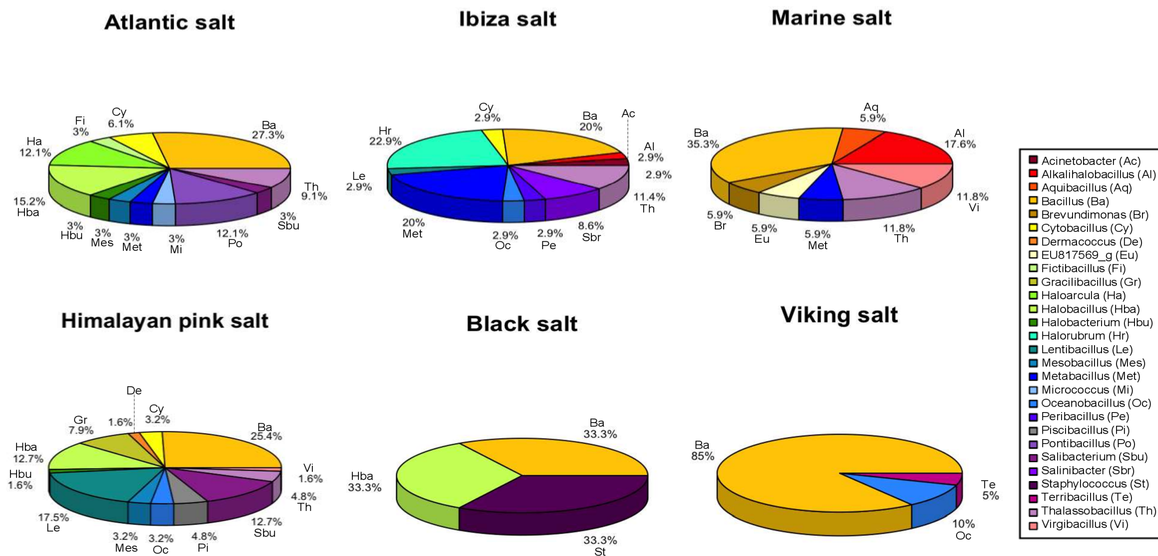

Figure 2. Microbial profiles of six table salts samples based on the identification of culturable isolates. The percentage indicates the value was calculated based on the total number of isolates in each table salt.

Finally, haloarchaea were isolated from three commercial table salts. Genus *Halobacterium* was isolated from Atlantic salt and Himalayan pink salt. However, *Haloarcula* sp. and *Halorubrum* sp. were only found in Atlantic and Ibiza salts, respectively. Taxon *Halorubrum,* with eight phylotypes belonging to three species, was the most frequent archaea in the Ibiza salt.

Culture-independent approach: Of seven selected commercial table salts, we were able to extract a sufficient amount of DNA in six of them. These six commercial salts, labelled as Atlantic salt, Ibiza salt, Himalayan pink salt, Hawaiian Black salt, Viking salt, and supermarket marine salt, are shown in Supplementary Fig. 1 and were analyzed through 16S rRNA gene sequencing. By using the Archaea-specific primers (see material and methods section), both Archaea and Bacteria were detected in the food-grade salts. The archaeal and bacterial diversity of the six table salts showed that the Ibiza salt sample displayed the highest biodiversity, with 335 identified amplicon sequence variants (ASVs) in total. In general, the main archaeal genera were *Halorubrum* (37.4 % in Atlantic salt), *Natronomonas* (12 % in Ibiza salt), *Halobellus* (11.2 % in Atlantic salt), *Haloquadratum* (9.2 % in Ibiza salt), *Haloplanus* (7 % in Atlantic salt) and *Halonotius* (7.5 % in supermarket marine salt). Other frequent archaea genera present in all samples were: *Natronomonas*, *Halolamina*, *Halonotius*, *Halapricum*, *Halobacterium*, *Haloarcula* and Uncultured Halobacterales (Fig. 3A). Salt samples from the Atlantic Ocean and Mediterranean Sea (Ibiza Island) were mainly composed of archaea (approximately 90 % and 70 % of the total microbial compositions, respectively). On the other hand, the supermarket marine salt sample (a salt whose production comes from the Odiel marshes in the province of Huelva, close to the Atlantic Ocean), displayed a high presence of bacterial genera such as *Salinibacter, Flavobacterium* or *Yoonia-Loktanella* with a frequency of 11.3 %, 12.9 % and 6.2 %, respectively.

The Himalayan pink salt, Black salt and Viking salt presented a taxonomic profile dominated by Bacteria. More than 85 % of their taxonomic composition corresponded to genera such as *Sulfitobacter*, *Flavobacterium*, *Bacillus*, *Enterococcus* or *Salinibacter*. However, it should be noted that genus *Sulfitobacter,* which was found in all these three samples, was very abundant (57.5 %) in the Himalayan pink salt, while in the others it did not exceed 3 %. Regarding the genus *Salinibacter,* we observed that it was more frequent in samples from the Mediterranean Sea than in the remaining salts. Viking salt also contained chloroplast sequences, as this salt has pepper and dry onion as ingredients.

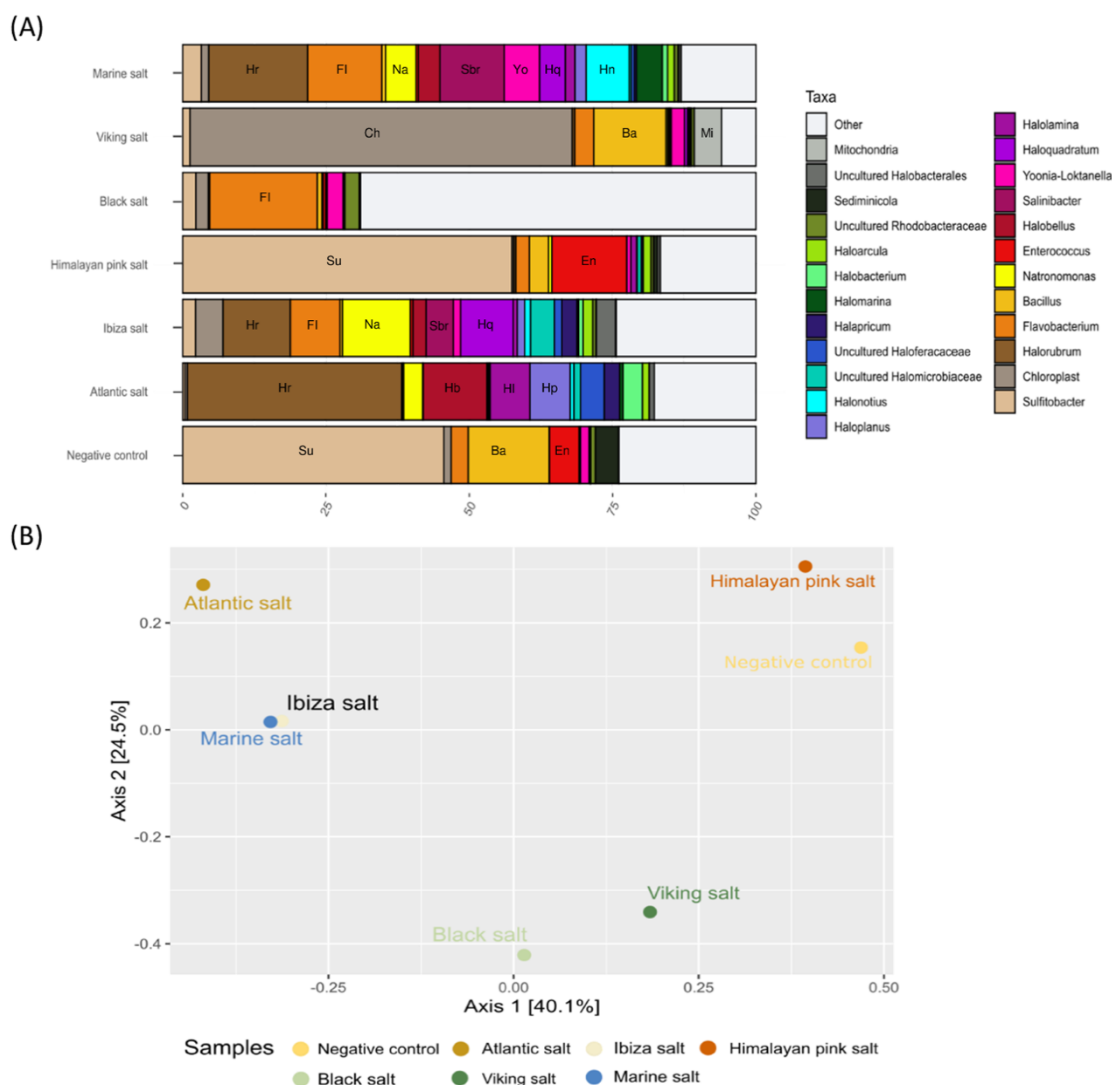

Figure 3. (**A**) Microbial composition of table salt samples after 16S rRNA gene sequencing. (**B**) Beta diversity (PCoA) based on Bray Curtis dissimilarity metric. Distances to the linear statistical correlation indicate the similarity of the microbial diversity of each sample affected by the origin of those salts.

Beta diversity analysis (PCoA) showed that samples tend to form three different groups (Fig. 3B). The first groups were composed by salts with mainly marine origins. The second group consisted of salts of non-marine origins. Finally, the Himalayan pink salt shared a similar taxonomic profile with the negative control for some genera. This clustering suggests that there is a correlation between the taxonomic profile similarities and the origin of the salt among pure marine salts.

We further investigated the distribution among the samples of Archaea and Bacteria with an average abundance higher than 1 % (Fig. 4). The variation in the taxonomic profiles was higher in the case of archaea than for bacteria. The higher number of Archaea was detected in samples coming from the Atlantic Ocean, the Ibiza Island and from the Odiel marshes. The most abundant taxon in Atlantic, Ibiza and supermarket marine salts was *Halorubrum,* followed by *Hallobellus*, *Natronomonas, Haloplanus* and *Haloarcula* (Fig. 4A). The genus *Natronomonas* presented a relative abundance of 11.8 % in the Ibiza salt sample. Nevertheless, in the other marine-salt samples, the abundances ranged between 3.3 % and 5.3 %. Finally, the genus *Haloarcula* was present in all three types of salt, but with values around 1.6 %.

Other relevant genera included *Halonotius* and *Halomarina,* which were especially abundant in the supermarket marine (7.5 % and 4.5 % of relative abundance) salt. In

addition, genus *Halolamina* (7 % in the Atlantic salt sample), and to a lower extent, an uncultured *Haloferacaceae* were detected. For the Ibiza salt sample, the genus *Haloquadratum* was the most abundant Archaea (9.2 %), followed by *Halobacterales* (5.6 %). Interestingly, the Himalayan pink salt was the unique non-marine sample in our collection where some Archaea were detected. These genera were *Halolamina* (as in the Atlantic and supermarket salt sample), *Haloparvum* (1 %) and some genera from family *Halobacteriaceae* (2.9 %).

Main bacterial genera for each sample were also analyzed (Fig. 4B). Except for *Sulfitobacter* and *Flavobacterium*, the rest of the genera displayed less than a 20 % abundance. The most frequent bacterial genera were *Sulfitobacter* (present in all samples, except in the Atlantic salt), *Bacillus* (12.7 % in Viking salt, but similar in Negative control), *Enterococcus* (13 % in Himalayan pink salt), Flavobacterium (mainly in the Black salt sample), and Salinibacter (only in the marine-like salts). It should be noted that the Atlantic was the only marine salt sample where no bacterial genera was over 1 % of relative abundance. The supermarket marine and the Ibiza salts were rich in the following genera: *Flavobacterium* (12.9 % and 8.6 %), *Salinibacter* (11.3 % and 4.8 %), *Yoonia-Loktanella* (6.2 % and 1.3 %), and *Sulfitobacter* (3.3 % and 2.3 %). The genus *Sphingorhabdus* was present in the Ibiza sample and in the Himalayan pink salt, a salt whose microbiome was mainly composed of *Sulfitobacter* (57.5 %) and *Enterococcus* (13 %).

Since the overall DNA concentration obtained from the salts was very low, a negative control of the extraction was also included and sequenced in order to shed light on the possible contaminations in the results of the salt samples.The negative control displayed high frequencies of the genera *Sulfitobacter* (45.6 %), *Bacillus* (14.1 %), *Enterococcus* (5.2 %), and, to a lesser extent, the genera *Flavobacterium, Yoonia-Loktanell.,* uncultured *Marinococcaceae*, *Halobacillus* and *Stenotrophomonas,* with relative abundances between 2.3 % and 4 %. Some genera were only found in some salts. For example, genus *Planococcaceae* was only detected in Viking salt, while Uncultured *Comamonadaceae*, *Dinghuibacter*, *Aminicenantales*, *Lysobacter*, *Thermus*, the clade candidates Sva0485 (deltaproteobacteria) and GN01 were only present in Black salt.

The proportion of Archaea in the original sample is usually lower than the abundance of Bacteria. Besides archaeal- specific primers, bacteria-specific primers were used to study both bacterial and archaeal communities. Although, there is a bias by using archaea-specific primers for amplifying bacteria and vice versa, results showed that bacterial profiles obtained by using archaea- and bacteria-specific primers were comparable. Nevertheless, the relative abundance of some genera differed due to the amplification bias. The main bacterial and archaeal genera identified with the bacteria-specific primers for the Viking, Ibiza and Atlantic salts are also shown in Supplementary Fig. 2.

Discussion

We report here a complete characterization of the bacterial and archaeal communities of six table salts by 16S rRNA gene sequencing analysis and culturomics. NGS analysis revealed that the three marine salts were rich in archaea; while Himalayan pink salt, Black salt, and Viking salt were rich in bacteria. It was evidenced that our collection of samples could be divided into two groups, one made up of salts from marine environments where the main presence of archaea stood out, and a group made up of non-marine salts with high relative abundances of bacteria.

The total DNA extraction for 16S rRNA gene we carried out resulted in very low amounts of DNA even after several steps of enrichment. Thus, we extracted the DNA from the salt-free sterile Milli-Q H_2O as the negative control, which was sequenced side by side with other samples, to analyze the kit possible microbial contamination and to identify possible background contaminations of the salt samples. The results for the negative control showed that *Sulfitobacter* sp., *Enterococcus* sp., *Sediminicola* sp., *Bacillus* sp., and *Flavobacterium* sp. were the most frequent taxa. These bacteria have previously been

reported as contaminants in DNA extraction kits and sequencing reagents (Glassing *et al*., 2016; Stinson *et al*., 2019). Other works have also found *Flavobacterium* sp. and *Bacillus* sp. in pure *Salmonella bongori* cultures, linked to contamination of the reagents (Salter *et al*., 2014). In the case of the genus *Sulfitobacter*, it seems likely that, when the control sample was processed alongside salt samples, the sample material contaminated the negative control, similarly to what previously observed (Edmonds and Williams, 2017); thus, this genus has a similar abundance with the Himalayan pink salt sample.

At the genus level, *Acinetobacter*, *Aquibacillus*, *Bacillus*, *Brevundimonas*, *Fictibacillus*, *Gracilibacillus*, *Halobacillus*, *Micrococcus*, *Oceanobacillus*, *Salibacterium*, *Salinibacter*, *Terribacillus*, *Thalassobacillus* (Bacteria) and *Haloarcula*, *Halobacterium*, and *Halorubrum* (Archaea) were identified with both culture-dependent and -independent methods.

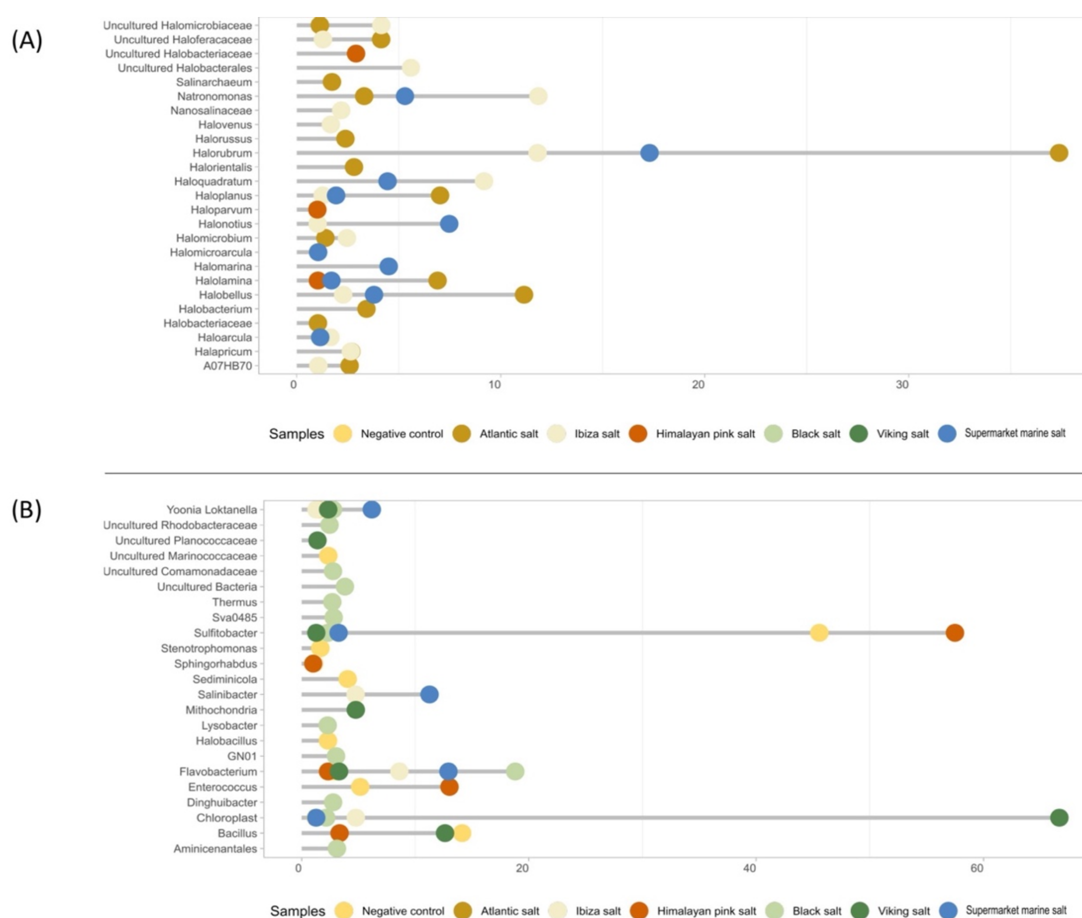

Figure 4. Main archaeal composition (**A**) and bacterial composition (**B**) of each table salt sample based on the 16S rRNA gene metagenomic analyses with archaea-specific primers. Only values higher than 1 % are shown to explain the biodiversity of the samples.

Microbiome sequencing revealed that those salts from marine origin shared a rather similar taxonomic profile. Archaeal taxa *Halorubrum*, *Halobacterium*, *Hallobellus*, *Natronomonas,* *Haloplanus*, *Halonotius*, *Halomarina*, and *Haloarcula*, were prevalent in these three salts. The presence of *Halorubrum* sp., *Halobacterium* sp., *Haloarcula* sp., and *Halonotius* sp. were previously reported in the taxonomic profile of La Baleine Coarse sea salt, Palm Island black salt (USA), food-grade salts from the Dead Sea (Israel) and Black Sea (Turkey) (Henriet *et al*., 2014). Moreover, a high diversity of archaeal genera *Halorubrum, Halobacterium, Haloarcula, Halonotius* and *Natromonas* in two Korean marine salts were previously reported (Gibtan *et al*., 2017). The presence of other archaeal taxa, such as *Halarchaeum*, *Halomicrobium*, and *Salarchaeum*, were also detected in those

Korean salts. This strongly suggests that the origin of salt plays a crucial role in establishing a similar microbial (mainly archaeal) population in table salts. Himalayan pink salt, Hawaiian black salt and Viking salt showed more heterogeneous taxonomic profiles. Non-marine salts are usually enriched by additional minerals or flavors such as Iron, hydrogen sulphate, activated charcoal and dry vegetables. Previous research by Henriet *et al.* (2014) also confirmed that the archaeal diversity in Himalayan pink salt is lower compared to marine salts. Further, genus *Sulfitobacter,* which belongs to the family *Rhodobacteraceae*, has previously been isolated from various habitats like the Mediterranean Sea, hypersaline lakes, and organisms such as starfish and sea-grass (Prabagaran *et al*., 2007). *Sulfitobacter* sp. was the most abundant genus in Himalayan pink salt, (57.5 % of the microbial population). This genus was previously detected in various marine ecosystems and considered as sulphur-oxidizing chemoheterotrophic species (Yoon, 2019). Although 16S rRNA gene sequencing results showed *Sulfitobacter* sp. in Himalayan pink salt, no viable bacteria from this genus were isolated by culture-based methods. A possible reason for that may link to the lack of particular ions or minerals in the prepared media, which could affect the growth of some species.

Based on microbiome sequencing results, *Halorubrum* sp. is the most abundant Archaea in the Atlantic salt, followed by *Halobellus*, *Halolamina* and *Haloplanus*. All these genera (belonging to the class *Halobacteria*) are present in the other salts originated from marine environments. *Halobacteria* is known to actively participate in processes of gene transfer and homologous recombination, and the population of the taxa in this class can increase very easily in saline and hyper-saline area (Fullmer *et al*., 2014). As this taxonomic group is predominant in aquatic environments, it is not surprising we detected these taxa within the Atlantic salt.

In the salt sample from Ibiza, the genus *Natronomonas* was the most frequent and abundant taxon, followed by *Haloquadratum*. The latter is frequent in solar salterns and is an extremely halophilic. Only one species of this genus has been described so far, and it was isolated from Spain and Australia (Burns *et al*., 2007). Moreover, the previous research reported that *Haloquadratum* often grows in a company of some extremely halophilic bacteria such as *Salinibacter* (Antón *et al*., 2013), which we also identified together with *Haloquadratum* in samples from the supermarket and Ibiza salts. Genus *Salinibacter* has been isolated from saltern crystallizer ponds in Mediterranean locations such as Alicante and the Balearic Islands (Antón *et al*., 2002), as well as in other extremely salted areas in Argentina (Viver *et al*., 2018) and Iran (Makhdoumi-Kakhki *et al*., 2012). Interestingly, this bacterium shares some archaeal properties of the family *Halobacteriaceae* that thrive in the same habitat (Oren, 2013). In summary, *Halorubrum*, *Natronomonas,* *Haloquadratum* and *Salinibacter* seem to be microbial markers for the salt samples obtained from marine environments.

The non-sea salt samples presented a taxonomic profile dominated by Bacteria. The Himalayan salt was not isolated from the sea; it was extracted from foothills in the Himalayan Mountain range, that once were seas. The most important genus in this sample was *Sulfitobacter*. It might be hypothesized that *Sulfitobacter* appears in such a significant amount in this sample due to the activity that this genus shows to oxidize sulfites (Sorokin, 1995), which are present in high concentrations in the Himalaya regain (Roy *et al*., 2020). Furthermore, this genus has previously been isolated from deep seawater (Song *et al*., 2019), where salinity conditions are similar to those in the Himalayan.

In the Black salt, the genus *Flavobacterium* stood out with 18.8 % of abundance, a similar value to that detected in the supermarket marine salt. Other frequent halophilic bacteria were *Sulfitobacter*, *Yoonia-Loktanella* or uncultured *Rhodobacteraceae.* The *Roseobacter* (*Yoonia-Loktanella*) group is a clade within the family *Rhodobacteraceae* that comprises a significant fraction of the total bacterial community on the ocean surface (Wirth and Whitman, 2018).

Another sample of the non-marine group is the Viking salt, which presents a high frequency for Chloroplasts (more than 66 % of its taxonomic profile) and Mitochondria (4.7 % of the total), which may be a consequence of its actual composition (it contains different vegetables). The genus *Bacillus* was also present in this sample. This genus contains certain salt-tolerant species (Sharma *et al*., 2015). Finally, both the Viking salt and Black salt presented very similar frequencies for *Sulfitobacter* or *Yoonia-Loktanella*.

Table salt could theoretically be carrier of human pathogens or health-promoting, probiotic strains, although the sensitivity of both to high NaCl concentrations has been previously reported (Ragul *et al*., 2017). In fact, by using 16S rRNA gene sequencing, some potentially pathogenic microbes such as *Enterococcus* sp. and *Brevundimonas* sp. were identified in almost all samples, whereas *Pseudomonas* sp. and *Lactobacillus* sp. were only detected in some table salts. No viable species from genera *Enterococcus* and *Lactobacillus* were isolated by culture-based methods. However, several other potential pathogens from different taxa were recovered in this research. From those, *Brevundimonas diminuta* and *Bacillus circulans* isolated from Himalayan pink salt and supermarket salt, respectively. They were previously reported as the potent pathogens causing infection in immunocompromised and cancer patients (Han and Andrade, 2005; Alebouyeh *et al*., 2011).

Another interesting, yet poorly explored, connection between the microbial contents of the table salt and consumers health is the presence of halophilic and halotolerant taxa in the human gut, whose origin has not been determined to date, to the best of our knowledge. Indeed, some halophilic and halotolerant microorganisms have previously been described by Seck *et al*. as the "Human halophilic microbiome" (Seck *et al*., 2018). At the genus level, it includes: *Alkalibacterium* sp., *Bacillus* sp., *Chromohalobacter* sp., *Flavobacterium* sp., *Gracilibacillus* sp., *Halobacillus* sp., *Haloferax* sp., *Halomonas* sp., *Kocuria* sp., *Methanomassiliicoccus* sp., *Nesterenkonia* sp., *Oceanobacillus* sp., *Paenibacillus* sp., *Paucisalibacillus* sp., *Planococcus* sp., *Planomicrobium* sp., *Pseudomonas* sp., *Salinisphaera* sp., *Sciscionella* sp., *Shewanella* sp., *Sporosarcina* sp., *Terribacillus* sp., *Thalassobacillus* sp., *Vibrio* sp., and *Virgibacillus* sp.. Another research by Oxley *et al*. reported that haloarchaea, beside methanogenic archaea, can be considered as members of the human microbiome, especially on the mucosa (Oxley *et al*., 2010). Of all these taxa, we identified in at least one of the table salts analyzed the genera: *Gracilibacillus* sp. (Himalayan pink salt), *Halobacillus* sp. (Atlantic salt, Himalayan pink salt and Hawaiian black salt), *Oceanobacillus* sp. (Ibiza salt, Himalayan pink salt and Viking salt), *Terribacillus* sp. (Viking salt), *Thalassobacillus* sp. (Atlantic salt, Ibiza salt, Himalayan pink salt and supermarket salt), and *Virgibacillus* sp. (Himalayan pink salt and supermarket marine salt). Although the present work was not designed to confirm the colonization of the human gut by these taxa, it is clear that table salt can be a source of some of the halophilic bacteria that characterize either healthy or diseased human guts (Seck *et al*., 2018, 2019).

Some archaeal products such as bacterioruberin, bacteriorhodopsin (BR), diether, and tetraether lipids are commercially available (Pfeifer *et al*., 2021). Moreover, the most common industrial enzymes (proteases, amylases, lipases , esterase) produced also by haloarchaea (Singh and Singh, 2017). Besides archaea, moderately and extremely halophilic bacteria can produce industrial hydrolytic enzymes. In this work, we have isolated some halophilic archaea and bacteria previously reported as excellent candidates for biotechnological applications. *Haloarcula hispanica* and *Haloarcula marismortui* both belonged to archaea (*Haloarcula sp.*) are considered as carotenoids and poly(3-hydroxybutyrate) (PHB) producers. PHB is commercially interesting as a primary substance for producing biodegradable plastics. Among the bacteria from our collection, *Bacillus megaterium*, isolated from Ibiza and Himalayan pink salt, is also considered a PHA producer (Mohanrasu *et al*., 2020). Further, *Bacillus flexus* was previously reported as species can degrade Polyvinyl chloride (PVC),

which is one of the most abundant petroleum-derived plastics on the earth (Giacomucci *et al*., 2019). Also, *Brevibacterium frigoritolerans* was isolated from Ibiza salt previously reported as a bacterium with the potential to biodegrade organophosphorus insecticides such as of phorate (Jariyal *et al*., 2015). Moderately halophilic bacterium, *Thalassobacillus devorans,* is also a promising candidate for the phenol biodegradation (Garcia *et al*., 2005).

Taken together, our results show that table salts harbor a relatively diverse set of halophilic microorganisms. Salts of marine origin were dominated by archaeal lineages whereas salts from salt mines or salt with added ingredients were dominated by bacteria. An important fraction of the identified microorganisms was culturable, and the biotechnological potential of some of them, as well as the potential pathogenicity of others suggests that table salt is, beyond a source or halophilic archaea, a relevant, poorly studied natural source of microbial diversity.

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Chapter III – Ice machine biofilm:

Biofilm clogging water pumps in ice machines: a case study of a reservoir of new bacterial taxa

Abstract

Microorganisms are ubiquitously distributed in nature, and they usually appear in the form of microbial associations, biofilms, attached to a variety of substrates, both natural and synthetic ones. Here we report the recurrent appearance of a biofilm in the drain pipe of a standard laboratory ice machine, and we describe, through culture-dependent and -independent techniques, the composition of this oligotrophic microbial community inhabiting this device. By using culturomics in eight different media at three temperatures, 25 different microbial strains were isolated and taxonomically identified. The 16S rRNA and ITS high-throughput sequencing analysis revealed that *Bacteroidota* and *Proteobacteria* were the most abundant bacterial phyla in the sample, followed by *Acidobacteriota* and *Planctomycetota*, while the fungal community was clearly dominated by the presence of an unknown genus from the *Didymellaceae* family. Alpha and beta diversity comparisons of the ice machine microbial community against that of other cold environments revealed a low similarity between samples. The recovery and analysis of high-quality metagenome-assembled genomes (MAGs) yielded a strikingly high rate of potential new species: All but one of the 18 analysed genomes corresponded to potential new species. From those, three MAGs could be classified up to the genus level, five were classified at the family level, three at the order level, two at the class level, and four at the phylum level. Our results reveal the surprising potential of a mechanic as a massive source of new microbial taxa.

This work is submitted to a scientific journal.

Introduction

Microorganisms are able to attach to surfaces and grow in biofilms. In fact, biofilms are the preferred mode of growth lifestyle for many microorganisms, as these strong and dynamic structures provide numerous advantages. Indeed, it has been calculated that as much as 40–80 % of cells on our planet reside within biofilms (Flemming and Wuertz, 2019). Biofilms are ubiquitously distributed in nature, from marine and continental waters to rocks, plant surfaces and artificial structures, and include both microbial cells and a complex extracellular polymeric substance (EPS) matrix made of polysaccharides, secreted enzymes, amphiphilic compounds, and other macromolecules (Flemming, Hans-Curt *et al.*, 2007). Microorganisms living in biofilms are found attached to a surface (rock, sand particles, metallic surfaces, plastic pipes, etc) rather than following a free -planktonic- growth. In comparison to their suspended counterparts, biofilm-associated cells count with enhanced adhesion/cohesion capabilities, nutritional sources and a vast metabolite exchange network for communication, protection, and joint defence (Santos *et al.*, 2018), although they also display a reduced growth rate, and a different regulation of specific genes (Donlan, 2002).

Environmentally, massive overgrowth of biofilms can lead to serious disturbances in the flow of water streams or biological fluids. Biofilms are well known for their ability to overgrow and clog artificial substrates such as intermittent sand filters, in which biofilm clogging as a consequence of heterotrophs growth occurred in the upper part of the system (Chen *et al.*, 2021). They can also be responsible for the clogging of water distribution systems in drip irrigation emitters of treated wastewater (Yan *et al.*, 2009); the obstruction and contamination of machines such as reverse-osmosis water systems used for haemodialysis (Cuevas *et al.*, 2020); or cause run ability problems in paper machines (Rasimus *et al.*, 2011). Biofilms in artificial environments and mechanical devices, particularly those with high water loads, can yield to risk of contamination by pathogens, for example in dishwashers (Raghupathi *et al.*, 2018; Zupančič *et al.*, 2018). Paradoxically, though, selected microbial populations can also be used to treat the effluents of those machines, such as the biofilter recently developed to treat dishwasher wastewater (Congestri *et al.*, 2020). Biofilms are also found in industrial and domestic washing machines, where, interestingly, the majority of the microbial isolates formed more biofilm than their reference strains in a study carried out with washing machines worldwide (Gattlen *et al.*, 2010).

In 2019, an ice machine of a molecular biology laboratory in Valencia, Spain, suffered from several episodes of water leakage because of clogging of its water pump. Later, two other machines of the same Institute (in different floors) started having the same problem. In all cases, a thick biofilm was found to totally clog the water reservoir of the water pump and part of the wastewater pipes. The invasiveness of this biofilm, its recalcitrance to a continuous chemical control treatment and the particularities of the artificial micro-niche in which it developed (oligotrophic, cold water) suggested that it may hold potential as a microbial niche and was thus subjected to the complete study of its microbial composition that we report here.

Materials and Methods

Sample collection: Two daily-use standard laboratory ice machines installed in two different floors at the Institute for Integrative Systems Biology (I2SysBio; 39.5167° N, 0.4231° W) with an approximate room temperature of 23 ºC started to clog in the summer of 2019. Since then, a treatment with Green Pantabs (Highside Chemicals Inc.; USA) consisting of quaternary ammonium chloride started to be applied every two weeks. However, machine failure due to clogging continued to occur. In August 2021, a piece of a pale, light brown, gummy biofilm was harvested from the out-flux pipe of one of the laboratory ice machines (ITV Ice Makers;

Spain; S/N: 17634427) and stored in a 50 ml tube at 4 ºC. The temperature and pH of the ice machine water reservoir and the sample were 5 ºC and 8.2±0.1, respectively.

Media and solutions: The following media and solutions were used for strain isolation from the ice machine biofilm. These were selected to cover a wide range of nutritional requirements in order to promote the growth of diverse bacterial and fungal species. Media composition was as follows. Reasoner's 2 Agar medium (R2A) contained in g/L: Peptone;1, Yeast Extract; 0.5, Dextrose/Glucose; 0.5, Soluble Starch; 0.5, Na_2HPO_4; 0.3, $MgSO_4.7H_2O$; 0.05, Sodium Pyruvate; 0.3, Agar; 15.0 and was adjusted to pH; 7.2±0.2. Lysogenic Broth medium (LB) contained in g/L: Tryptone; 10, Yeast Extract; 5, NaCl; 10, Agar; 15 and was adjusted to pH; 7.2±0.2. Trypticase Soy Broth medium (TSA) contained in g/L: Tryptone; 15.0, Soya Peptone; 5.0, NaCl; 5.0, Agar; 15.0 and was adjusted to pH; 7.2±0.2. Marine Agar medium (MA) contained in g/L: H_3BO_3; 0.022, NH_4NO_3; 0.0016, $CaCl_2$; 1.8, $SrCl_2$; 0.034, Yeast Extract; 1.0, Iron Citrate; 0.1, $MgCl_2$; 8.8, Bile Salt n°3; 5.0, KBr, 0.55, NaCl; 19.4, NaF; 0.0024, $NaHCO_3$; 0.16, Na_2HPO_4; 0.008, Na_2SiO_3; 0,004, Na_2SO_4; 3.24. Agar; 15.0 and was adjusted to pH; 7.6±0.2 (PanReac Química SLU; Spain). Yeast Mold Agar medium (YM) contained in g/L: Yeast Extract; 3.0, Malt Extract; 3.0, Dextrose; 10.0, Peptone Soybean; 4.0, Agar;15.0 and was adjusted to pH; 6,2±0.2. Columbia Agar medium (CA) contained in g/L: Special Peptone; 23.0, Starch;1.0, NaCl; 5.0, Agar;10.0 and was adjusted to pH; 7.3±0.2 (Oxoid Ltd., UK). Brain Heart Infusion Agar medium (BHI) contained in g/L: Brain Heart Infusion Solids; 17.5, Peptone; 10.0, Glucose; 2.0, NaCl; 5.0, Na_2HPO_4; 2.5, Agar; 15.0 and was adjusted to pH; 7.4±0.2 (VWR International bvba, EC). Efm2 medium was prepared by adding 1 % Glucose, 0.3 % Saccharose, and 1.5 % Agar to 42 g M17 Broth medium (SIGMA ALDRICH-MERCK; Spain) with the following compositions (g/L): Tryptone; 2.5, Meat Peptone; 2.5, Soya Peptone; 5.0, Yeast Extract; 2.5, Meat Extract; 5.0, Sodium glycerophosphate; 19.0, $MgSO_4$; 0.25, Ascorbic acid; 0.5 and Lactose; 5.0, adjusted to pH; 7.0±0.2. Phosphate Buffer Saline (PBS) solution consisted of g/L: NaCl; 8.0, KCl; 2.0, Na_2HPO_4; 1.44, KH_2PO_4; 0.24. pH; 7.4.

Strain isolation and identification: Immediately after collecting the sample from the out-flux pipe of the laboratory ice machine, a fraction of the biofilm was transferred to a 1.5 ml tube and physically smashed by using an adapted plastic pestle and then vortexing to obtain a homogenized mixture. Serial dilutions were prepared using PBS and plated on eight different media (R2A, LB, TSA, MA, YM, CA, BHI, Efm2). The plates were incubated for at least 15 days at 25 °C, 10 °C and 4 ºC under aerobic conditions. Single colonies were periodically isolated and re-streaked on new plates until pure strains were obtained. These were cryo-conserved in 15 % glycerol at –80 C°.

For taxonomic identification, a colony of pure culture was resuspended in 100 µL of sterile MilliQ-water and subjected to three consecutive heat shock cycles (99 °C; 3 min and 4 °C; 3 min). This mixture was used to amplify either the 16S rRNA gene or the ITS region. The 16S rRNA PCR was performed using the *Taq* PCR Master Mix Kit (Qiagen, Germany), and the following PCR cycle: initial denaturation at 94 °C for 3 min; 30 cycles of amplification (30 s at 94 °C, 30 s at 50 °C, 1 min 30 s at 72 °C); and 10 min of extension at 72 °C, using the universal primers that amplify the 16S of ribosomal RNA gene 8F (5'- AGA GTT TGA TCC TGG CTC AG -3') and 1492R (5'- CGG TTA CCT TGT TAC GAC TT -3'). To amplify the ITS2 hypervariable region of the fungal nuclear ribosomal DNA (rDNA), PCRs were carried out using the primers 3F (5'- GCA TCG ATG AAG AAC GCA GC -3') and 4R (5'- TCC TCC GCT TAT TGA TAT GC -3') and the following PCR cycle: initial denaturation at 95 °C for 3 min; 30 cycles of amplification (30 s at 94 °C, 30 s at 53 °C, 30 s at 72 °C); and 10 min of extension at 72 °C. The amplification of the targeted regions was confirmed by gel electrophoresis (1.2 % agarose in 0.5X Tris-Borate-EDT buffer) and the PCR products were purified using a mixture of isopropanol and 3M potassium acetate (10:1 v/v). Amplicons were sequenced by Sanger

sequencing (Eurofins Genomics; Germany). All the sequences were manually trimmed before comparing them against the EzBioCloud (https://www.ezbiocloud.netN) and NCBI online databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi). EzBioCloud was used to taxonomically identify the closest type strains for the bacterial isolates, whereas for the fungal strains NCBI was used applying the following constrains: Nucleotide Blast; rRNA/ITS databases, 16S ribosomal RNA sequences or Internal Transcribed spacer region (ITS), limit to sequences from type material. Additionally, in order to gather information regarding the ecology of the isolated strains, the trimmed sequences were compared against the NCBI standard nucleotide collection (nr/nt).

Metagenomic DNA isolation: A physical pre-treatment was applied to break down the biomass before DNA extraction. Then, the homogenized mixture was treated using the DNeasy PowerSoil kit (Qiagen, Germany) following the manufacturer's instructions. The DNA was eluted in 20 µL of MilliQ water pre-warmed at 65 ºC. The extracted DNA was quantified using Qubit dsDNA HS Assay kit (Qubit 2.0 Fluorometer, Q32866).

Metataxonomic analysis: In order to study the bacterial and fungal communities present in the biofilm, the extracted metagenomic DNA was used to amplify the hypervariable region V3-V4 of the 16S ribosomal RNA gene and the ITS2 hypervariable region of the fungal nuclear ribosomal DNA (rDNA). The conserved regions V3 and V4 (459 bp) of the 16S rRNA gene were amplified using the following forward and reverse primers: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG -3′ and 5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C -3′, and the following PCR cycle: initial denaturation at 95 °C for 3 min; 25 cycles of amplification (30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C); and 5 min of extension at 72 °C (Satari *et al.*, 2020). The DNA amplicon libraries of the ITS2 region were generated using the primers ITS3-F_KYO2 (18S SSU 2029–2046) and ITS4_KYO1 (5.8 2390–2409) (Toju *et al.*, 2012), and a PCR cycle consisting of: initial denaturation at 95 ºC for 3 min, 28 cycles of amplification (30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C), and 5 min of a final extension step at 72 ºC. In both cases, the amplification was carried out using the KAPA HiFi HotStart ReadyMix PCR kit (KK2602). The 16S rRNA and ITS2 amplicons were mixed with Illumina sequencing barcoded adaptors (Nextera XT index kit v2, FC-131-2001), and libraries were normalized and merged. The pools with indexed amplicons were loaded onto the MiSeq reagent cartridge v3 (MS-102-3003) and spiked with 10 % PhiX control to improve the sequencing quality, that was finally conducted using paired-ends on an Illumina MiSeq platform (2 × 300 bp) in the Foundation for the Promotion of Health and Biomedical Research of the Valencian Community (Fisabio) (Valencia, Spain).

The raw Illumina sequences were loaded into Qiime2 (v. 2021.2.0) (Bolyen *et al.*, 2019). The quality of the sequences was checked using the plugin Demux and the Qiime2-integrated DADA2 pipeline was used for trimming and joining the sequences, removing chimeras and detecting amplicon sequence variants (ASVs) (> 99.9 % of similarity). The taxonomy of each sequence variant was determined via the classify-Sklearn module from the feature-classifier plugin, employing SILVA (v. 138) (Quast *et al.*, 2013) and UNITE (v. 8.2) (Nilsson *et al.*, 2019) as reference databases for 16S rRNA and ITS2 taxonomic assignment, respectively. Additionally, the most abundant ASVs resulting from ITS sequencing were classified with BLAST against the NCBI's ITS database. Results were analysed with the phyloseq R package (McMurdie and Holmes, 2013).

In order to assess the similarity of the ice machine biofilm to previously reported ones in terms of bacterial profile, a beta diversity analysis was carried out including this sample and others originating from cold, freshwater, tap water and artificial environments. The detailed list of the samples included in this analysis is available in Table S2. This meta-analysis was performed using the phylogeny-

based weighted UniFrac metrics (Lozupone *et al.*, 2006).

Shotgun metagenomics analysis: The extracted DNA was prepared for whole genome sequencing by sonication. The obtained DNA fragments were polished, A-tailed, ligated with the following adaptors 5'- AGA TCG GAA GAG CGT CGT GTA GGG AAA GAG TGT AGA TCT CGG TGG TCG CCG TAT CAT T -3' and 5' -GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GGA TGA CTA TCT CGT ATG CCG TCT TCT GCT TG -3' and amplified by PCR. The PCR products were purified with the AMPure XP system for library preparation, and the size distribution of the libraries was checked by Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and quantified by real-time PCR. Sequencing was conducted using Illumina NovaSeq 6000 platform (2 × 150 bp).

Adaptors were trimmed from raw reads with Cutadapt (v.3.4) (Martin, 2011), and quality filtering was performed with BBDuk, from the BBTools package (Bushnell B., https://sourceforge.net/projects/bbtools/ updated January 2, 2018). Quality-checked (QC) reads were mapped against the *Homo sapiens* reference genome (GRCh38.p13, https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.39) using Bowtie2 (v. 2.3.5.1) (Langmead and Salzberg, 2012) to detect and filter any human-related reads. The quality of the reads was checked before and after the filtering using FastQC (v. 0.11.9) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc).

Taxonomy was assigned to QC reads via Centrifuge (v. 1.0.3) (Kim *et al.*, 2016) using a database that contained reference sequences from *Bacteria*, *Archaea*, virus and human (https://genome-idx.s3.amazonaws.com/centrifuge/p_compressed %2Bh %2Bv.tar.gz, last updated 12/06/2016). A Centrifuge custom database was created by using centrifuge-download and centrifuge-build commands to obtain and format the GenBank fungi reference sequences. This database was used for evaluating the fungal fraction of the sample.

QC reads were then assembled with MEGAHIT (v. 1.2.9) (Li *et al.*, 2015), and assembly statistics were obtained with QUAST (v. 5.0.2) (Gurevich *et al.*, 2013). The filtered reads were mapped to the assembled contigs using Bowtie2 and a sorted BAM file was obtained with SAMtools (v. 1.13) (Li *et al.*, 2009). Contig abundance statistics were obtained using the jgi summarize bam contig depth script from MetaBAT pipeline (Kang *et al.*, 2019). Then, binning was carried out by MetaBAT (v. 2.12.1) (Kang *et al.*, 2019), and MaxBin (v. 2.2.7) (https://www.sourceforge.net/projects/maxbin2/) using the default parameters. Metagenome-assembled genomes (MAGs) were selected by using DAS Tool (v. 1.1.3) (Sieber *et al.*, 2018), which compares the quality of the MAGs recovered by the binning tools and chooses the best ones. The quality of those MAGs was further evaluated with CheckM (v. 1.1.3) (Parks *et al.*, 2015).

MAG taxonomic classification and novelty were determined with Microbial Genomes Atlas MiGA Online (Rodriguez-R *et al.*, 2018) using the type material genomes collection (last updated 2021/04/12). The Average Nucleotide Identity (ANI) of the MAGs with their closest relatives was calculated using JspeciesWS (Richter *et al.*, 2016). Finally, prokka (v. 1.14.6) (Seemann, 2014) was used for annotating the MAGs.

Scanning Electron Microscopy (SEM): The structural and morphological characteristics of the ice machine biofilm were analysed through SEM. Small fragments of the biofilm were fixed by immersion into Karnovsky's Fixative (2 % paraformaldehyde, 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer; pH 7.4) overnight, then gently washed with sterile deionized water and a series of ethanol solutions (30, 50, 70, 90, and 100 % ethanol absolute; PanReac, AppliChem). Samples were incubated for 48 hours in the desiccator prior to embedding them in resin using carbon tape. Sputtering was done using Gold/Palladium (Au/Pd) particles and samples were examined under the Field Emission Scanning Electron Microscope (FE-SEM) Hitachi S4800 (SCSIE, University of Valencia).

Results

In this work, we describe the microbial composition of a biofilm that colonised the drain pipe of a standard laboratory ice machine (Fig. 1A). SEM analysis of the biofilm revealed a mixed community of bacteria and fungi embedded in an EPS-like matrix (Fig. 1B-E). Thus, both culturomics and metagenomics analysis focused on the isolation and identification of both fungi and bacteria.

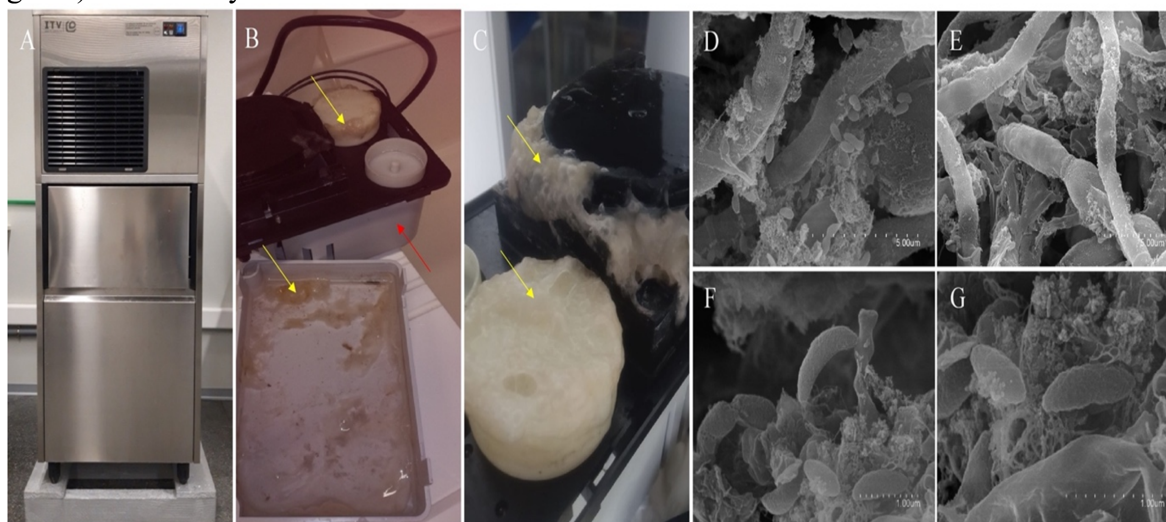

Figure 1. (**A**) The standard laboratory ice machine sampled in this work, (**B** and **C**) The biofilm formed around the out-flux pipes of the laboratory ice machine. Yellow arrows point to the biofilm, and red arrows to the ice machine water reservoir. (**D**, **E**, **F**, and **G**) The images from the Field Emission Scanning Electron Microscope (FE-SEM) of the biofilm show microbial cells within the EPS matrix.

Strain collection: A set of 25 microbial strains (18 bacterial and seven fungal strains) were isolated and taxonomically identified from the ice machine biofilm by culture-dependent techniques. In total, 20 of these strains were isolated at 25 ºC, while seven were isolated after incubation at 10 ºC and eight were obtained by culturing at 4 ºC (Fig. 2, Table S1 and Table S2). At genus level, the microorganisms isolated at 25 ºC belong to the genera *Acidovorax*, *Acinetobacter*, *Bacillus*, *Chryseobacterium*, *Delftia*, *Hydrogenophaga*, *Methylobacterium*, *Nocardia*, *Prolinoborus*, *Rhodococcus*, *Aspergillus*, *Neomicrosphaeropsis*, and *Penicillium*. At the species level, the bacterial strains isolated at 25 ºC were closely related to *Acidovorax temperans*, *Acinetobacter lwoffii, Bacillus toyonensis*, *Chryseobacterium hispalense*, *Delftia acidovorans*, *Hydrogenophaga palleronii*, *Methylobacterium marchantiae*, *Nocardia asteroides*, *Nocardia neocaledoniensis, Prolinoborus fasciculus*, *Rhodococcus cercidiphylli*, *Rhodococcus fascians*, and the fungal strains to *Aspergillus austroafricanus*, *Neomicrosphaeropsis italica*, and *Penicillium citrinum*. At 10 ºC the isolates fall within the genera *Bacillus*, *Peribacillus*, *Pseudomonas*, *Sphingomonas*, *Briansuttonomyces*, *Cadophora*, and *Vishniacozyma* while the genera *Flavobacterium*, *Pseudomonas*, *Sphingomonas*, *Cadophora*, *Filobasidium*, and *Vishniacozyma* were isolated at 4 ºC (Table S1 and Table S2). There were shared strains among all the temperatures used for incubation. Strains belonging to the genera *Pseudomonas*, *Sphingomonas*, and *Cadophora* were isolated at all the aforementioned temperatures, while *Flavobacterium* sp. was obtained at 4 and 25 ºC, *Briansuttonomyces* sp. and *Bacillus sp.* were cultured at 10 and 25 ºC), and *Vishniacozyma* sp. was isolated both at 4 and 10 ºC. Those strains that were isolated on multiple media but whose 16S rRNA gene sequence was 100% identical were considered as identical clones. Details on the media in which each strain was isolated can be found in Table S1 and Table S2 for bacteria and fungi, respectively.

In addition to the taxonomic identification of the isolated strains, a BLAST against the NCBI standard nucleotide collection (nr/nt) allowed to shed light into their ecology. Interestingly, the closest strain to isolate IM-2b was an *Acidovorax* strain isolated from water

purifiers, the closest strain to isolate IM-32a was a clone closely related to *Hydrogenophaga* species that thrived in a freshwater biofilm and an environmental *Methylobacterium* closely related to IM-41 was isolated from a freshwater lake in Antarctica. The closest neighbours to strains IM-38 and IM-42 were *Rhodococcus* strains isolated in the Baltic Sea and freshwater RAS systems as well as in cloud water respectively, the closest strains to IM4-31 were *Sphingomonas* clones found in water and a drinking water system, and the closest clone to IM4-X was an uncultured bacterium from a kart aquifer. In the case of the fungal strains, the closest strain to isolate IM10-13 was a *Phoma* strain isolated from the sediment of a Canadian glacier; the closest neighbour to IM4-10 was a *Cadophora* strain isolated from a former coal-spoil site in the Arctic, and the closest neighbour to strain IM4-11 was a *Filobasidium* strain located in the waters of a mesotrophic lake (Table S3). Overall, these environments are characterised by the low temperatures and the low availability of nutritional resources similar to the ice machine drain pipe environment. The closest environmental neighbours of the rest of the strains isolated from the ice machine pipe drain were associated to soil, human and plants (Table S3).

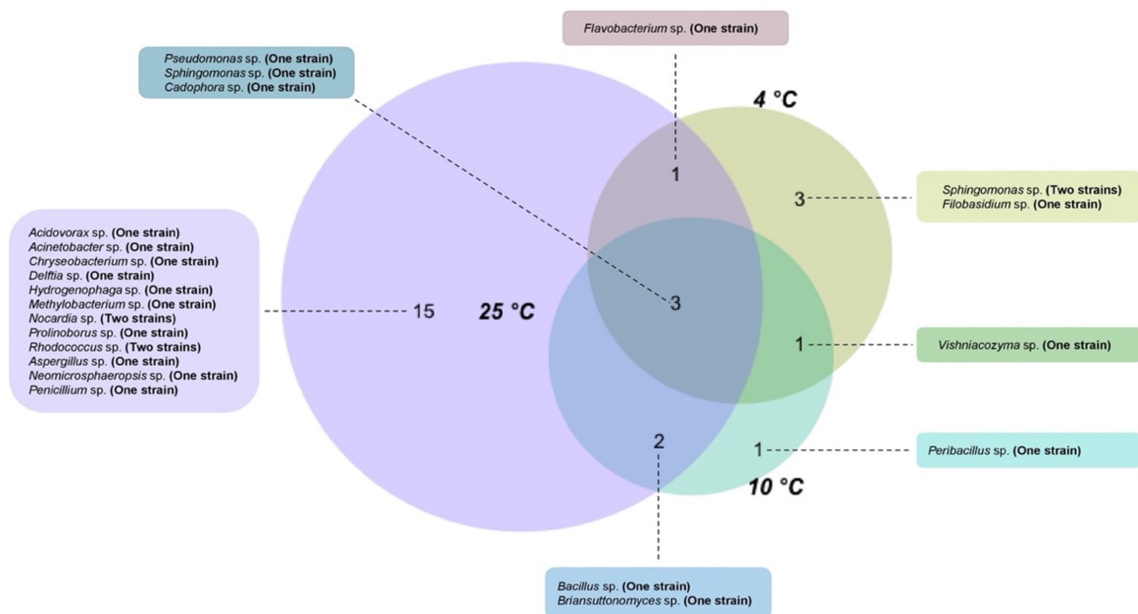

Figure 2. Venn diagram displaying the shared and unique isolated microorganisms among the three different temperatures. A higher diversity of isolates was observed at 25 ºC in comparison to the other two incubating temperatures.

Microbial community in the ice-machine biofilm analysed through NGS: In parallel to the culture-dependent study, the microbial community within the biofilm was analysed by 16S rRNA and ITS high-throughput sequencing. After cleaning of reads, a total of 106,886 16S rRNA sequences and 149,989 ITS sequences were available for further analyses. The entire diversity of this sample was captured, as the rarefaction curves were saturated (Supplementary Fig. 2). *Bacteroidota* and *Proteobacteria* were the most abundant bacterial phyla in the sample, both with a relative abundance greater than 40 %. Two other phyla, *Acidobacteriota* and *Planctomycetota*, showed an abundance greater than 10 % (Fig. 3A). At the genus level, *Sediminibacterium* predominated with a relative abundance higher than 40 %, followed by *Hydrogenophaga* and *Methyloversatilis*, both with a relative abundance greater than 10 % (Fig. 3B). Results from ITS sequencing revealed that the fungal community was clearly dominated by the phylum *Ascomycota*, with almost 100 % relative abundance (Fig. 3C). This high abundance is almost entirely explained by the presence of an unknown genus from the *Didymellaceae* family, whose relative abundance exceeded 98 % (Fig. 3D). A BLAST analysis of the ITS sequences from the most abundant ASVs was done to elucidate which members of this family were more present in

the biofilm. Three of these five ASVs (>98 % of total abundance; Table S8) aligned with equal level of significance to different species from *Didymellaceae* family, such as *Didymella boeremae*, *Ascochyta phacae*, *Neomicrosphaeropsis italica* or *Briansuttonomyces eucalypti*, although the similarity was below 99.5 % in all the cases; therefore, it was not possible to identify the specific *Didymellaceae* species in the sample. *Cadophora luteo-olivacea* and *Vishniacozyma victoriae* were also detected in low abundances after the BLAST analysis.

The comparison of the ice machine microbial community with other microbiomes in terms of beta diversity revealed that the unweighted UniFrac dissimilarity metric between this sample and all others was high (Table S6). This suggested that the selected samples presented marked differences in the bacterial composition.

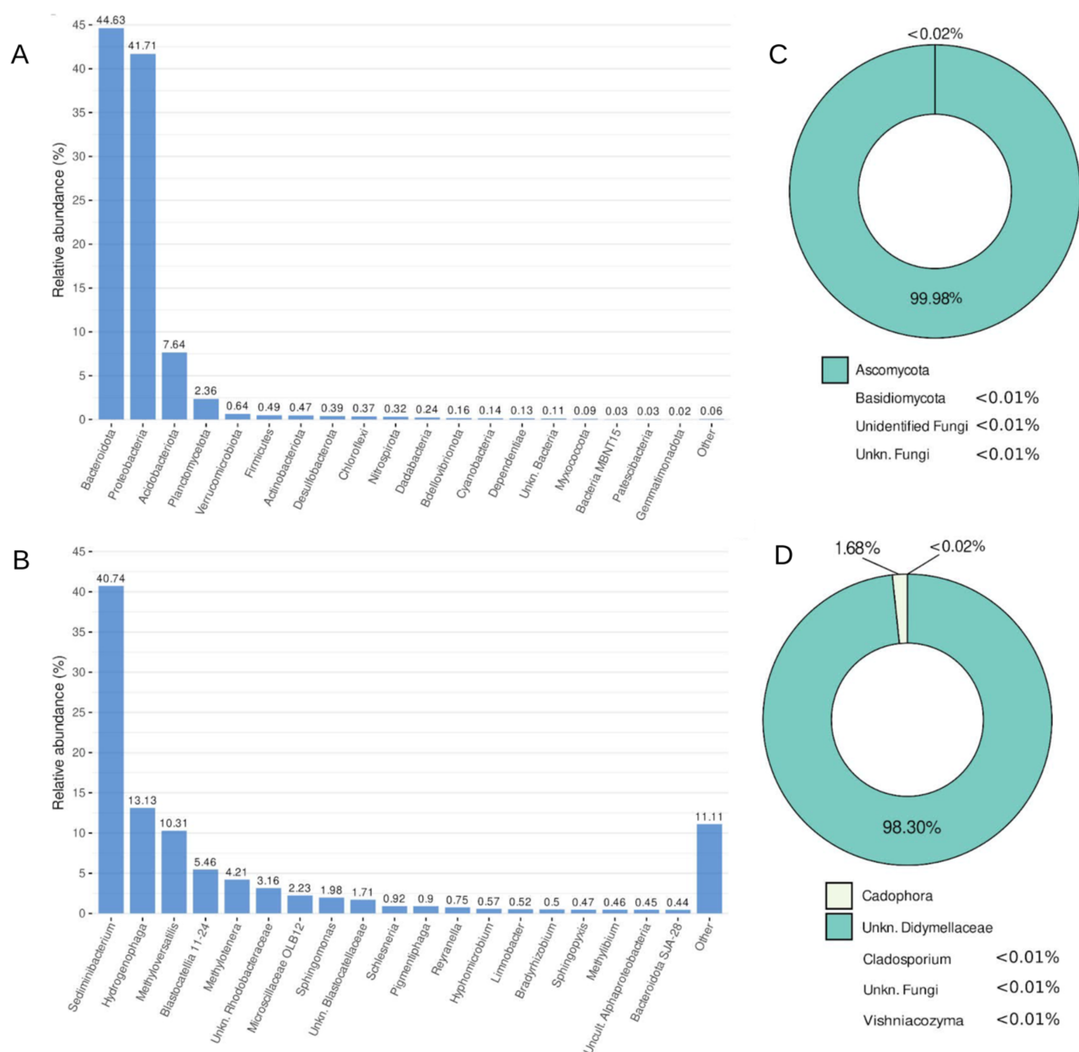

Figure 3. Taxonomic composition of the (**A**) bacterial community detected in the sample at phylum and (**B**) genus level; and the fungal community at (**C**) phylum level and (**D**) genus level.

The Principal Coordinate Analysis (PCoA) plot (Fig. 4) showed that the most similar samples to that of the ice machine biofilm were two biofilm samples coming from biological air scrubbers in a pig housing facility and an Arctic soil crust sample. In fact, despite the low similarity between samples, up to six genera identified through NGS (*Pseudoxanthomonas*, *Ferruginibacter*, unkn. *Comamonadaceae*, *Clostridium sensu stricto 1*, unkn. *Microbacteriaceae* and *Brevundimonas*) were shared by these four samples (Fig. 5), while other 51 genera found in the ice machine microbiome were also found in at least one of the other samples. Finally, the alpha diversity analysis proved that the ice machine sample,

despite showing a high number of observed ASVs, is relatively less diverse, that is, more homogeneous in its composition (Supplementary Fig. 1).

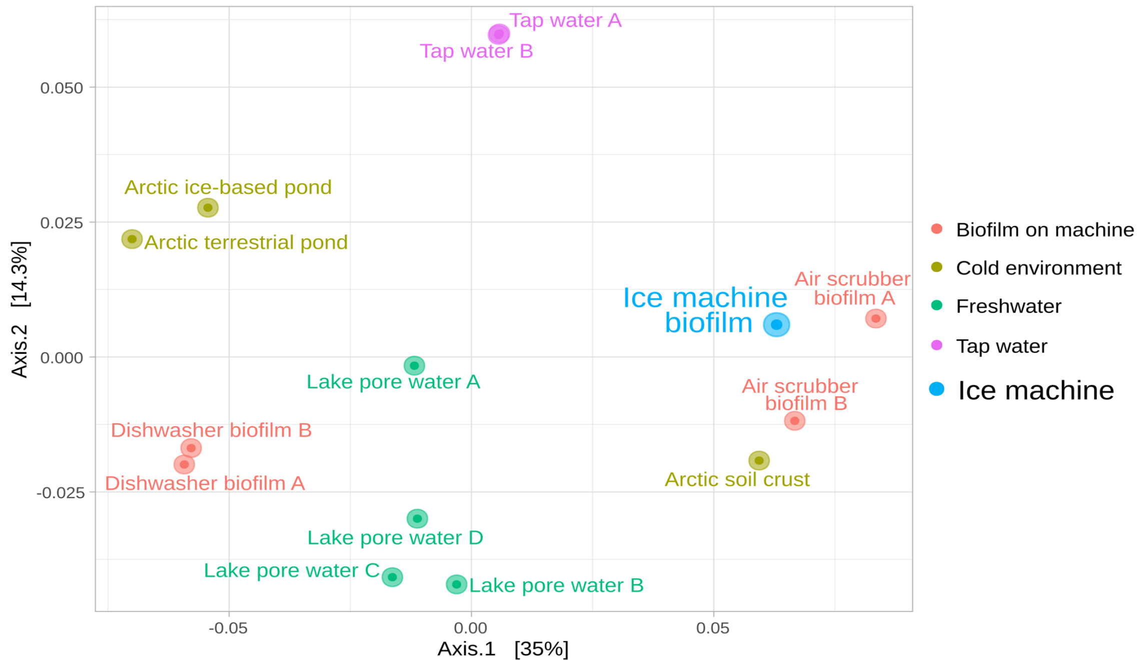

Figure 4. PCoA of all the samples included in the beta diversity analysis. See Table S4 for additional information about the samples.

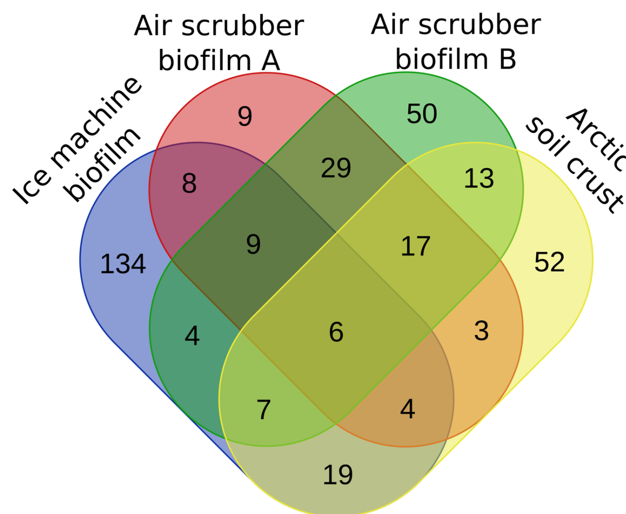

Figure 5. Venn diagram displaying the number of shared and unique genera among the ice machine biofilm and the biomes more closely related. Only genera with relative abundance greater than 0.01 % were included in this analysis.

In order to complement the taxonomic information of the microbial community, shotgun metagenomic sequencing was also performed. Overall, the microbial profiles obtained by amplicon and shotgun sequencing were similar (Fig. 3 and 6). Although the most abundant bacteria according to the 16S rRNA gene sequencing (*Sediminibacterium*) was not detected by the metagenomic analysis. *Methyloversatilis* and *Hydrogenophaga* were identified as majoritarian taxa by both techniques (Fig. 3B). *Stagonosporopsis*, which belongs to the *Didymellaceae* family, was the most abundant genus among eukaryotes (Fig.

5A). This is in concordance with the results obtained from the ITS sequencing analysis (Fig. 3D), in which members of the *Didymellaceae* family were also found to be predominant. Finally, most of the metagenomic reads belong to the kingdom *Bacteria* (76.9 %) while less than a quarter correspond to *Fungi* (Fig. 6B).

Shotgun reads were assembled, and binning was applied in order to recover metagenome-assembled genomes (MAGs) from the pool of contigs. A total of 47 and 52 bins were obtained with MaxBin2 and MetaBAT2, respectively. After running Das Tool, those non-redundant bins with the highest score (18) were selected (Table S5). Following the defined standards by Bowers *et al*. (2017), eight of the isolated genomes could be considered as high-quality MAGs (completeness > 90 % and contamination < 5 %). Among them, four have a completeness >99 %. The other ten were medium-quality MAGs (completeness > 50 % and contamination < 10 %), where four of them have a completeness greater than 80 % and contamination lower than 5 %. Each MAG was taxonomically classified using MiGA and then the Average Nucleotide Identity (ANI) index to the closest phylogenetic neighbour was calculated. Surprisingly, all the MAGs were classified as potential new species with an ANI value <95 % (following Richter and Rosselló-Móra 2009, standards), except MAG 14 which was identified as *Mycobacterium gordonae* DSM 44160^T with an ANI value of 98.32 % (Table S7). Interestingly, only three MAGs could be classified up to the genus level by MiGA. Moreover, five MAGs were classified at the family level, three at the order level, two at the class level, and four at the phylum level. It is worth highlighting that MAG 002 was classified as *Sediminibacterium,* although this genus was not identified by Centrifuge (Fig. 6). Moreover, MAG 52 revealed the presence of a strain of the genus *Sphingomonas* closely related to *Sphingomonas faeni*, and, through culture dependent techniques, two strains closely related to *Sphingomonas faeni* with similarity lower than the threshold that determines a new species (98.7 %) (Chun *et al.*, 2018) were isolated.

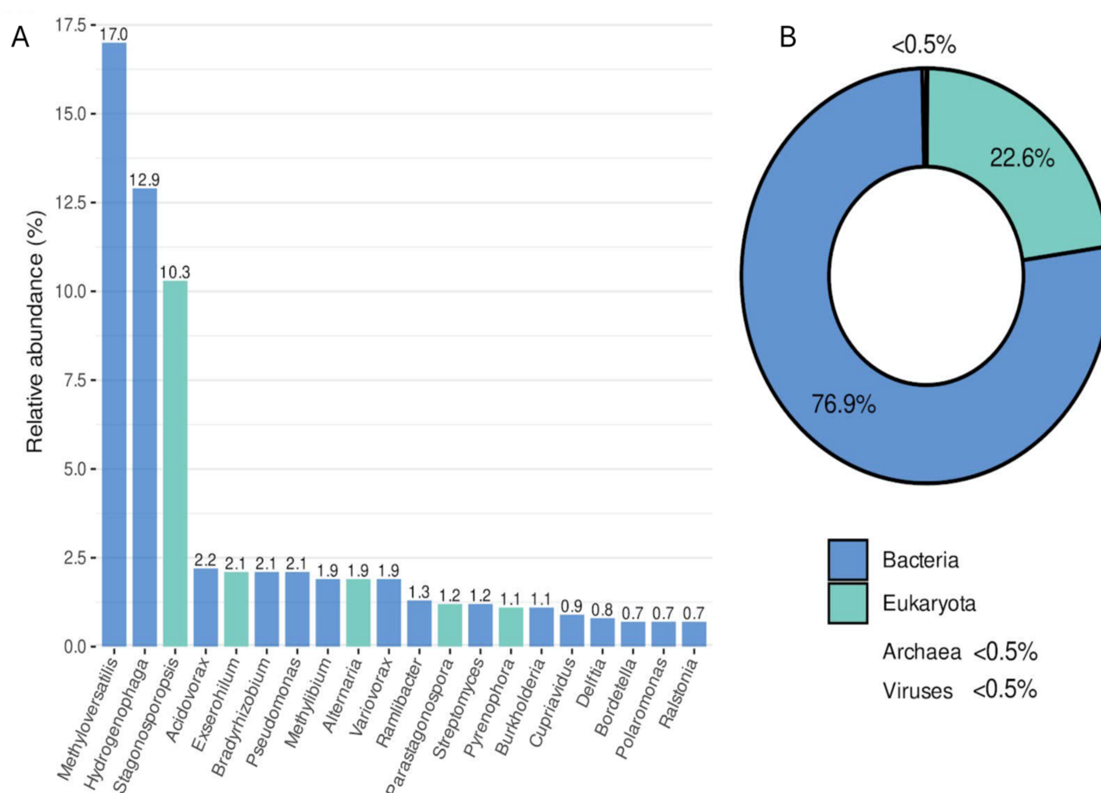

Figure 6. Taxonomic classification of the microbial community present in the ice machine biofilm at the (**A**) genus (only the 20 most abundant genera are shown) and (**B**) kingdom level.

Discussion

In this work, we report the taxonomic characterisation of a massively growing microbial biofilm developing in the drain pipe of a standard laboratory ice machine. This microbial community, that formed a mucus, gummy biofilm from the drain pipe down to the pump's vessel, developed under cold oligotrophic conditions even after continuous chemical treatments. The biofilm, consisting of bacteria and fungi as revealed by SEM (Fig. 1), was analysed through both culture-dependent and -independent analysis.

A set of media covering a wide range of nutritional requirements in combination with three different incubation temperatures allowed the isolation of 18 bacterial and seven fungal strains. The 20 strains isolated at 25 ºC were closely related to previously described mesophilic species, although two of them were also isolated in this study at 10 ºC, one of them at 4 ºC and three of them at all three temperatures (Fig. 2). Only one and three strains were strictly isolated at 10 ºC and 4 ºC respectively. The strain that was isolated only at 10 ºC is closely related to the mesophilic *Peribacillus simplex*, two of the strains isolated at 4 ºC belonged to the genus *Sphingomonas* from which most members also grow under mesophilic conditions, and a fungal strain closely related to the mesophilic yeast *Filobasidium magnum*. We were able to isolate strains belonging to the phylum *Bacteroidetes*, *Proteobacteria* and *Ascomycota*, although other non-abundant species belonging to the phylum *Actinobacteria* and *Firmicutes* were also isolated.

Since culturomics is well known for yielding only a fraction of the actual microbial diversity, a culture independent NGS approach was carried out through high throughput 16S rRNA and ITS gene sequencing. The presence of some species we could culture such as *Neomicrosphaeropsis italica*, *Briansuttonomyces eucalypti*, and the low-abundant species *Cadophora luteo-olivacea* and *Vishniacozyma victoriae* in the ice machine biofilm were also confirmed by the metagenomics analysis. Despite the high abundance of *Sediminibacterium* species (more than 40 % of the bacterial population according to the NGS results) and the favourable culturing conditions _ *Sediminibacterium* spp. have been previously isolated from sediments using R2A medium (Qu and Yuan, 2008; Song *et al.*, 2017) _ it was not possible to isolate any strain from this genus. Specific abiotic factors such as pH (pH 5.5-6.0 for *S. lactis*) and salt concentration (3-10 % (w/v) NaCl concentration for *S. halotolerans*) might be the cause of these results. *Hydrogenophaga* sp. and *Methyloversatilis* sp. were also abundant in the ice machine biofilm population (approximately 10-13 % relative abundance), and a member of the first one was retrieved by culture-dependent techniques. Members of these two genera have a worldwide distribution, although some species were previously isolated from different aquatic environments such as hot springs (Lin *et al.*, 2017) and wastewater (Yoon *et al.*, 2008) as well as in Antarctica [https://eol.org; (Schauer *et al.*, 2011; Sannino *et al.*, 2017)]. Both have been reported to be psychrotrophic and psychrotolerant (Lambo and Patel, 2006, 2007; Li *et al.*, 2016). Regarding the fungal community that colonised the ice machine drain pipe, with a relative abundance higher than 98 %, this was completely dominated by fungi from the family *Didymellaceae.* Although through culture dependent techniques it was possible to isolate two members of this family (*Neomicrosphaeropsis italica* and *Briansuttonomyces eucalypti*), it was not possible to identify by ITS high throughput sequencing the main *Didymellaceae* species in the sample.

With the aim of complementing the taxonomic information described above, shotgun metagenomic sequencing was also performed. The microbial profiles obtained were similar to those captured by amplicon sequencing (Fig. 3 and 6). Although the most abundant genus in the 16S rRNA gene analysis (*Sediminibacterium*) was not detected in the taxonomic profiling by shotgun sequencing, MAG 002 was indeed classified as a possible member of this taxonomic group (Table S7). We noticed that Centrifuge databases did not contain *Sediminibacterium* genomes, thus explaining why this genus was not detected in the taxonomic profiling but it was actually

recovered after metagenomic assembly. In order to find out the abundance of this genus, filtered reads were mapped against MAG 002, and an overall alignment rate of 99.8 % was obtained. This confirmed that *Sediminibacterium* was among the most abundant genus in the microbial community. Other MAGs recovered after binning were also classified as members of taxonomic groups that were phylogenetically related to the taxa detected by metagenomic and metataxonomic profiling (Fig. 3B and 6A; Table S7), with the exception of MAG 014 (classified as *Mycobacterium gordonae*). Interestingly, this species was previously identified in samples of tap water and different water distribution systems (Millar and Moore, 2020). Taxonomic analysis of the MAGs that were recovered from the metagenomic sample demonstrated that bacteria that colonize the ice machine held a great taxonomic novelty, according to phylogenomic indices. Indeed, some MAGs could be only classified at the phylum or class level. Altogether, our analyses have proved that the ice machine microbiome is a diverse, unexplored microbial niche with a surprisingly high prevalence of previously undescribed bacterial species.

Moreover, we studied the uniqueness of the ice machine microbial community in terms of beta diversity by comparing it with other microbiomes. The beta diversity analysis carried out in a previous work revealed that biofilms in biological air scrubbers in a pig housing facility and Arctic soil crust were more similar to the ice machine biofilm than other samples from fresh and tap water included in the analysis. Nevertheless, large differences were detected when comparing these microbiomes with the microbial community inhabiting the ice machine. For example, *Sediminibacterium*, the most abundant genus in the ice machine biofilm, was only detected in tap water and freshwater samples always under 2 % abundance. As for *Hydrogenophaga* and *Methyloversatilis*, these genera were absent or appeared in minor abundances in all samples besides the ice machine one. Apparently, it is unusual to find biomes with such a high abundance of these genera, although its joint presence has been found in dental unit waterlines (Yoon and Lee, 2019), which are also enclosed areas with direct contact with water. In this later case, the relative abundances of these genera were substantially lower than in the ice machine.

Overall, and despite the conditions in which the biofilm developed, all the strains that we isolated were closely related to mesophilic microorganisms. Similarly, the species with a higher relative abundance according to the NGS analysis (*Sediminibacterium*, *Methyloversatilis*, *Hydrogenophaga* and *Stagonosporopsis*) were mesophilic as well. Although previous research reported that some isolated bacteria and fungi such as *Methylobacterium marchantiae* and *Vishniacozyma victoriae* can grow in a wide range of temperatures (Schauer *et al.*, 2011; Garcia *et al.*, 2012), the biofilm could provide shelter and protection for some mesophilic microorganisms, that could remain metabolically inactive. Some environmental bacteria such as *Acidovorax temperans*, *Bacillus toyonensis, Delftia acidovorans, Hydrogenophaga palleronii, Methylobacterium marchantiae*, and *Pseudomonas lactis*, were previously described as microorganisms which tend to form biofilms (Kim *et al.*, 2012; Doerges and Kutschera, 2014; Rema Tara *et al.*, 2014; Verplaetse *et al.*, 2017; Quintieri *et al.*, 2020). Indeed, future work could focus on analysing temperature growth ranges of all the strains to elucidate whether they are psychrophiles or low temperature-tolerant mesophiles. Sporogenesis may also be playing a key role, as some species closely related to the strains present in the ice machine drain pipe such as *Bacillus toyonensis* and *Peribacillus simplex* were previously reported as spore-forming bacteria (Zhang *et al.*, 2017; Bressuire-Isoard *et al.*, 2018; Quintieri *et al.*, 2020). Most certainly, a possible explanation for a higher biodiversity of the isolated species at 25 ºC compared to the other two temperatures may relate to the high relative abundance of spore-forming bacteria in the biofilm.

From an environmental perspective, strains closely related to the ones isolated from the ice machine such as *Acidovorax* sp., *Hydrogenophaga* sp., *Methylobacterium* sp.,

Pseudomonas sp., *Rhodococcus* sp., *Sphingomonas* sp., and *Filobasidium* sp. have previously been isolated in aquatic environments such as water purifiers, fresh water, pharmaceutical wastewater, cloud water, and a drinking water distribution system. Moreover, among the fungal strains *Briansuttonomyces* sp. and *Cadophora* sp., some strains were previously detected in different sites in the Arctic and species of the *Didymellaceae* family have been identified as cold-adapted microorganisms in Antarctica (Canini *et al.*, 2021), and forests in the north of Poland (Kwaśna *et al.*, 2021). In almost all these natural and artificial environments, nutritional resources are scarce, suggesting that the uniqueness of the ice machine microbiome could result from the effect of both selective pressures simultaneously, cold and nutrient shortage. Psychrooligotrophic fungi are polyextremophiles capable of slowly growing and reproducing at temperatures that range from −20 °C to +10 °C in environments with very low levels of nutrients. Interestingly, previous research revealed that the mycelia of *Briansuttonomyces* sp. and *Cadophora* sp. strains acts as a network for the exchange of nutrients and more particularly elements such as carbon (Finlay and Read, 1986; Crous and Groenewald, 2016; Di Francesco *et al.*, 2021).

Fungi are also present in artificial and semi-artificial environments such as drains in kitchens and bathrooms, sills beneath condensation-prone windows (Adams *et al.*, 2013), air-conditioning filters (Anas *et al.*, 2016), and dishwashers (Zalar *et al.*, 2011) due to their ability to colonise nutrient depleted environments also subjected to drastic temperature changes with the EPS matrix supports the development of a diverse but unique bacterial community.

In this case study, we describe the microbial composition of a biofilm that recurrently developed in a standard laboratory ice machine. Metataxonomic and metagenomics analysis indicate that the microbiome of the ice machine drain pipe biofilm is composed mainly of one or few fungal members closely related to the *Didymellaceae* family, and a relatively diverse bacterial community, dominated at the phylum level by *Bacteroidota* and *Proteobacteria*, and at the genus level by *Sediminibacterium*, *Hydrogenophaga* and *Methyloversatilis*. Moreover, alpha and beta diversity analysis demonstrate that the microbiome is complex and different to those of other environments (although it shares some important genera). At a higher taxonomic resolution (the one that MAGs provide), we have found that bacteria found in the ice machine correspond to a number of new species, which we have not been able to culture to date.

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Chapter IV – *Sagittula salina* sp. nov., isolated from marine waste

Abstract

A novel Gram-stained-negative, non-motile, halophilic bacterium designated strain M10.9X^T was isolated from the inner sediments of an aluminium can collected from the Mediterranean Sea (València, Spain). Cells of strain M10.9X^T were rod-shaped and occasionally formed aggregates. The strain was oxidase-negative and catalase-positive, and showed a slightly psychrophilic, neutrophilic, and slightly halophilic metabolism. The phylogenetic analyses revealed that strain M10.9X^T was closely related to *Sagittula stellata* E-37^T and *Sagittula marina* F028-2^T. The genomic G+C content of strain M10.9X^T was 65.2 %. The average nucleotide identity and digital DNA-DNA hybridization values were 76.6 % and 20.9 %, respectively, confirming its adscription to a new species within the genus *Sagittula*. The major cellular fatty acids were $C_{18:1}$ $\omega 7c$/$C_{18:1}$ $\omega 6c$ and $C_{16:0}$. The polar lipids consisted of M10.9X^T phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, an unidentified glycolipid, an unidentified phospholipid, and an unidentified lipid. According to a polyphasic study, strain M10.9X^T represents a novel species of the genus *Sagittula* for which the name *Sagittula salina* sp. nov. (type strain M10.9X^T= DSM 112301^T= CECT 30307^T) is proposed.

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Introduction

The genus *Sagittula* was first described by González *et al*. (1997) and reclassified within the *Rhodobacteraceae* family by Lee *et al.* (2012). At the time of writing, the genus *Sagittula* is composed of only two species: *S. stellata* and *S. marina*, both isolated from marine environments and promising strains with bioremediation capacities (Gonzalez *et al*., 1997; Lee *et al*., 2012; Frank *et al*., 2018). In the present research, we describe the polyphasic characterization of strain M10.9X^T, which was isolated from the inner sediment of a wasted aluminium can during the study on the microbial diversity of marine waste. Anthropic residues distributed worldwide represent a major environmental problem and constitute new ecological niches which may harbour potential new microbial species.

Material and Methods

Strain M10.9X^T was isolated from the inner sediment of a wasted can collected from the Malva-rosa beach, on the western Mediterranean Coast (València, Spain) (39° 27' 48.3" N 0° 19' 07.6" E), during a study of the microbial communities associated to marine wasted residues (Vidal-Verdú *et al*., 2021). The sediment was resuspended in phosphate-buffered saline (PBS) (1x, pH 7.4) and 50 µL were then spread on Marine Agar (MA) (Laboratorios Conda S.A., Spain. Ref: 1059). The plates were incubated at 18 °C for a week. Strain isolation was carried out by re-streaking on fresh media until pure cultures were obtained. Cell suspensions in Marine Broth (MB) (Laboratorios Conda S.A., Spain. Ref: 1217) supplemented with 15 % glycerol (v/v) were cryopreserved at –80 °C. A polyphasic approach was followed in order to determine the taxonomic status of strain M10.9X^T. After isolation, the analysis of the 16S rRNA gene sequence in EzBioCloud revealed that the strains *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T were closely related. Therefore, these strains were selected as comparative strains. Unless otherwise specified, the reference strains *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T, from the DSMZ (German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Braunschweig, Germany), and the strain M10.9X^T were grown simultaneously on MA media at 30 °C.

The phenotypic characterization of strain M10.9X^T was carried out after a week of growth at 30 °C. Gram staining test was performed with KOH 3 % (w/v), recording viscosity as a positive result for Gram-negative bacteria (Tanner *et al*., 2020; Busse *et al*., 2021). In order to test oxidase activity, a commercial oxidase test stick for microbiology (PanReac AppliChem, Barcelona, Spain) was used following the manufacturer's instructions. Hydrogen peroxide 30 % (v/v) was used to test catalase activity, by recording bubble formation as a positive result (Tanner *et al*., 2020). The motility of the strain was studied using the hanging-drop method (Bernardet *et al*., 2002). Growth at different temperatures (4, 10, 16, 20, 24, 30, 37, 40 and 42 °C) and salt tolerance was checked on Zobell agar medium (ZoBell, 1941) that contained the following ingredients (g per litre in modified artificial seawater): Bacto peptone, 5; yeast extract,1; ferric citrate, 0.1; and Bacto agar, 15. The modified artificial seawater contained (g per litre): NaCl, 0–100; $MgSO_4.7H_2O$, 5.94; $MgCl_2.6H_2O$, 0.64; KCl, 4.53; and $CaCl_2$, 1.3 (Lee *et al*., 2012). Growth at different pH values (5.0-10.0 at intervals of 0.5 pH unit) was tested by culturing the strain in MB buffered with MES (pH 5.0-6.5), HEPES (7.0-8.5) and CHES (9.0-10.0) at a final concentration of 10 mM. The ability to grow under anaerobic was determined with the BD GasPak™ EZ pouch system (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Growth under microaerophilic conditions was also tested by BD GasPak™ EZ Pouch System for microaerophilic conditions (Bacton, Dickinson and Company; USA). Carbon source assimilation and enzymatic activities were assessed using the API 20 NE and API ZYM system strips (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions, replacing saline solution 0.9 % by 3.5 % w/v Sea Salts solution (Sigma-Aldrich, USA. Ref: S9883-500G) for cell suspension preparation. BIOLOG GEN III MicroPlates (BIOLOG Inc., Hayward, CA, USA) were also used to test carbon source assimilation.

For fatty acids analysis, strains M10.9X^T, *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T were grown on MA at 30 °C for 48 h. The analysis was carried out following the protocol recommended by MIDI Microbial Identification System (version 6.1, MIDI, Inc, Newark, DE, USA) (Sasser, 1990; MIDI, 2008). Fatty acids were analysed on an Agilent 6850 gas chromatography system and using the TSBA6 method following the manufacturer's instructions.

DNeasy® PowerSoil® kit (Qiagen, Germany) was used for genomic DNA extraction according to the manufacturer's instructions but incubating at 65 °C after adding C1 reagent. Whole 16S rRNA gene PCR was carried out with universal primers 8F (5'- AGA GTT TGA TCC TGG CTC AG -3') (Edwards *et al*., 1989) and 1492R (5'- GGT TAC CTT GTT ACG ACTT -3') (Stackebrandt and Liesack, 1993) following the protocol described by Molina-Menor *et al*. (Molina-Menor *et al*., 2021). MEGA X software v.10.1.7 was used to construct a phylogenetic reconstruction based on the 16S rRNA gene sequences. The trees were constructed by maximum-likelihood (ML) method (Felsenstein, 1981) and neighbour-joining (NJ) method (Saitou and Nei, 1987). For the ML and the NJ trees, the T92+G+I evolutionary model and the Kimura two-parameter model were used, respectively. Bootstrap analysis was used in order to assess the reliability of the branch patterns based on 500 and 1000 replicates, respectively, for the ML and NJ trees (Felsenstein, 1985).

The draft genome of strain M10.9X^T was sequenced with Illumina NovaSeq 6000 system (2 × 150 bp paired-end sequencing). The genomic DNA was randomly fragmented by sonication. DNA fragments were then end polished, A-tailed, and ligated with the full-length adapters of Illumina sequencing. PCR amplification was carried out with P5 and indexed P7 oligos. AMPure XP system was used to purify PCR products as the final construction of the libraries. The size distribution of the libraries was checked by Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA), and quantified by real-time PCR. The quality of the sequence reads was assessed with the FastQC tool (v0.11.5) (Andrews, www.bioinformatics.babraham.ac.uk/projects/fastqc/). The '--isolate' mode in SPAdes (3.14.1) (Bankevich *et al*., 2012) was used for genome assembly of paired reads. Assembly statistics were calculated with QUAST (v.5.0.2) (Gurevich *et al*., 2013). Completeness and contamination levels were evaluated with CheckM (v.1.1.3) (Parks *et al*., 2015). Genome annotation was carried out using the RAST tool kit (RAStk) (Brettin *et al*., 2015) integrated in PATRIC v.3.6.8. In order to identify the closest type strains of strain M10.9X^T and to calculate the dDDH genomic index, the draft genome was uploaded to TYGS (Meier-Kolthoff and Göker, 2019). Average Nucleotide Identities (ANIb) were calculated with JSpecies (Richter *et al*.,2016) according to BLAST between genome pairs. The phylogenomic tree reconstruction based on a multiple alignment of a set of 92 housekeeping genes was conducted with UBCG (v.3.0) (Na *et al*., 2018). FastTree was used to infer the phylogenetic relationships. The reliability of the branch patterns was assessed using bootstrap analysis based on 100 replicates as well as with gene support indices.

The morphological characteristics of the strain M10.9X^T was analysed through Scanning Electron Microscopy (SEM). A fresh overnight culture of the strain M10.9X^T in Marine Broth (MB) was centrifuged and cells were fixed by resuspending them in 1 ml Karnovsky's Fixative (2 % paraformaldehyde, 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer; pH 7.4) overnight. Cells were harvested by centrifugation at 4 ºC for 10 min at 7500 rpm and washed with sterile deionized water and a series of ethanol solutions (30, 50, 70, 90, and 100 %; Pan Reac, AppliChem). The sample was filtered through a polycarbonate membrane filter with the 0.1 um pore size (FILTER-LAB PC Membrane filter; MPC0010013N) and incubated for 48 h in the desiccator prior to embedding them in resin using carbon tape. Sputtering was done using Gold/Palladium (Au/Pd) particles and samples were examined under the Field Emission Scanning Electron Microscope (FE-SEM) Hitachi S4800 (SCSIE, University of Valencia).

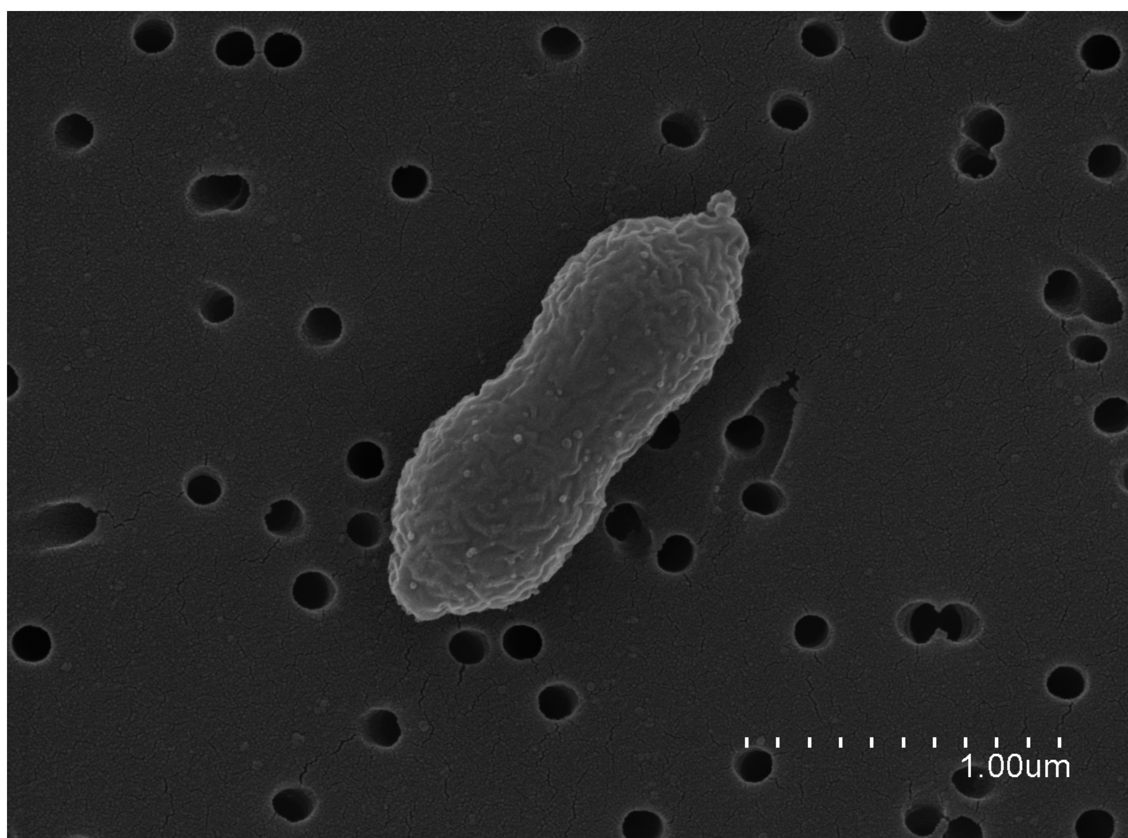

Figure 1. Field Emission Scanning Electron Microscope (FE-SEM) shows the general morphology of a cell of strain M10.9X^T after growth in Marine Broth (MB) at 30 °C, 180 rpm for an overnight. Size bar corresponds to 1 µm.

Results

Strain M10.9X^T was aerobic, oxidase-negative, catalase-positive, Gram-stained-negative, non-motile with a rod-shaped (approximately 0.5 µm in diameter and 1.0-2.0 µm in length) exhibited polarity (Fig. 1) and aggregated occasionally. Colonies on MA medium were light cream, circular, convex, with entire margins, smooth and displayed a diameter of 1-2 mm after 5 days of incubation at 30 ºC.

Strain M10.9X^T was able to grow between 16 and 37 ºC (optimum at 30 ºC) and showed NaCl tolerance up to 5.0 % (w/v). The M10.9X^T was not able to grow on the media without salt. This strain can, therefore, be considered as halophilic based on the salt requirement shows for optimum growth (Kushner, 1987; Amoozegar *et al.*, 2019), which is in accordance with the data reported for other species within this genus. Strain M10.9X^T was able to grow between 5.5 and 9.0 pH units (optimum at 6.0-7.5).

Strain M10.9X^T was negative for acid phosphatase and gelatin hydrolysis, in contrast to *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T, which were positive for these activities. Moreover, strain M10.9X^T was positive for *N*-acetyl-*β*-glucosaminidase, whereas *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T showed a negative response to it. In contrast to strains M10.9X^T and *S. marina* DSM 102235^T, *S. stellata* DSM 11524^T showed a positive response to urease, *α*- and *β*-glucosidase. In API 20 NE strips, all three strains were able to assimilate D-glucose, D-mannose, D-mannitol, D-maltose, potassium gluconate and malic acid. All three strains were negative for the assimilation of capric acid and trisodium citrate. The utilization of L-arabinose was only positive for strain M10.9X^T, while *N*-acetyl-glucosamine and adipic acid were utilized by strain M10.9X^T and *S. marina* DSM 102235^T. Moreover, strain M10.9X^T could not assimilate phenylacetic acid (Table 1). BIOLOG GENIII MicroPlates revealed that strain M10.9X^T was able to oxidise 48 carbon sources after 72 h, in contrast to strains *S. stellata* DSM 11524^T and *S. marina* DSM

102235^T, which were able to oxidise 67 and 69 carbon sources, respectively (Suppl. Table 1). This suggests that strains *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T display a more polytrophic metabolism than strain M10.9X^T.

The almost complete 16S rRNA gene sequence (1410 bp) of strain M10.9X^T was obtained and deposited in the DDBJ/ENA/GenBank under the accession number MW785249. According to EzBioCloud database (https://www.ezbiocloud.net/), the closest type strains of strain M10.9X^T were *Sagittula stellata* E-37^T, *Maliponia aquimaris* CECT 8898^T, *Mameliella alba* DSM 26384^T, *Ponticoccus litoralis* CL-GR66^T and *Sagittula marina* F028-2^T with 97.81 %, 97.74 %, 97.37 %, 96.34 % and 96.22 % of similarity, respectively. After isolation, the analysis of the 16S rRNA gene sequence in EzBioCloud revealed that the strains *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T were closely related. Therefore, these strains were selected as comparative strains, which were obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures (Leibniz Institute, Germany).

The phylogenetic tree based on the maximum-likelihood (ML) and neighbour-joining (NJ) methods showed that strain M10.9X^T was closely related to *S. stellata* E-37^T (Fig. 2 and Supplementary Fig. 1). Although the relationship between the reference strains and *S. marina* DSM 102235^T was not supported by high bootstrap values due to the low resolution of the 16S rRNA gene sequence, the phylogenomic inference confirmed the inclusion of strain M10.9X^T within the clade of *Sagittul*a (Fig. 3).

Table 1. Differential phenotypic characteristics between strain M10.9X^T and the other members of the genus *Sagittula*. Strains: 1, M10.9X^T; 2, *S. marina* DSM 102235^T; 3, *S. stellata* DSM 11524^T. Data for reference strains were analysed parallelly in the present study. +, positive; -, negative. All strains are positive for alkaline phosphatase, esterase (C4), leucine arylamidase, esterase lipase (C8), valine arylamidase and naftol-AS-BI-phosphydrolase, D-glucose, D-mannose, D-mannitol, D-maltose, potassium gluconate and malic acid. All strains are negative for lipase (C14), cystine arylamidase, trypsin, α-chymotripsin, α-galactosidase, β-galactosidase, α-manosidase, α-fucosidase, nitrate reduction and indole production.

	Strain 1	Strain 2	Strain 3
Source	Inner sediment of aluminium can	Sea water	Coastal environment
G+C content (%)	65.2	61.6	65.0
Growth at/in			
Temperature range (°C)	16-37	16-37	16-40
pH range	5.5-9.0	5.5-9.0	6.5-9.0
NaCl tolerance (%, w/v)	1.0-5.0	1.0-8.0	1.0-8.0
Carbon source utilization (API 20NE)			
L-arabinose	+	-	-
***N*-acetyl-glucosamine**	+	+	-
Adipic acid	+	+	-
Phenylacetic acid	-	+	+
Enzymatic activity (API 20NE)			
Urease	-	-	+
Gelatin	-	+	+
Enzymatic activity (API ZYM)			
Acid phosphatase	-	+	+
***α*-glucosidase**	-	-	+
***β*-glucosidase**	-	-	+
***N*-Acetyl-*β*-glucosaminidase**	+	-	-

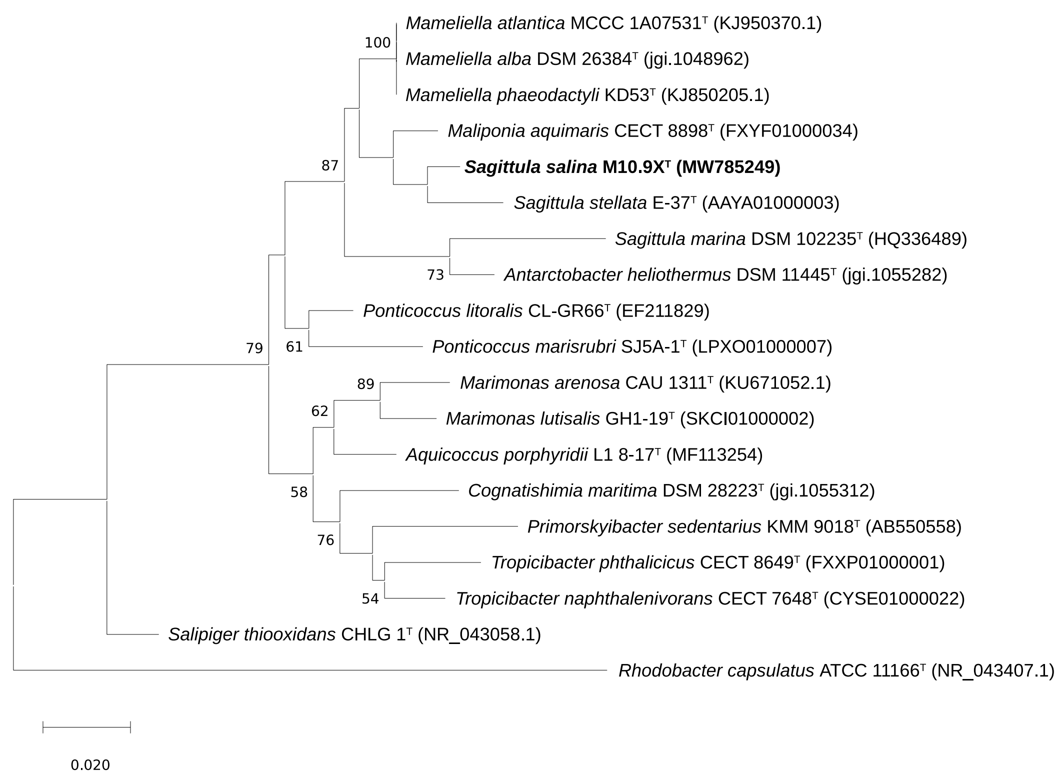

Figure 2. Maximum Likelihood phylogenetic tree showing the relationship between strain M10.9X^T and other members of the family *Rhodobacteraceae* based on 16S rRNA gene sequences. The evolutionary model of nucleotide substitution used was T92+G+I model. Numbers at the nodes indicate bootstrap percentages based on 500 replicates (values below 50% are not indicated). Scale bar 0.020 substitutions per nucleotide position. *Rhodobacter capsulatus* ATCC 11166^T (NR_043407.1) was used as an outgroup.

The draft genome of strain M10.9X^T consisted of 147 contigs (4800470 bp total length). The genomic G+C content was 65.2 %. There were 4717 predicted coding sequences (CDSs), of which 3189 were predicted as proteins with functional assignments. The completeness value and contamination level of the draft genome were 99.7 % and 1.3 %, respectively. This contamination value is low enough to consider the draft genome for further analysis.

The strain M10.9X^T 16S rRNA gene sequence was checked by using BLAST tool (BLASTn) integrated in PATRIC v.3.6.12 compared to the 16S rRNA gene sequence retrieved from the complete genome sequence of strain M10.9X^T with 100 % similarity.

The phylogenetic tree based on the maximum-likelihood (ML) and neighbour-joining (NJ) methods showed that strain M10.9X^T was closely related to *S. stellata* E-37^T (Fig. 2 and Supplementary Fig. 1), The 16S rRNA gene based phylogenetic inference revealed that the genus *Sagittula* did not form a monophyletic group, independently from the algorithm used, as *S. marina* showed a paraphyletic position in both ML and NJ trees. Although the relationship between the reference strains and *S. marina* DSM 102235^T was not supported by high bootstrap values due to the low resolution of the 16S rRNA gene sequence, the phylogenomic inference confirmed the inclusion of strain M10.9X^T within the clade of *Sagittul*a (Fig. 3).

A phylogenomic tree based on a set of 92 housekeeping gene sequences was constructed in order to obtain a more accurate phylogenetic inference of strain M10.9X^T. Strain M10.9X^T clustered with S. marina DSM 102235^T. All three *Sagittula* strains formed a monophyletic group, which was supported by high bootstrap and GI values.

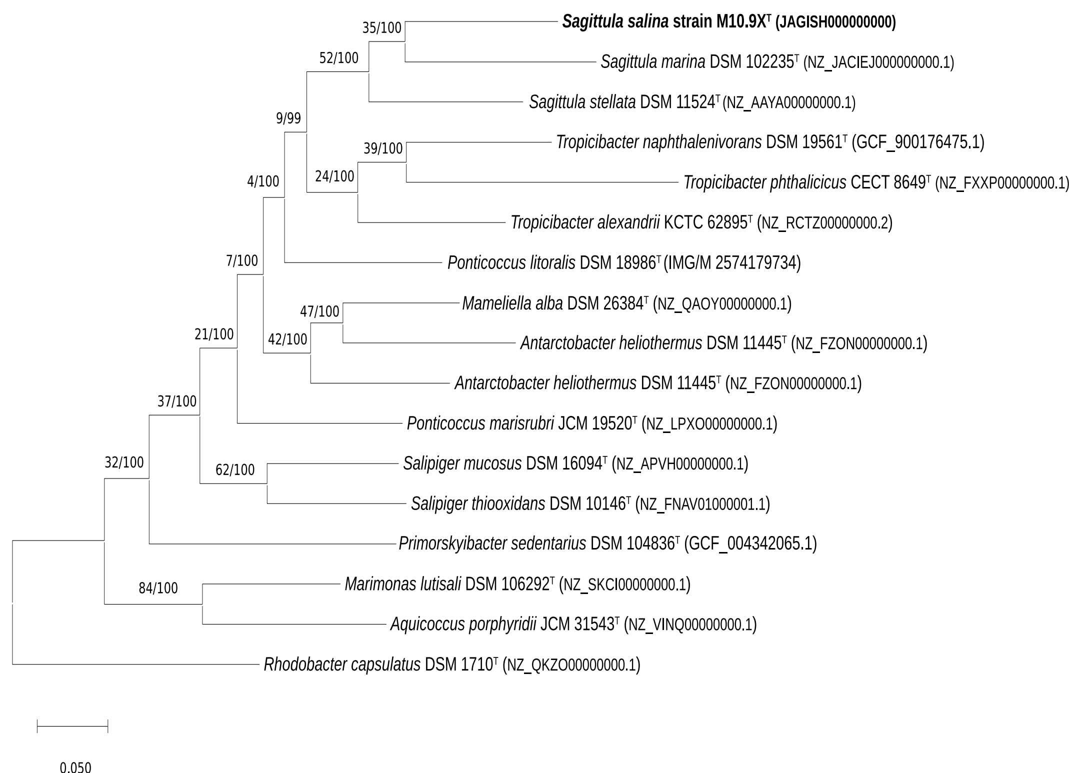

Figure 3. Phylogenomic tree showing the position of strain M10.9X^T among other members of the family *Rhodobacteraceae* based on a multiple alignment of a set of 92 housekeeping genes. Bootstrap analysis was carried out based on 100 replicates. Gene support indices (max. value; 92) and percentage bootstrap values (max. value; 100) are given at nodes. Scale bar shows 0.050 substitutions per site. *Rhodobacter capsulatus* DSM 1710^T was considered as an outgroup.

The dDDH and ANIb values between strain M10.9X^T and other type strains of the genus *Sagittula* were compared (Suppl. Table 2). The dDDH values of strain M10.9X^T against *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T were 20.9 % and 20.2 %. The ANIb values of strain M10.9X^T against *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T were 76.6 %, and 75.6 %, respectively. These values are in accordance with the threshold established to consider strain M10.9X^T as a new species, which are 70 % (Meier-Kolthoff *et al.*, 2013) and 95 % (Richter and Rosselló-Móra, 2009) respectively, for dDDH and ANIb.

Analysis of polar lipids for strain M10.9X^T was carried out by the Identification Service, DSMZ-German Collection of Microorganisms and Cell Cultures (Leibniz Institute, Germany). The strain M10.9X^T is able to synthesise phosphatidylglycerol (PG), phosphatidylethanolamine (PE), an unidentified aminolipid, an unidentified glycolipid, an unidentified phospholipid and an unidentified lipid. The polar lipid profile of strain M10.9X^T is in accordance with the data previously reported by Lee *et al.* (Lee *et al.,* 2013) for *S. marina* F028-2^T, which was also able to synthesise phosphatidylglycerol, and phosphatidylethanolamine and two unidentified aminolipids, an unidentified phospholipid and six unidentified lipids.

The major fatty acid of strain M10.9X^T was summed feature 8 ($C_{18:1}$ $\omega 7c$/$C_{18:1}$ $\omega 6c$) (84.4 %). Moreover, there were also high amounts of $C_{16:0}$ (11.1 %). This is in accordance with the profiles displayed by other species within the genus *Sagittula*, which were also dominated by summed feature 8 and $C_{16:0}$ (Table 2). This confirms the adscription of strain M10.9X^T to the genus *Sagittula.*

According to the phenotypic, genomic, and phylogenetic analysis carried out in the present study, there is evidence to support the inclusion of strain M10.9X^T as a new species within the genus *Sagittula*, for which the name *Sagittula salina* sp. nov. is proposed.

Table 2. Fatty acid analysis (%) of strain M10.9X^T and the strains of the genus *Sagittula*. Strains: 1, M10.9X^T; 2, *S. marina* DSM 102235^T; 3, *S. stellata* DSM 11524^T. tr, < 1.0%; -, not detected.

	Strain 1	Strain 2	Strain 3
Saturated			
$C_{16:0}$	11.11	7.26	12.65
$C_{18:0}$	1.34	1.67	2.16
$C_{19:0}$ cyclo $\omega 8c$	-	-	5.57
Unsaturated			
$C_{18:1}$ $\omega 7c$ 11-methyl	-	4.67	3.42
Hydroxylated			
$C_{12:0}$ 2-OH	-	tr	tr
$C_{12:1}$ 3-OH	2.45	3.32	3.27
Summed Features*			
Sum in Feature 8	84.37	81.67	71.96

*Groups of fatty acids that cannot be resolved reliably from another fatty acid by the chromatographic system were labelled as ‘summed features. Sum in Feature 3 corresponds to $C_{16:1}$ $\omega 7c$/$C_{16:1}$ $\omega 6c$; Sum in Feature 8 corresponds to $C_{18:1}$ $\omega 7c$/$C_{18:1}$ $\omega 6c$.

Description of *Sagittula salina* sp. nov.

Sagittula salina (sa.li’na. N.L. fem. adj. *salina*, salty, referring to the marine environment from which the strain was isolated).

Colonies are light cream, circular, convex, entire, smooth with 1-2 mm diameter after five days of incubation at 30 ºC. Cells are Gram-stained-negative, non-motile, rod-shaped (approximately 0.5 µm in diameter and 1.0-2.0 µm in length) exhibited polarity and aggregation is occasionally observed. This species grows under aerobic and microaerophilic conditions, but no growth is observed in anaerobiosis. Growth occurs at 16-37 ºC (optimum 30 ºC), pH 5.5-9.0 (6.0-7.5), and tolerates 1.0-5.0 % (w/v) NaCl. Alkaline phosphatase, esterase (C4), leucine arylamidase, esterase lipase (C8), valine arylamidase, naftol-AS-BI-phosphydrolase, *N*-acetyl-*β*-glucosaminidase and esculin hydrolysis are detected. Lipase (C14), cystine arylamidase, trypsin, *α*-chymotripsin, acid phosphatase, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *β*-glucosidase, *α*-manosidase, *α*-fucosidase, nitrate reduction, indole production, glucose fermentation, arginine dihydrolysis, esculin hydrolysis, gelatinase and urease are not detected. In API 20 NE test, this species is positive for the assimilation of D-glucose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, malic acid, L-arabinose and adipic acid; and negative for the assimilation of capric acid, trisodium citrate and phenylacetic acid. Using BIOLOG GENIII MicroPlates, this species is positive for the utilization of D-raffinose, *α*-D-glucose, pectin, D-mannose, D-mannitol, D-galacturonic acid, methyl pyruvate, *γ*-amino-butyric acid, D-maltose, D-fructose, D-arabitol, L-alanine, L-galactonic acid lactone, D-lactic acid methyl ester, *α*-hydroxy-butyric acid, D-trehalose, *β*-methyl-D-glucoside, D-galactose, myo-inositol, L-arginine, D-gluconic acid, L-lactic acid, *β*-hydroxy-D,L-butyric acid, D-cellobiose, D-salicin, 3-methyl-D-glucoside, glycerol, D-glucuronic acid, gentiobiose, *N*-acetyl-D-glucosamine, D-fucose, D-glucose-6-PO_4, glucuronamide, *α*-keto-glutaric acid, sucrose, *N*-acetyl-*β*-D-mannosamine, L-fucose, D-fructose-6PO_4, D-malic acid, propionic acid, D-turanose, L-rhamnose, quinic acid, L-malic acid, inosine, L-serine, D-saccharic acid, bromo-succinic acid and negative for the utilization of D-sorbitol, gelatin, p-hydroxy-phenylacetic acid, tween 40, dextrin, *α*-D-lactose, glycyl-L-proline, D-melibiose, L-aspartic acid, citric acid, *α*-keto-butyric acid, L-glutamic acid, acetoacetic acid, L-histidine, mucic acid, *N*-acetyl-D-galactosamine, D-aspartic acid, L-pyroglutamic acid, acetic acid, stachyose, *N*-acetyl neuraminic acid, D-serine and formic acid. The major cellular fatty acids are $C_{18:1}$ $\omega 7c$/$C_{18:1}$ $\omega 6c$ and $C_{16:0}$. The polar

lipids consist of M10.9X^T phosphatidylglycerol (PG), phosphatidylethanolamine (PE), an unidentified aminolipid, an unidentified glycolipid, an unidentified phospholipid, and an unidentified lipid. The type strain M10.9X^T (DSM 112301^T= CECT 30307^T) was isolated from the inner sediments of an aluminium wasted can, which was collected from the western Mediterranean coast (Malva-rosa beach, València, Spain). The DNA G+C content of the type strain is 65.2 %. The 16S rRNA gene sequence and the genome DDBJ/ENA/GenBank accession numbers are MW785249 and JAGISH000000000, respectively.

Data Availability: The 16S rRNA gene sequence of strain M10.9X^T has been deposited in DDBJ/ENA/GenBank under the accession number MW785249. The genomic sequence of strain M10.9X^T has been deposited under the DDBJ/ENA/GenBank accession number JAGISH000000000.

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Abbreviations

MA – Marine Agar
MB – Marine Broth
ANIb – Average Nucleotide Identity
dDDH – Digital DNA Hybridization
ML – Maximum Likelihood
NJ – Neighbour Joining
T92 – Tamura 3-parameter

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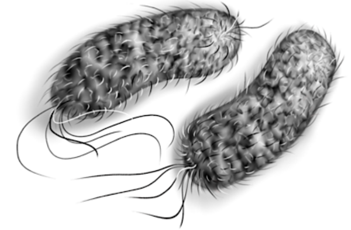
General Discussion



Our research on the wasted chewing gum bacteriome received the Ig Nobel prize in ecology in September 2021. Receiving this prize had a significant effect on the impact of the work. Metrics available here: https://www.nature.com/articles/s41598-020-73913-4/metrics.

Microbial ecology can only be understood based on selective pressure. Selective pressures correspond to physicochemical (abiotic) or biological (biotic) factors that can promote or inhibit a microbial cell or population in natural and artificial habitats (Dini-Andreote *et al.*, 2014; Tobias-Hünefeldt *et al.*, 2021). Biotic factors such as competition, predation, symbiosis and parasitism have been broadly studied in various niches and micro-habitats (Pacheco and Segrè, 2019; Setubal *et al.*, 2020). The present thesis focuses on the microbial population of some artificial and semi-artificial environments, which, although may be presumed mesophilic, and human-associated taxonomically, are in fact often extremophilic microbial niches, whose microbiota is shaped by abiotic factors such as desiccation, irradiation, salinity, low or high temperatures, low water activity, and diminished nutrient availability.

We have characterized here, for the first time, the bacteriome of disposed gum wads through a multi-omics approach. Also, the natural biodegradation of the wasted chewing gum composition, especially by focusing on the biodegradation of the main ingredients such as natural and artificial sweeteners and gum base (Konar *et al.*, 2016). Gum base can be produced from either natural or synthetic polymers. The latter has recently received more attention by the chewing gum industries because of cost, availability, and consistency of the synthetic polymers (Hartel *et al.*, 2018). The results of our research revealed that wasted chewing gums display a moderate diversity of naturally occurring, surface-colonising bacterial population with variations among the analysed wasted chewing gum sampled in several countries. The shared bacterial population at the genus level consisted of *Sphingomonas*, *Kocuria*, *Blastococcus*, *Deinococcus*, and *Skermanella*; other abundant genera *Nesterenkonia* and *Hymenobacter*. Interestingly, all these genera were previously reported as a part of the microbial communities of biofilms in various sun-exposed natural and artificial surfaces, especially solar panels (Dorado-Morales *et al.*, 2016; Ren *et al.*, 2020). One of the shared ecological pressures in all those areas is UV irradiation which was previously described as the selective pressure for some microorganisms such as *Sphingomonas*, *Deinococcus sp. and Hymenobacter sp.* (Dorado-Morales *et al.*, 2016; Tanner *et al.*, 2018; Maeng *et al.*, 2020). It is thus conceivable that a common selection pressure in those environments, in the shape of sun irradiation/UV light, lies behind the similar taxonomic and ecologic profiles of both

photovoltaic panels and pavement-attached wasted chewing gums.

One of the most interesting findings of our work with wasted chewing gum is the precise monitoring we were able to make of the microbial succession taking place in the discarded gum, which consists of a smooth transition between a typical oral microbiome to a surface-associated, environmental one. Indeed, the oral microbiome present in the freshly chewed gums was first gradually substituted by transient taxa such as *Cellulomonas* spp. and *Rubellimicrobium,* which did not persist until the end of the experiment. *Cellulomonas* spp. was previously distinguished by the ability to secrete multiple polysaccharide-degrading enzymes (Ontañon *et al.*, 2021). Discarded gum samples analysed between the sixth and ninth weeks after being placed on an outdoor pavement showed more diversity and richness in terms of environmental genera than the oral bacteria *Streptococcus* sp., *Corynebacterium* sp., *Haemophilus* sp., and *Rothia* sp. Also, *Craurococcus* spp. and *Sphingomonas* spp. were detected in discarded gum samples during this period and remained in the community until the end of the experiment. Furthermore, a Principal Coordinate Analysis (PCoA) proved that the bacterial communities in the wad samples fell into three separated clusters during the 12 weeks of the experiment, indicating a clear correlation between taxonomic profile and time of outdoor exposition. Finally, the succession ended with a mature population of environmental bacterial taxa such as *Actinobacteria*, *Blastococcus, Pseudomonas,* and *Bacillus*. This is not the first time our laboratory has studied microbial successions in artificial environments. Previously, we characterized the bacterial colonization of coffee machines (Vilanova *et al.*, 2015). In the case of the coffee machine, the microbial colonization started by the genus *Pantoea*, followed by other genera such as *Bacillus*, *Terribacillus*, *Paenibacillus*, and followed by a second wave of *Curtobacterium*, *Sphingobium*, *Acinetobacter*, *Pseudomonas*, and *Enterococcus* during the late phase of the experiment. However, *Curtobacterium* spp. could not persist by the end of the experiment. Of those taxa, *Acinetobacter* spp. and *Pseudomonas* spp. were also important players in our chewing gum study. Both have previously been reported as aromatic polymer-degrading bacteria (Meliani and Bensoltane, 2014; Wang *et al.*, 2020). Both genera are also capable to form biofilm under harsh conditions (Vásquez-Ponce *et al.*, 2017; Irankhah *et al.*, 2019). In another research in our lab, the microbial succession of the solar panels was studied within a two-year monitoring (Tanner *et al.*, 2020). First colonizers of the solar panel surface were genera *Lactobacillus*, *Bacillus*, *Sphingomonas* and *Hymenobacter*, which *Sphingomonas* showed the upward trend and became stable after several weeks. Transient taxa such as *Lactobacillus* sp., *bacillus* sp., and *Stemphyllium* sp. arrived and colonized the surface in the first and mid phases of experiment. They were later substituted besides other microorganisms such as *Rubellimicrobium*, *Modestobacter*, *Skermanella Microbispora* by the end of the process. This microbial succession previously reported by our group on solar panels, where four groups, each consisting of two bacterial pairs; (1) *Sphingomonas* and *Deinococcus*, (2) *Arthrobacter* and *Blastococcus*, (3) *Cellulomonas* and *Rubellimicrobium*, and (4) *Skermanella* and *Microbispora* displayed a similar upwards and downwards trend over time. Interestingly, at least in two cases the result of the bacterial colonizers on the wasted chewing gum was the same as these findings. The *Cellulomonas*/*Rubellimicrobium* couple emerged at the mid-phase, and *Arthrobacter*/*Blastococcus* couple also appeared at the late monitored phase. Intriguingly, co-appearance of a Arthrobacter/Blastococcus was previously observed in other poly-extreme ecosystems such as High-Altitude Andean Lakes (HAALs) where the ecosystem suffers from the high UV radiation (Rasuk *et al*., 2017), and extreme hyper-arid soil of several deserts in different geographical locations of the world (Idris *et al*., 2017; Sun *et al*., 2018).

Some of the main bacteria identified in the wasted gums (*Sphingomonas, Hymenobacter* and *Deinococcus)*, have previously been reported as very resistant to

desiccation and radiation conditions (Porcar *et al.*, 2018; Tanner *et al.*, 2018), especially by biofilm formation, which represents a successful survival strategy for microorganisms (Prakash *et al.*, 2003). Biofilm allows the microbial population to deal better with stress and become more resistant to unfavourable environmental conditions such as low water activity and nutrient deprivation. For biofilm formation, Extracellular Polymeric Substances (EPSs) have a crucial role in environmental interactions, such as attachment of cells adhesion to surfaces results in the stabilization and proliferation of the cells in a new environment (Flemming and Wingender, 2010). Moreover, EPSs help microbial interactions between cells by aggregation that assist the communication between those microbial cells (Sheng *et al.*, 2010). EPSs were previously reported in *Sphingomonas* sp., *Hymenobacter* sp., and *Deinococcus* sp. (Baker M. G. *et al.*, 2010; Frösler *et al.*, 2017; Gulati and Ghosh, 2017). It is thus hypothesized that those taxa also produce EPS-based biofilms on/in wasted gums, facilitate interactions within the community, that contribute to their persistence in time in such an adverse environment. The genus *Sphingomonas* was one of the early arrivals that colonized the wasted chewing gums and remained stable towards the end of the monitoring. It can be hypothesized that the primary colonizers of the wasted chewing gum or any other surfaces may modify surface characteristics, allowing subsequent colonization of secondary microorganisms. Interestingly, *Sphingomonas* spp. also can degrade the polycyclic aromatic chains which is a part of the gum base (Leys *et al.*, 2004). On the other hand, previous research revealed that the production of EPSs by *Hymenobacter* sp. clearly increases the interaction of the cell with the surfaces and extending the pH range in a way that the cells can be able to adsorb more cations from the environments (Baker M. G. *et al.*, 2010). Changing the pH range by *Hymenobacter* sp. modifies the abiotic factors and probably enables the second microbial wave to get advantages over the novel favourable conditions. Due to the limitation of the analysis of a single sample gum wads microbiome, further research for studying the behaviour of the sun-exposed microbial communities is needed to confirm all these hypotheses.

NGS strategies were coupled with culturomics, and we set a collection of bacteria from the closely related genera *Curtobacterium*, *Pantoea*, *Microbacterium*, *Pseudomonas*, *Paenibacillus*, *Arthrobacter*, *Serinicoccus*, *Aureimonas*, *Bacillus*, *Sphingomonas*, *Agrococcus*, *Terribacillus, Cellulosimicrobium*, and *Williamisia*. Most of the isolated strains from wasted chewing gum were carotenoid producers such as *Curtobacterium* spp., *Pantoea* spp., *Microbacterium* spp., *Pseudomonas* spp., *Arthrobacter* spp., *Agrococcus* spp., and *Cellulosimicrobium* spp., it can be hypothesized that carotenoid production contributes to survival by overcoming oxidative pressure of sun-exposed surfaces. Carotenoids are colourless to yellow, orange, and red pigments mainly constituted by C40 lipophilic isoprenoids, produced by some microorganisms such as algae, yeast, fungi, bacteria and haloarchaea (Alcaíno *et al.*, 2016; Yabuzaki, 2017). Carotenoids, as antioxidant compounds, play a crucial role in protecting cells, especially in high intense irradiation by quenching of singlet oxygen (Wagner and Elmadfa, 2003; Ram *et al.*, 2020). However, the role of carotenoids is not only limited to saving microorganisms from photodynamic death, but it can also protect the cells by reducing hydroperoxides into stable compounds, preventing free radicals' formation, and inhibiting the auto-oxidation chain reaction. Moreover, carotenoids can act as metal chelators and convert iron and copper toxic ions into safe molecules (Galasso *et al.*, 2017). Previous research in our laboratory also showed that microorganisms from sun-exposed environments such as photovoltaic panels are able to produce different carotenoids (Tanner *et al.*, 2019). As hinted before, genera *Curtobacterium*, *Arthrobacter*, *Pantoea*, *Microbacterium*, and *Sphingomonas* are carotenoid producers, isolated also from the disposed wads.

Another group of extremophilic microorganisms requiring a high salts concentration is that of "Halophiles". Halophilic microorganisms cannot survive

without salts. The diversity of halophilic microorganisms and their adaptation mechanisms to deal with the high salinity in natural and semi-artificial hypersaline and saline environments have received considerable attention (Pandey and Sharma, 2021). Solar saltern is one of the semi-artificial hypersaline environments constructed for the harvesting of salt for human daily consumption. Several studies have been conducted on different geographical located solar salterns around the world that revealed the microbial diversity of solar salters are dominated by unique or mixed microbial communities (Konstantinidis *et al.*, 2022). In the present thesis, we described the microbiome of table salts from solar salterns and originating from landmines.

Archaea and bacteria were detected in the food-grade salt samples by culture-dependent and -independent techniques. Various media with a broad range of salinity and different nutritional and trace elements and long-term incubation allowed us to set a collection of microorganisms from table salts. Interestingly, genus *Bacillus* was the most abundant genera which previously its sporogenesis was well studied (Meeske *et al.*, 2016; Bressuire-Isoard *et al.*, 2018). Sporogenesis is a survival mechanism, applying by many microorganisms to overcome unfavourable conditions such as salinity, low water availability and nutritional resources. Although microbial competition in shortage of nutritional recourses results in the survival of some microorganisms in a population, later, microorganisms with the sporogenesis potential have more chance to survive as vegetative dormant cells for very long periods (Milanesi *et al.*, 2015).

As expected, we isolated strains from the table salts that were closely affiliated to moderately halophilic bacterial genera such as *Gracilibacillus*, *Halobacillus*, *Lentibacillus*, *Metabacillus*, *Piscibacillus*, *Salibacterium*, and *Thalassobacillus*. Previous studies reported species of these genera could optimally grow on the media supplemented with 10 - 20 % (w/v) NaCl (García *et al*., Jeon *et al*., Namwong *et al*., 2005; Tanasupawat *et al*., 2007; Romano *et al*., 2008, Tang *et al*., Amoozegar *et al*., 2009; Mehrshad *et al*., 2013; Kim *et al*., Vishnuvardhan Reddy *et al*., 2015; Gan *et al*., 2020). Apart from moderately halophilic bacteria, extremely halophilic bacteria *Salinibacter ruber* and haloarchaea were also isolated from several table salts, which their demand for high salinity were well studied in the previous research (Antón *et al.*, 2002; Bowers and Wiegel, 2011; Antunes *et al.*, 2017). High salinity can also affect other abiotic factors such as osmotic pressure due to the interaction of water molecules with Na^+ and Cl^- ions which results in the low water activity (Tim, 2002). Besides low water availability, nutritional resources are generally limited to a narrow range in the table salt packages which may affects establishing a particular microbial community.

Our results based on high-throughput sequencing revealed that salts from marine origin shared a similar microbial profile dominated mainly by archaeal taxa *Halorubrum*, *Halobacterium*, *Hallobellus*, *Natronomonas,* *Haloplanus*, *Halonotius*, *Halomarina*, and *Haloarcula*. As hinted before, the origin of salt plays a crucial role in establishing a similar microbial population in table salts. A possible reason for that can be referred to “Forchhammer’s Principle”. This principle is also known as the principle of constant composition that explains the composition of open oceans is remarkably stable and similar not only based on the total salinity but also for the relative proportions of the constituents within and between the open aquatic systems (Solan and Whiteley, 2016). Sodium chloride is one of the main mineral contents of the open oceans, exchanging and reaching stability between all geographically located open aquatic systems based on the Forchhammer’s Principle. Therefore, in the case of salt samples with the marine origin, salinity as the abiotic factor significantly impacts the microbial populations, resulting in the high presence of haloarchaea, which thrives at high range of salinity. On the other hand, all granular salts with non-marine origins were enriched with additional minerals or flavours such as iron, hydrogen sulphate, activated charcoal or dry vegetables, which showed a more heterogeneous taxonomic population dominated by bacteria, which is consistent with a plant- or ingredient-based origin of such

bacterial load over an original archaeal pool. In other words, very likely bacteria originating from those ingredients were inoculated into the salt during the production process. Additionally, there are other possible explanations for the prevalence of bacteria over archaea in those samples. In fact, the salt enriched with dried vegetables (Viking salt) has less sodium chloride content. Although high salinity (above 20 - 25 % w/v NaCl) is the main factor for the survival of haloarchaea (Bowers and Wiegel, 2011), the sodium content for these salts was lower than the threshold. Therefore, salinity as the abiotic factor is the main selective pressure for establishing the microbial population in the table salt packages along with the presence of other ingredients, which may act as an inoculant of bacterial taxa into a basically archaea-rich pool originally present in pure salt samples.

While there is a limitation for the isolation of many microorganisms, the NGS analysis is a powerful tool to study the microbial diversity in a population (Vincent *et al.*, 2017). Here, we can highlight the presence of genus *Sulfitobacter* in Himalayan pink salt with approximately 60 % relative abundance of the microbial population, while no viable species from this genus were isolated by culturomics. One possible reason for that is the lack of particular nutritional resources for the growth of *Sulfitobacter* sp. Another reason may link to physicochemical parameters such as pH, temperature, water, and oxygen availability that affect the growth of the particular *Sulfitobacter* species. Genus *Sulfitobacter* plays a crucial role in the sulphur cycle by is oxidizing sulfites and producing large amounts of organosulfur and atmospheric activated compounds such as dimethylsulfoniopropionate (DMSP) and dimethyl sulfide, respectively (Zeng *et al.*, 2020). The sulphur cycle is almost always coupled with the carbon and nitrogen cycle, which reveals that secondary metabolites of some microorganisms can be a nutritional resource for others. This can make a huge difference in the case of the growth of the microbial cells with co-metabolic pathways.

As it was the case with the microbial collection we set from chewing gum, most of the isolated halophilic and halotolerant microorganism from table salts presented colourful colonies on the presence of different NaCl concentrations. From our collection, bacteria affiliated with the genera *Bacillus, Halobacillus*, *Micrococcus*, *Virgibacillus,* and all archaeal strains which were identified as *Haloarcula* sp*., Halobacterium* sp*., and Halorubrum* sp. were represented a wide range of pale yellow to dark orange colonies on different media. Previous research also showed that the type strains of these genera are carotenoid producers (Antón *et al.*, 2002; Wieser *et al.*, 2002; Romano *et al.*, 2008; Yoon *et al.*, 2010; Wu *et al.*, 2014; Daroonpunt *et al.*, 2019). It is possible that, to cope with the harsh effect of salinity, microorganisms use some alternative mechanisms such as carotenoid production (Wang *et al.*, 2017). The production of carotenoids has been monitored in a variety of halophilic bacteria and archaea. Carotenoids from haloarchaea have received more attention since most archaeal species can produce a higher amount of carotenoids compared to bacteria. Moreover, high salinity results in low water availability and desiccation. In a multi-stress environment such as table salt, pigment production might release some osmoregulation resources, in order to cope better with salinity and desiccation and prevent cells from harmful damages (Mueller *et al.*, 2005).

Previous research on different table salts from various geographical origins have focused mainly on the study of haloarchaea (Henriet *et al.*, 2014; Gibtan *et al.*, 2017). However, our research is the first report on the isolation of halophilic and halotolerant bacteria which constituted a massive portion of the table salts microbiome. Intriguingly, our research was the first work concluding that the origin of the salts is one of the most significant parameters correlating with a particular microbial community in the salt. In the previous study by Henriet *et al.*, the archaeal community of the table salts from different locations was studied by both culture-dependent and independent techniques. Their research also resulted in the isolation of a small group of halophilic and halotolerant bacteria closely affiliated with *Salinibacter ruber*, *Halobacillus alkaliphilus* and *Pontibacillus marinus*, *Actinopolyspora halophila*, and *Staphylococcus hominis* only

from a small fraction of their salt samples. Later, Gibtan and colleagues (2017) studied the bacterial and archaeal diversity in four table salts by 16S rRNA gene sequencing. In our research, we combined both methods to study the archaeal and bacterial communities in order to present the complete microbial draft for the analysed table salt samples. Our results reveal that bacterial community is a largest proportion of the table salt microbiome originating from non-marine ecosystems.

Besides wasted food or food ingredients, other human-associated samples can be home of potentially interesting microbial populations. Previous research in our lab showed that man-made devices such as coffee-machine and solar panels harbour their own microbial communities (Vilanova *et al.*, 2015; Porcar *et al.*, 2018; Tanner *et al.*, 2018). In the present thesis, we also studied the microbiome of another man-made device, a standard laboratory ice machine, which produces artificial ice for laboratory-based experiments. Since summer 2019, two standard laboratory ice machines in I2SysBio have started clogging by a gummy, thick biofilm. The temperature of the ice machine water reservoir being rather low (around 5 ºC); the pH of the water reservoir mildly alkaline (pH 8.2±0.1), and the nutritional recourses very limited, decided to study the microbial diversity of the biofilm growing in such a particular environment by using culture-dependent and -independent techniques. The bacterial strains isolated _by standard microbial culturing_ from an ice machine biofilm closely affiliated to genera *Acidovorax, Bacillus*, *Chryseobacterium*, *Delftia*, *Flavobacterium*, *Hydrogenophaga*, *Methylobacterium*, *Nocardia*, *Peribacillus, Pseudomonas, Prolinoborus*, *Rhodococcus*, *Sphingomonas*. At the species level the fungi also identified as *Aspergillus austroafricanus*, *Cadophora luteo-olivacea, Briansuttonomyces eucalypti*, *Filobasidium magnum, Neomicrosphaeropsis italica, Penicillium citrinum*, and *Vishniacozyma victoriae*. Among all, a higher diversity of microbial strains was observed at 25 ºC versus two other incubating temperatures (4 and 10 ºC).

16S rRNA and ITS high-throughput sequencing revealed that *Sediminibacterium* sp., with a relative abundance higher than 40 %, was the most frequent genus, followed by other genera, *Hydrogenophaga* sp. and *Methyloversatilis* sp. Moreover, the fungal community was dominated by the presence of an unknown genus from the *Didymellaceae* family, with an overwhelming relative abundance of more than 98 % of the fungal reads of the microbial community. Also, BLAST analysis of the ITS sequences from the most abundant ASVs showed that *Didymella boeremae*, *Briansuttonomyces eucalypti*, *Ascochyta phase*, *Neomicrosphaeropsis italica*, and to a lesser extent *Cadophora luteo-olivacea*, and *Vishniacozyma victoriae* were present in the ice machine biofilm.

Based on the shotgun metagenomic sequencing, the most frequent reads belonged to the kingdom Bacteria while less than a quarter was correspond to Fungi. *Methyloversatilis* and *Hydrogenophaga* were also identified as important taxa by shotgun metagenomic sequencing. Although the centrifuge databases did not contain *Sediminibacterium* genomes, it was recovered after metagenomic assembly, which confirmed that *Sediminibacterium* was also among the most abundant genera in the microbial community of the biofilm.

In nature, biofilms are commonly formed by the cooperation of multispecies aerobic, anaerobic, phototrophic, heterotroph and chemotrophic microorganisms, which improves stress tolerance, biomass production, increases signalling and metabolic cooperation among the community. As the number of species increases in the biofilm, the interactions within the multispecies community affect the carbon and nitrogen flows (Tan *et al.*, 2017). Carbon and nitrogen cycles play a key role in the biofilms metabolic pathways (Mhatre *et al.*, 2014). Previous research in the freshwater ecosystems revealed that heterotrophs are able to metabolize organic carbon to carbon oxide, which assimilates by other microbial partners in a biofilm, making biofilm independent from the external carbon resources (Battin *et al.*, 2016). In the case of artificial devices such as the ice machine, biofilm suffers from the limitation of carbon and other nutritional resources, making an artificial oligotrophic environment. An

example of the oligotrophic environments in nature is pure waters, which are the habitats of oligotrophs; a group of microorganisms can thrive under a narrow range of nutrition (Yin *et al.*, 2019). Low nutrition availability is environmental stress for many microorganisms, while oligotrophic microorganisms can deal with this stress well. Under oligotrophic conditions, biofilms act as a protective mechanism for oligotrophs and protect their survival and reproduction (Yin *et al.*, 2019). In oligotrophic niches, biofilm formation has more ecological advantages than living in planktonic form, including efficient nutrient recycling due to the facilitation of syntropy and metabolite exchange. For instance, previous research on the deep waters terrestrial aquifers revealed that biofilms dominated by hydrogen-fed autotrophs can fix nitrogen, which partially reverts the original oligotrophic conditions (Wu *et al.*, 2017). In nature, some biofilms consist of a diversity of microbial cells representing self-sustainable systems due to the complete biogeochemical cycles of carbon, nitrogen, phosphorus, even iron and sulphur (Tan *et al.*, 2017). Among all, nitrogen and phosphorous cycles are often coupled with the carbon cycle, significantly impacting biofilm stability (Battin *et al*., 2016). The ice machine biofilm we have characterised in the present thesis, as an oligotrophic biofilm, is an intriguing, relatively closed microbial niche, which might be largely independent of external nutritional resources due to the cooperation of different species.

The ice machine microbial pool is composed of taxa previously found in similar environments. A member of the genus *Sediminibacterium*, *Sediminibacterium goheungense*, was previously isolated from low nutritional habitats such as a freshwater reservoir. The temperate for isolation and optimum temperature for the growth were reported at 30 and 37 ºC, respectively (Kang *et al.*, 2014). Although previous research by Ayarza *et al.* (2015) revealed that *Sediminibacterium* sp. can either grow planktonically or by self-aggregation, and biofilm formation, the nutrient concentration and redox conditions of the environment might have effects on each type of growth (Ayarza *et al.*, 2015). Regardless of a high relative abundance of *Sediminibacterium* sp. in our samples, this genus was not recovered by culture-based methods which revealed the defects of the culture-dependent methods. Moreover, previous research reported that the genus *Sediminibacterium* is one of the most abundant genera of the water pipelines, in which it has a key role for the corrosion by iron oxidation in the water distribution systems (Wang *et al.*, 2012). This may be related to a role of this genus in the iron cycle. Another study on the biodegradation of plastics such as vinyl chloride (VC) reported a microbial community closely affiliated with *Nocardioides, Sediminibacterium, Aquabacterium, Variovorax* and, to a lesser extent, *Pseudomonas,* which can use carbon directly from VC, or have an impact on VC metabolites indirectly (Wilson *et al.*, 2016). Recently, research by Huang *et al*. revealed that the genus *Sediminibacterium* constituted approximately 10 % of the microbial community of the biofilms on another plastic polymer, polyethylene, in a freshwater ecosystem (Huang *et al.*, 2022). Considering that *Sediminibacterium* was abundant in the ice machine biofilm, and that the ice machines pipelines and water reservoirs are made of metal and plastic, it might be hypothesized that *Sediminibacterium* might affect the carbon cycle by biodegradation of plastic-like materials in an oligotrophic environment. However, due to a few available studies on oligotrophic biofilms and the limitations of our research, further research is needed to confirm this hypothesis.

In the low nutrition environments such as freshwater, genera *Hydrogenophaga* spp., *Methyloversatilis* spp., and *Sphingomonas* spp. have been reported to stay within the biofilms rather than in planktonic forms (Zhang *et al.*, 2014). The high abundance of *Hydrogenophaga* sp. in the natural nutrient-deprived ecosystems revealed that *Hydrogenophaga* species such as *Hydrogenophaga laconesensis*, has an oligotrophic capacity (Mantri *et al.*, 2016). Additionally, previous research by Ruiz-Lopez *et al.* hypothesized that *Hydrogenophaga* species are capable to hydrolyse water and use hydrogen metabolism as an energy source

(Ruiz-Lopez *et al.*, 2020). Furthermore, in case of *Methyloversatilis* spp., members of this genus are methanotrophs and can oxidise methanol (Kuloyo *et al.*, 2020). Methane and methanol oxidation is often coupled with the reduction of nitrate and nitrite in the denitrification process (Vigneron *et al.*, 2017). As it previously discussed by Vigneron *et al*., a possible reason for the high presence of *Methyloversatilis* spp. in oligotrophic groundwater and aquifers ecosystems _as well as the ice machine biofilm_ is probably related to the ability of this genus to couple methane and methanol oxidation to nitrate and nitrite reduction (Vigneron *et al.*, 2017), and its impact on biogeochemical cycles.

In this research, the microbial content of the ice machine biofilm was also compared with other microbial communities that inhabit in cold environments based on the beta diversity (Bray-Curtis dissimilarity metric). Regardless of the similarity between those close cold-adapted biofilms and ice machine biofilm, there is a huge difference in terms of the microbial diversity of the ice machine sample, which reveals that the microbial diversity of the ice machine is novel and unique. The recovery and analysis of high-quality metagenome-assembled genomes (MAGs) resulted in a strikingly high rate of potentially new species. From those 18 analysed genomes, three MAGs could be classified up to the genus level; five were classified at the family level; three at the order level; two at the class level; and four at the phylum level, except one which was identified as a known species: *Mycobacterium gordonae* DSM 44160^{T}. Among all those MAGs analysed to the genus level, we successfully isolated one strain closely affiliated to *Sphingomonas faeni* by culture-based methods. These results demonstrate that the ice machine has a surprisingly high prevalence of previously undescribed bacterial species, which may hold interest in biotechnological applications, especially under oligotrophic conditions.

The ice machine is not only an oligotrophic but also a psychro-alkaliphilic environment (the temperature and pH of the ice machine water reservoir and the sample were 5 ºC and 8.2±0.1, respectively), where the ice machine dynamic microbial community, consisting of several novel species, formed under the poly-extreme selective pressures of low temperature, slightly alkaline pH, and oligotrophy. An example of the poly-extreme environment is deep cold marine habitats, where microbial communities represent different adaptation mechanisms to cope with the low temperature, high pressure, and oligotrophy. Recently, cold-adaptive enzymes and cryoprotectants have been detected and extracted from those microbial communities, suggesting the poly-extreme natural environments represent microbial diversity, with various biotechnological applications (Vester *et al*., 2015; Poli *et al*., 2017).

Finally, we also described in the present thesis a new species of human debris-associated bacterium, of the genus *Sagittula*, designated *Sagittula salina* strain M10.9X^{T}. This strain was isolated from the inner sediment of a wasted aluminium can from the Malva-rosa beach (Vidal-Verdú *et al*., 2021). Marine waste such as plastic and aluminium are distributed worldwide, representing a major environmental problem, and constituting new ecological niches that may harbour potential new microbial species. In previous studies, two species of the genus *Sagittula* were isolated from aquatic environments and reported as promising candidates with bioremediation capacities (Gonzalez *et al.*, 1997; Lee *et al.*, 2013; Frank *et al.*, 2018). Therefore, we carried out a polyphasic characterization of the strain M10.9X^{T} in order to uncover the possible potential of this microorganism from bioremediation and bioprospecting point of view.

For a long time, the famous microbiological tenet "*Everything is everywhere, but, the environment selects*" by Baas Becking (1934) has been used as the question-raising starting point for lots of research on microbial diversity and distribution (De Wit and Bouvier, 2006). In the present thesis, we studied the microbial diversity of several semi-artificial and artificial environments considering the Baas Becking hypothesis. Here, we have also discussed the microbial distribution and colonization of those environments and explained the impact of the

environmental physicochemical factors such as desiccation, UV irradiation, nutrient deprivation, low temperature, *etc*. on the microbial contents of man-made microbial habitats. Therefore, "Everything is everywhere" points to the notable distribution of microorganisms all over the world, whereas "the environment selects" reveals that only microorganisms with the particular adaptation mechanisms can survive and thrive in the new extreme environments, and after stability, they can establish their own populations in the new micro-niches (Fondi *et al*., 2016). Our results therefore support Bass Becking thesis on the primary role of selective pressure at the microinches level as a key factor shaping microbial worlds.

General Conclusions

In the present thesis, the microbial communities of several semi-artificial and artificial environments have been studied using culture-dependent and independent techniques. Although the conclusions of each particular environment are discussed in detail in the corresponding chapters, the general conclusions of our research are listed here:

-The NGS analysis allowed us to describe the microbial communities of a food-grade semi-artificial condiment (table salts), undescribed synthetic product (the disposed gum wads), and anthropized machine (the standard laboratory ice machine). As a result, several microbial species with potential applications in the biotechnological process have been detected and identified.

-Culturomics has been used to isolate and characterise individual strains of particular interest as the complementary approach to the NGS analysis. Some of the isolates have a variety of applications in bioprospecting. The isolates from the wasted chewing gums show a promising potential for the bioremediation of the disposed sticky plastic-like chewing gum residues. Also, the isolates from the food-grade salts have received a noticeable interest in producing antioxidant biomolecules such as carotenoids. Furthermore, bacteria isolated from the ice machine are proper candidates for studying the oligotrophic environments, and those isolates corresponding to a new species may have a biotechnological interest.

-The present thesis studied the succession of the environmental microorganism over the trapped oral microbiome in the wasted chewing gums. The result consists of a smooth transition between a typical oral microbiome to a surface-associated, environmental one, which revealed that the oral microbiome persists in the wasted chewing gum even after several weeks of outdoor exposition. Our findings have applications in forensics and contagious disease control disciplines.

-The physicochemical properties of the analysed environments have been extensively discussed in this thesis. We described those extreme selective pressures; UV-irradiation, desiccation on/in the wasted chewing gums, salinity, low water availability in the table salts, low temperature, and nutrient availability in the ice machine, which shape the particular microbial communities, harbouring specific survival mechanisms including spore and carotenoids production to survive and colonize the environments.

-In connection with the role of abiotic pressures in establishing a microbial community in an environment, our results support Bass Becking's thesis of the primary role of selective pressures as a key factor that shapes microbial worlds.

Future Perspective: a personal view

The present thesis aimed to study the microbial community of several semi-artificial and artificial environments that suffer from multi-stress conditions. Although our research on the microbiome of the man-made products presents engaging results and interesting conclusions, there are still a few possible open questions, we would like to answer in the close future. As the time limitation of a PhD thesis doesn't allow us to exceed further, here, we list some future possible approaches, which could be interesting topics to study.

In chapter I, the microbial community of the wasted chewing gums attached to the outdoor sun-exposed environments was studied. However, it would be an interesting subject to investigate the wasted chewing gum microbiome in indoor areas. In this case, the colonization of the indoor wasted chewing gum could be compared with our research, and the shared microbial taxa, with the possible biodegradation capacities, would be uncovered under both conditions. Studying the proteomics of the wasted chewing gum microbiome could also be interesting, which could provide more information on the wasted chewing gum biodegradation.

In chapter II, we showed that the table salt bacteriome could be a source of halophilic halotolerant bacteria that characterize either healthy or diseased human guts. Another interesting research could be the connection between the microbial contents of the table salts and human microbiota, as the origin of the halophilic human gut microbiome has not been determined so far. It could be interesting to study whether the bacteria ingested along with salt consumption affect the microbial gut content and, ultimately, the health of the consumers.

In chapter III, our results showed that the ice machine biofilm is a multispecies microbial community, with the collaboration of previously described aerobic, anaerobic, heterotrophs, chemotrophs, etc., which suggests that the ice machine biofilm is probably relatively independent of external nutrient resources. However, it would be more fruitful if the microbial content of different layers of the ice machine biofilm could be studied in order to test the microbial special distribution. It is also recommended that the ice machine biofilm metabolomics be studied to understand the role of each microbial taxa in the nutrition cycles through this oligotrophic biofilm.

Finally, in chapter IV, we suggest that the aluminium corrosion by *Sagittula salina* strain M10.9X^T would be investigated as this strain was isolated from the inner sediment of aluminium waste. This could present more information on the role of the genus *Sagittula* in biochemical cycles.

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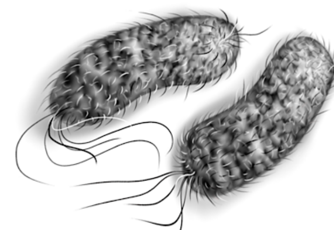
Resumen en castellano



Introducción

La Tierra es el hogar de una gran biodiversidad de microorganismos, que recientemente se ha estimado en 10^{12} o más taxones microbianos que habitan nuestro planeta. Durante décadas, se han explorado los ambientes naturales para estudiar sus comunidades microbianas, así como los mecanismos de adaptación específicos de las especies que los componen y sus potenciales aplicaciones biotecnológicas. No solo los entornos naturales sino también los artificiales tienen interés como fuentes de diversidad microbiana. Investigaciones anteriores sobre el microbioma de los productos hechos por el hombre revelaron que los dispositivos artificiales con un diseño y un procedimiento de operación estándar representan presiones selectivas particulares, como ocurre con los paneles solares, cafeteras, lavadoras, lavavajillas, sistemas de refrigeración por aire y sistemas de calentamiento de agua, que dan como resultado la selección de un microbioma que no cambia significativamente dentro de las variaciones geográficas o climáticas. Sin embargo, esos ambientes artificiales y sus comunidades microbianas han recibido poca atención.

En la presente tesis doctoral, estudiamos el microbioma de varios ambientes artificiales y semiartificiales, especialmente aquellos con presiones selectivas particulares como la desecación, la radiación ultravioleta, la salinidad, la privación de nutrientes, las temperaturas extremas, la actividad de agua y las variaciones de pH. Nuestros resultados mostraron que, además del microbioma asociado al ser humano, que puede constituir una fracción considerable de los dispositivos artificiales, los consorcios microbianos ambientales no patógenicos acabn estableciéndose en esos ambientes. Entre todas esas comunidades microbianas, algunas especies son extremófilas y algunas pueden tolerar las condiciones extremas de su entorno, albergando una variedad de mecanismos de supervivencia, como la esporulación y/o la formación de biopelículas. Algunos otros han desarrollado evolutivamente mecanismos adaptativos intercelulares e intracelulares para hacer frente a las duras condiciones ambientales.

Objetivos

El objetivo de esta tesis doctoral es caracterizar las comunidades microbianas de algunos ambientes artificiales y semiartificiales que se encuentran bajo constantes presiones selectivas particulares tales como irradiación UV, desecación, salinidad, privación de nutrientes, baja temperatura y disponibilidad de oxígeno. Para ello, se utilizaron análisis NGS y cultarómicos para describir las comunidades microbianas asociadas con chicles desechados, sales de mesa y la máquina de hielo de un laboratorio. Además, estudiamos la sucesión de la comunidad microbiana en la colonización de productos sintéticos como la goma de mascar desechada. Se identificaron especies microbianas asociadas con productos artificiales como goma de mascar, sales de mesa y dispositivos completamente manufacturados, como la máquina de hielo, y hemos discutido su potencial para aplicaciones biotecnológicas. También aislamos y caracterizamos microorganismos

individuales de esos ambientes utilizando medios y métodos estándar para determinar su actividad biológica. Una nueva especie del género Sagittula, previamente aislada del desecho marino (una lata de aluminio) se caracterizó mediante enfoques fenotípicos multiómicos.

Capítulo I - Metodología, resultados y discusión

En esta disertación, hemos estudiado el bacterioma de los chicles desechados, los cuales son a menudo desechados indebidamente en calles, terrazas, aceras o pavimentos exteriores de cualquier tipo. Generalmente, los chicles se componen de dos fases: la fase insoluble en agua (base de goma) y la fase soluble en agua, que puede estar hecha de azúcar o alcoholes de azúcar como polioles. El componente principal de cualquier goma de mascar es la base de goma (20-30 %), que no es comestible ni digerible, se puede producir a partir de polímeros naturales o sintéticos y tiene propiedades similares a las del plástico. Los chicles desechados a menudo se consideran contaminantes ambientales y tienen además un componente estético muy negativo. Estudios anteriores muestran que el ADN del consumidor y el microbioma oral pueden quedar atrapados en el residuo pegajoso, pero la supervivencia del microbioma oral a lo largo del tiempo solo ha recibido una atención limitada hasta la fecha.

Por primera vez, hemos estudiado el contenido microbiano de los chicles desechados muestreados en diferentes lugares del mundo, así como la distribución de bacterias según la profundidad (en tres fases: la superficie, la capa intermedia y la base del chicle, en contacto con el sustrato) y hemos realizado un estudio dinámico para descubrir la sucesión microbiana que tiene lugar en el chicle durante las primeras semanas después de que sea descartado en una superficie al aire libre. Además, hemos evaluado la capacidad de biodegradación de los ingredientes de la goma de mascar de una colección de cepas bacterianas que aislamos de los residuos de la goma de mascar.

Las sucesiones microbianas a partir del bacterioma oral atrapado en el proceso de masticación en la goma de mascar desperdiciada mostraron que el bacterioma oral fue gradualmente sustituido, en los chicles descartados sobre el pavimento, por bacterias ambientales durante tres meses de exposición al aire libre. Mientras que el grupo del microbioma salival se detectaron principalmente como géneros predominantes *Streptococcus*, *Corynebacterium*, *Haemophilus* y *Rothia*, bacterias ambientales transportadas por el aire como *Acinetobacter* spp., *Sphingomonas* spp. *Pseudomonas* spp. y *Rubellimicrobium* spp. colonizaron la goma de mascar desperdiciada transcurridas varias semanas. De hecho, el microbioma oral presente en los chicles recién masticados primero fue sustituido por taxones transitorios como *Cellulomonas* spp. y *Rubellimicrobium*, que no persistieron hasta el final del experimento. Es interesante recalcar que *Cellulomonas* spp. se ha descrito por las capacidades de secretar múltiples enzimas degradadoras de polisacáridos. Por tanto, se puede plantear la hipótesis de que estos colonizadores primarios del chicle desperdiciado pueden modificar las características de la superficie, permitiendo la colonización posterior de microorganismos secundarios.

Curiosamente, varios chicles desechados recolectados en todo el mundo contienen un típico bacterioma ambiental de biopelícula subaérea, caracterizado por especies como *Sphingomonas* spp., *Kocuria* spp., *Deinococcus* spp. y *Blastococcus* spp., que están habitualmente presentes en ambientes naturales y artificiales expuestos al sol, como paneles solares, y que suelen estar caracterizados por la formación de biofilms o biopelículas. La biopelícula permite que la población microbiana afronte mejor el estrés y se vuelva más resistente a las condiciones ambientales desfavorables, como la baja actividad del agua y la privación de nutrientes. Además, algunos de los miembros de esos géneros pueden degradar las cadenas aromáticas policíclicas que forman parte de la base de la goma.

En nuestrio trabajo, se estableció una colección de cepas bacterianas de chicles desechados y se analizó la capacidad de biodegradación de diferentes ingredientes de chicles por parte de los aislados. Nuestros resultados sugieren que la bioaumentación de algunas de las cepas que

caracterizamos puede usarse como una estrategia de biorremediación para eliminar los residuos de chicle de los pavimentos contaminados. También mostramos que no hay diferencias significativas en términos de las comunidades microbianas de las diferentes capas de chicle, lo que sugiere que las propiedades fisicoquímicas de un chicle no cambian a lo largo de su profundidad y los factores selectivos como son la radiación de UV y la actividad del agua forman una comunidad bacteriana particular. En conjunto, nuestros resultados sugieren que las bacterias ambientales desempeñan un papel en la biodegradación natural de la goma de mascar y también pueden ser una fuente de cepas con propiedades de biodegradación. Esta investigación fue el primer informe que caracterizó, desde un enfoque holístico, la composición bacteriana del chicle desechado, y recibió el premio IgNobel de ecología a las investigaciones sorprendentes en septiembre de 2021.

Capítulo II - Metodología, resultados y discusión

También estudiamos el microbioma de otro entorno semiartificial, las sales de mesa, producidas en diferentes salinas marinas y continentales. En los hábitats salinos naturales, las arqueas y las bacterias halófilas son las comunidades microbianas predominantes. Esos ambientes salinos son en los que se produce, por cristalización, la sal de mesa comercial. La mayoría de las sales comerciales se refinan y se muelen finamente antes de su distribución. Por lo tanto, esos microorganismos pueden quedar atrapados en las inclusiones fluidas durante el proceso de cristalización y seguir siendo viables después de la extracción y el envasado. Hay muy pocos informes sobre el microbioma de la sal de mesa y tienden a centrarse en los taxones de arqueas. Sin embargo, el presente trabajo describe una caracterización completa del bacterioma de dichas sales, donde se estudió la diversidad de microorganismos halotolerantes y halófilos en seis muestras comerciales de sal de mesa mediante técnicas dependientes e independientes del cultivo. Se obtuvieron tres sales de mesa de origen marino: del Océano Atlántico, del Mediterráneo (Isla de Ibiza) y de las marismas del Odiel (sal marina de Mercadona). También se utilizaron otras sales complementadas con ingredientes minerales y nutricionales: sal rosa del Himalaya, sal negra de Hawai y una sal vegetal seca conocida como sal Vikinga.

Independientemente del origen de las sales, las especies de géneros bacterianos como *Flavobacterium*, *Bacillus* y *Yoonia-Loktanella* fueron los taxones más abundantes, mientras que las arqueas más frecuentes fueron *Natronomonas*, *Halolamina*, *Halonotius*, *Halapricum*, *Halobacterium*, *Haloarcula* y *Halobacterales*. Curiosamente, los resultados de la secuenciación del gen 16S rRNA revelaron que las sales de origen marino muestran una taxonomía de arqueas similar, pero con variaciones significativas entre los géneros. Los taxones *Halorubrum*, *Halobacterium*, *Hallobellus*, *Natronomonas*, *Haloplanus*, *Halonotius*, *Halomarina* y *Haloarcula* prevalecieron en las sales marinas. Además, el género *Salinibacter* se detectó solo en sales de origen marino.

La sal rosa del Himalaya, la sal negra de Hawai y la sal Vikinga mostraron perfiles taxonómicos más heterogéneos dominados por bacterias. Estas sales no marinas a menudo se enriquecen con minerales o saborizantes adicionales, como hierro, sulfato de hidrógeno, carbón activo y vegetales secos. La sal del Himalaya se extraía de las estribaciones de la cordillera del Himalaya que alguna vez fueron mares. El género más importante en esta muestra fue *Sulfitobacter*. Podría plantearse la hipótesis de que *Sulfitobacter* aparece en una cantidad tan significativa en esta muestra debido a la actividad que muestra este género para oxidar los sulfitos, que están presentes en altas concentraciones en la región del Himalaya. Otra muestra del grupo no marino es la sal Vikinga, que presenta una alta frecuencia de cloroplastos (más del 66 % de su perfil taxonómico) y mitocondrias (4.7 % del total), lo que puede ser consecuencia de su composición actual (contiene diferentes vegetales). El género *Bacillus* también estuvo presente en esta muestra. Este género contiene ciertas especies tolerantes a la sal. Además, se puede suponer que las bacterias que se originan a partir de minerales o sabores adicionales se inocularon en la sal durante el proceso de producción. Además, existen otras posibles explicaciones para la prevalencia de bacterias sobre arqueas en esas muestras. De hecho, la sal enriquecida con vegetales secos tiene

menos contenido de cloruro de sodio. La alta salinidad (por encima del 20 - 25 % p/v de NaCl) es el factor principal para la supervivencia de las haloarqueas. Por lo tanto, la salinidad como factor abiótico es la principal presión selectiva para establecer la población microbiana en los paquetes de sal de mesa junto con la presencia de otros ingredientes.

Como era de esperar, aislamos cepas de las sales de mesa que estaban estrechamente relacionadas con bacterias (en general, moderadamente) halófilas. Se estableció una colección de 76 especies de bacterias y haloarqueas halotolerantes y halófilas mediante el cultivo en diferentes medios con una amplia gama de salinidad y composición de nutrientes. La comparación de los resultados de la metataxonómica y la culturómica del gen 16S rRNA reveló que los microorganismos cultivables *Acinetobacter*, *Aquibacillus*, *Bacillus*, *Brevundimonas*, *Fictibacillus*, *Gracilibacillus*, *Halobacillus*, *Micrococcus*, *Oceanobacillus*, *Salibacterium*, *Salinibacter*, *Terribacillus*, *Thalassobacillus*, *Haloarcula*, *Halobacterium* y *Halorubrum* se identificaron al menos en una muestra por ambos métodos. Aunque la mayor parte del microbioma de las sales de mesa eran bacterias halófilas y arqueas, algunos microorganismos potencialmente patógenos como *Enterococcus* sp. y *Brevundimonas* sp. se identificaron en casi todas las muestras, mientras que bacterias potencialmente consideradas como beneficiosas como *Lactobacillus* sp. solo se detectaron en algunas sales de mesa. Esto sugiere que la sal de mesa puede ser una fuente de algunas de las bacterias halotolerantes que se caracterizan como microorganismos promotores de la salud o patógenos, cuya conexión con la salud del consumidor debería recibir más atención. Por otro lado, se ha monitorizado la producción de carotenoides en una variedad de bacterias y arqueas aisladas. La mayoría de los microorganismos aislados de la sal de mesa son productores de carotenoides. Los carotenoides son pigmentos que pueden ser incoloros, amarillos, naranjas o rojos, y están constituidos principalmente por isoprenoides lipofílicos C40, como compuestos antioxidantes y desempeñan un papel crucial en la protección de las células, especialmente al reducir los hidroperóxidos en compuestos estables, prevenir la formación de radicales libres, inhibir la reacción en cadena de auto-oxidación y en la irradiación de alta intensidad mediante la extinción del oxígeno monoatómico. Además, los carotenoides pueden actuar como quelantes de metales y convertir los iones tóxicos de hierro y cobre en moléculas no tóxicas. Parece que, en un entorno de estrés múltiple como la sal de mesa, la producción de pigmento podría ser un mecanismo de supervivencia, especialmente para los microorganismos halotolerantes, frente a la salinidad y la desecación y probablemente evitar que las células sufran daños irreversibles.

Capítulo III - Metodología, resultados y discusión

Además de los alimentos o residuos alimentarios, los dispositivos artificiales pueden albergar poblaciones microbianas potencialmente interesantes. En esta tesis, también estudiamos el microbioma de una máquina de hielo de laboratorio estándar, cuya bomba de extracción de agua estaba obstruida por una biopelícula espesa y gomosa. Describimos la composición de esta comunidad microbiana oligotrófica que habita este dispositivo a través de técnicas independientes y dependientes de la cultura. Mediante el uso de culturómica en ocho medios diferentes a tres temperaturas psicrófilas y mesófilas, se aislaron e identificaron taxonómicamente 25 cepas microbianas diferentes. Las cepas bacterianas aisladas de la biopelícula de máquina de hielo están estrechamente afiliadas a los géneros *Acidovorax*, *Bacillus*, *Chryseobacterium*, *Delftia*, *Flavobacterium*, *Hydrogenophaga*, *Methylobacterium*, *Nocardia*, *Peribacillus*, *Pseudomonas*, *Prolinoborus*, *Rhodococcus*, *Sphingomonas*. A nivel de especie los hongos también se identificaron como *Aspergillus austroafricanus*, *Cadophora luteo-olivacea*, *Briansuttonomyces eucalypti*, *Filobasidium magnum*, *Neomicrosphaeropsis italica*, *Penicillium citrinum* y *Vishniacozyma victoriae*. Entre todos, se observó una mayor diversidad de cepas microbianas a 25 ºC frente a otras temperaturas de incubación; 4 y 10 ºC. Aquí, la esporogénesis puede desempeñar un papel clave, ya que algunas especies estrechamente relacionadas con las cepas presentes en la tubería de drenaje de la máquina de hielo, como los géneros *Bacillus* y *Peribacillus*, se han descrito anteriormente como bacterias formadoras de esporas. Una posible explicación de la mayor

biodiversidad de las especies aisladas a 25 ºC en comparación con las otras dos temperaturas puede estar relacionada con la alta abundancia relativa de bacterias formadoras de esporas en la biopelícula.

El análisis de secuenciación de alto rendimiento 16S rRNA e ITS reveló que *Bacteroidota* y *Proteobacteria* eran los filos bacterianos más abundantes en la muestra, seguidos de *Acidobacteriota* y *Planctomycetota*, mientras que la comunidad fúngica estaba claramente dominada por la presencia de un género desconocido de la familia *Didymellaceae*. A nivel de género, la comunidad bacteriana se caracterizó por la presencia de *Sediminibacterium* sp., con una abundancia relativa superior al 40 %, seguida de otros géneros, *Hydrogenophaga* sp. y *Methyloversatilis* sp. A pesar de la gran abundancia de especies de *Sediminibacterium* según los resultados de la NGS y las condiciones de cultivo favorables, que se utilizaron previamente para aislar *Sediminibacterium* spp. de otros ambientes oligotróficos, no fue posible aislar ninguna cepa de este género. Esto puede ser debido a la falta de factores abióticos específicos, como el pH ácido y la concentración de sal, que son necesarios para algunos miembros de este género, como *Sediminibacterium lactis* y *Sediminibacterium halotolerans*, respectivamente. En los ambientes de bajo contenido en nutrientes como es ciertas aguas continentales, los géneros *Hydrogenophaga* spp. y *Methyloversatilis* spp. se ha reportado que permanecen dentro de las biopelículas en lugar de en formas planctónicas. La alta abundancia de *Hydrogenophaga* sp. en los ecosistemas oligotróficos naturales reveló que especies de *Hydrogenophaga* como *Hydrogenophaga* laconesensis, tienen una capacidad de adaptación a la vida oligotrófica. Investigaciones anteriores también sugirieron que *Hydrogenophaga* sp. son capaces de hidrolizar agua, utilizando el metabolismo del hidrógeno como fuente de energía, lo que podría afectar la composición microbiana de la biopelícula.

El análisis de coordenadas principales (PCoA) mostró que las muestras más similares a la biopelícula de la máquina de hielo eran dos muestras de biopelícula provenientes de depuradores de aire biológicos en una instalación de alojamiento porcino, así como una muestra de biocrust del suelo del Ártico. De hecho, a pesar de la baja similitud entre las muestras, estas cuatro muestras compartían hasta seis géneros (*Pseudoxanthomonas*, *Ferruginibacter*, unknown *Comamonadaceae*, *Clostridium sensu stricto 1*, unknown *Microbacteriaceae* y *Brevundimonas*) identificados mediante NGS, mientras que en los otros 51 géneros encontrados en el hielo el microbioma de la máquina también se encontró en al menos una de las otras muestras. Además, el análisis de diversidad alfa demostró que la muestra de la máquina de hielo, a pesar de mostrar un alto número de ASV observados, es relativamente menos diversa, es decir, más homogénea en su composición. Finalmente, las comparaciones de diversidad alfa y beta de la comunidad microbiana de la máquina de hielo con la de otros ambientes fríos revelaron una baja similitud entre las muestras.

Para complementar la información taxonómica de la comunidad microbiana, también se realizó secuenciación metagenómica (shotgun). A partir de la secuenciación metagenómica de shotgun, las lecturas más frecuentes correspondieron al reino Bacteria mientras que menos de una cuarta parte correspondió a hongos. *Sediminibacterium*, *Methyloversatilis* e *Hydrogenophaga* también se identificaron como principales taxones de biopelículas mediante secuenciación metagenómica. Además, *Stagonosporopsis sp*, perteneciente a la familia *Didymellaceae*, fue el género más abundante entre los eucariotas. Esto está en concordancia con los resultados obtenidos del análisis de secuenciación ITS, en el que también predominaron los miembros de la familia *Didymellaceae*.

La recuperación y el análisis de genomas ensamblados en metagenoma (MAG) de alta calidad arrojaron una tasa sorprendentemente alta de nuevas especies potenciales en la máquina de hielo. Cada MAG se clasificó taxonómicamente usando MiGA y luego se calculó el índice de Identidad Promedio de Nucleótidos (ANI) al vecino filogenético más cercano. Sorprendentemente, todas las MAG se clasificaron como especies potencialmente nuevas con un valor ANI <95 %,

excepto MAG 14 que se identificó como *Mycobacterium gordonae* DSM 44160^T con un valor ANI de 98,32 %. En general, tres MAG se pudieron clasificar hasta el nivel de género, cinco se clasificaron para nivel de familia, tres a nivel de orden, dos a nivel de clase y cuatro a nivel de phylum. Además, con una resolución taxonómica más alta que la proporcionada por los MAG, hemos encontrado que las bacterias detectadas en la máquina de hielo corresponden a varias especies nuevas, que no hemos podido cultivar hasta la fecha.

Como mostraron los resultados de la metataxonómica y la metagenómica, la biopelícula de la máquina de hielo está formada por la cooperación de microorganismos aeróbicos, anaeróbicos, heterótrofos y quimiotrópicos de múltiples especies, lo que podría mejorar la tolerancia al estrés, la producción de biomasa, aumentar la señalización y la cooperación metabólica entre la comunidad. A medida que aumenta el número de especies en la biopelícula, las interacciones dentro de la comunidad de múltiples especies afectan a los flujos de carbono y nitrógeno. En este caso, probablemente cada población microbiana tiene un papel crucial dentro de la comunidad en los ciclos biogeoquímicos. La singularidad del microbioma de la máquina de hielo podría resultar del efecto de ambas presiones selectivas simultáneamente, el frío y la escasez de nutrientes. Los microorganismos psicooligotróficos son capaces de crecer y reproducirse lentamente a temperaturas que oscilan entre -20 °C y +10 °C en ambientes con niveles muy bajos de nutrientes. En este caso, las sustancias exopolisacáridas bacterianas (EPSs) y los micelios fúngicos podrían actuar como una red de intercambio de nutrientes y más particularmente de elementos como el carbono y el nitrógeno. Por tanto, se puede plantear la hipótesis de que la biopelícula oligotrófica de la máquina de hielo es un nicho microbiano relativamente cerrado, que podría ser en gran medida independiente de los recursos nutricionales externos debido a la cooperación de diferentes especies.

Además de la oligotrofia, la máquina de hielo también es un entorno psicro-alcalino debido a que la temperatura del depósito de agua de la máquina de hielo era bastante baja (alrededor de 5 ºC); y el pH era ligeramente alcalino (pH 8,2 ± 0,1). Así que, en resumen, la comunidad microbiana dinámica de la máquina de hielo consta de varias especies nuevas, formadas bajo presiones selectivas poliextremas de baja temperatura, pH ligeramente alcalino y oligotrofia. Recientemente, se han detectado y extraído enzimas adaptables al frío y crioprotectores de comunidades microbianas oligo-psicrofílicas, lo que sugiere que los entornos poliextremos representan diversidad microbiana, con diversas aplicaciones biotecnológicas. Dado que el microbioma de la máquina de hielo comprende nuevas especies y puede tolerar este estrés multiextremo, esto sugiere que la máquina de hielo tiene una prevalencia sorprendentemente alta de especies no descritas anteriormente, que pueden tener interés en aplicaciones biotecnológicas, especialmente en condiciones oligotróficas y/o poliextremófilas.

Capítulo IV - Metodología, resultados y discusión

Por otro lado, en la presente tesis doctoral, describimos una nueva especie de bacteria asociada a desechos que fue aislada del sedimento interior de una lata de aluminio desechada de la playa de la Malvarrosa. Los desechos marinos como el plástico y el aluminio se distribuyen en todo el mundo, lo que representa un problema ambiental importante y constituye nuevos nichos ecológicos que pueden albergar nuevas especies microbianas. Por ello, llevamos a cabo una caracterización polifásica de la cepa M10.9X^T, para descubrir el posible potencial de este microorganismo desde un punto de vista de biorremediación y bioprospección. La cepa M10.9X^T era una bacteria halófila, inmóvil, teñida de Gram negativa con forma bacilar y células agregadas ocasionalmente formadas. Las colonias en medio de Marine Agar (MA) eran de color crema claro, circulares, convexas, con márgenes enteros, lisas y presentaban un diámetro de 1-2 mm después de 5 días de incubación a 30 ºC. La cepa era oxidasa negativa y catalasa positiva y mostró un metabolismo ligeramente psicrofílico, neutrofílico y ligeramente halófilo. Además, el contenido genómico de G+C de la cepa M10.9X^T fue del 65,2 %. Los valores promedio de identidad de nucleótidos y de hibridación digital ADN-ADN fueron de 76,6 % y 20,9 %, respectivamente, lo

que confirma su adscripción a una nueva especie dentro del género Sagittula. Los principales ácidos grasos celulares fueron C18:1 *ω*7c/C18:1 *ω*6c y C16:0. Los lípidos polares consistían en fosfatidilglicerol M10.9X^T, fosfatidiletanolamina, un aminolípido no identificado, un glicolípido no identificado, un fosfolípido no identificado y un lípido no identificado. El árbol filogenómico basado en un conjunto de 92 secuencias de genes domésticos se agrupó con las dos cepas de Sagittula (*S. stellata* DSM 111524^T y *S. marina* DSM 102235^T), formaron un grupo monofilético, lo cuál fue respaldado por los valores elevados de Bootstrap y GI. Los análisis filogenéticos revelaron que la cepa M10.9X^T estaba estrechamente relacionada con el género *Sagittula* y representa una nueva especie de este género para la cual se ha propuesto el nombre *Sagittula salina* sp. nov. (cepa tipo M10.9X^T= DSM 112301^T= CECT 30307^T). El género *Sagittula* fue descrito por primera vez en 1997 y reclasificado dentro de la familia *Rhodobacteraceae* en 2012. Otras dos especies del género Sagittula (*Sagittula stellata* y *Sagittula marina*) fueron aisladas de ecosistemas acuáticos y reportadas como cepas prometedoras con capacidades de biorremediación.

Conclusión

En conclusión, en la presente tesis discutimos las presiones selectivas de los ambientes artificiales y semiartificiales descritos, especialmente los factores fisicoquímicos (abióticos) que podrían promover o inhibir una célula o comunidad microbiana. Durante mucho tiempo, el famoso principio microbiológico " *Todo está en todas partes, pero el entorno selecciona*” de Baas Becking (1934) se ha utilizado como punto de partida para cuestionar muchas investigaciones sobre diversidad y distribución microbiana. En la presente tesis hemos estudiado la diversidad microbiana de varios ambientes semiartificiales y artificiales considerando dicha hipótesis de Baas Becking. Aquí, también hemos discutido la distribución microbiana y la colonización de esos ambientes, así como el impacto de los factores fisicoquímicos ambientales como la desecación, la radiación ultravioleta, la privación de nutrientes, la baja temperatura, etc., sobre el contenido microbiano de los hábitats microbianos creados por el hombre. Aunque la primera comunidad microbiana de cualquier entorno puede influir en factores abióticos como el pH, la condición redox y la disponibilidad de nutrientes, nuestros resultados respaldan la tesis de Bass Becking sobre el papel principal de la presión selectiva a nivel de micronicho ecológico como un factor clave que da forma a los mundos microbianos que prosperan en los ambientes creados por el hombre.

Appendix A
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The wasted chewing gum bacteriome

Leila Satari[1], Alba Guillén[1], Àngela Vidal-Verdú[1] & Manuel Porcar[1,2]✉

Here we show the bacteriome of wasted chewing gums from five different countries and the microbial successions on wasted gums during three months of outdoors exposure. In addition, a collection of bacterial strains from wasted gums was set, and the biodegradation capability of different gum ingredients by the isolates was tested. Our results reveal that the oral microbiota present in gums after being chewed, characterised by the presence of species such as *Streptococcus* spp. or *Corynebacterium* spp., evolves in a few weeks to an environmental bacteriome characterised by the presence of *Acinetobacter* spp., *Sphingomonas* spp. and *Pseudomonas* spp. Wasted chewing gums collected worldwide contain a typical sub-aerial biofilm bacteriome, characterised by species such as *Sphingomonas* spp., *Kocuria* spp., *Deinococcus* spp. and *Blastococcus* spp. Our findings have implications for a wide range of disciplines, including forensics, contagious disease control, or bioremediation of wasted chewing gum residues.

Chewing gums may have been used for thousands of years, since wood tar from the Mesolithic and Neolithic periods have been found with tooth impressions, which suggests a role in teeth cleaning as well as its usage as early adhesives[1,2]. The first modern chewing gum was introduced in the market in the late 19th[3] and chewing gums are today vastly consumed worldwide: it is estimated that Iran and Saudi Arabia are the countries with the highest chewing gum consumption, where 80% of the population are regular chewing gum consumers[4]. Moreover, global online surveys on gum intake conducted in Europe[5] and United States[6] displayed similar chewing gum patterns among them, where more than 60% of adolescents and adults had chewed gums in the last 6 months before the survey and the mean intakes ranged from 1 to 4 pieces of chewing gum per day. Significantly, Hearty et al. (2014)[5] reported the lowest chewing gum intake (46%) in the United Kingdom. Finally, the value of chewing gum trade has been estimated as more than 30 billion U.S. dollars in 2019[7].

Chewing gums are generally composed of two phases: the water-insoluble phase (gum base) and the water-soluble phase, which can be made of sugar (sugar chewing gums) or sugar alcohols such as polyols (sugar-free chewing gums). Some chewing gums present a solid coat, which is involved in flavour release as well as in chewing gum protection to physicochemical agents, that can be specified as a third phase[8]. The main component of any chewing gum is the gum base (20–30%), that is not edible, nor digestible, but allows chewing, during which added flavours and sweeteners are released. Indeed, chewing gum can be chewed for a long period without any structural modifications because of the water-insoluble property of the gum base[9]. Gum base can be produced from either natural polymers, such as latex or waxes, or synthetic polymers, particularly polyvinyl acetate (15–45%)—a key ingredient in chewing gum formulation—and synthetic elastomers (10–30%) including co-polymers of butadiene-styrene, isobutylene-isoprene as well as, polyethylene, polyisobutylene and polyisoprene[8]. Hence, this inert part of the formula constitutes the support for the water-soluble components which consist of: (i) sweeteners, whether sugar or sugar alcohols that constitute the 60% of the chewing gum; (ii) humectants, such as glycerine; (iii) antioxidants, supplemented to avoid oxidation of other ingredients; (iv) colours, flavours and organic acids, added to define an specific taste of the chewing gum; and (v) optionally, "active" ingredients such as nicotine in chewing gums as an alternative to smoking[8,9]. The particular amounts of these components in the chewing gum formula are a well-kept secret of each confectionery industry. As hinted before, sweeteners comprise more than half of the chewing gum composition. Sucrose, dextrose and glucose syrup are the most frequently used in sugar-containing gums. However, most of chewing gums present in the European market are sweetened with polyols (sugar alcohols) such as xylitol, sorbitol, mannitol, maltitol and isomalt[10,11] as well as artificial sweeteners such as aspartame[12], all of which being labelled as sugar-free chewing gums. The effect of the sugar-free chewing gums in the control of dental disease, salivary pH, and the oral microbiome has been reported[10,13–15].

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Beyond Archaea: The Table Salt Bacteriome

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Commercial table salt is a condiment with food preservative properties by decreasing water activity and increasing osmotic pressure. Salt is also a source of halophilic bacteria and archaea. In the present research, the diversity of halotolerant and halophilic microorganisms was studied in six commercial table salts by culture-dependent and culture-independent techniques. Three table salts were obtained from marine origins: Atlantic Ocean, Mediterranean (Ibiza Island), and Odiel marshes (supermarket marine salt). Other salts supplemented with mineral and nutritional ingredients were also used: Himalayan pink, Hawaiian black, and one with dried vegetables known as Viking salt. The results of 16S rRNA gene sequencing reveal that the salts from marine origins display a similar archaeal taxonomy, but with significant variations among genera. Archaeal taxa *Halorubrum*, *Halobacterium*, *Hallobellus*, *Natronomonas*, *Haloplanus*, *Halonotius*, *Halomarina*, and *Haloarcula* were prevalent in those three marine salts. Furthermore, the most abundant archaeal genera present in all salts were *Natronomonas*, *Halolamina*, *Halonotius*, *Halapricum*, *Halobacterium*, *Haloarcula*, and uncultured *Halobacterales*. *Sulfitobacter* sp. was the most frequent bacteria, represented almost in all salts. Other genera such as *Bacillus*, *Enterococcus*, and *Flavobacterium* were the most frequent taxa in the Viking, Himalayan pink, and black salts, respectively. Interestingly, the genus *Salinibacter* was detected only in marine-originated salts. A collection of 76 halotolerant and halophilic bacterial and haloarchaeal species was set by culturing on different media with a broad range of salinity and nutrient composition. Comparing the results of 16S rRNA gene metataxonomic and culturomics revealed that culturable bacteria *Acinetobacter*, *Aquibacillus*, *Bacillus*, *Brevundimonas*, *Fictibacillus*, *Gracilibacillus*, *Halobacillus*, *Micrococcus*, *Oceanobacillus*, *Salibacterium*, *Salinibacter*, *Terribacillus*, *Thalassobacillus*, and also Archaea *Haloarcula*, *Halobacterium*, and *Halorubrum* were identified at least in one sample by both methods. Our results show that salts from marine origins are dominated by Archaea, whereas salts from other sources or salt supplemented with ingredients are dominated by bacteria.

Keywords: table salt microbiome, halotolerant bacteria, halophilic bacteria, haloarchaea, 16S rRNA gene sequencing analysis

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MICROBIOLOGY SOCIETY
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Sagittula salina sp. nov., isolated from marine waste

Leila Satari[1], Esther Molina-Menor[1], Àngela Vidal-Verdú[1], Javier Pascual[2], Juli Peretó[1,2,3] and Manuel Porcar[1,2,*]

Abstract

A novel Gram-stain-negative, non-motile, halophilic bacterium designated strain M10.9X^T was isolated from the inner sediment of an aluminium can collected from the Mediterranean Sea (València, Spain). Cells of strain M10.9X^T were rod-shaped and occasionally formed aggregates. The strain was oxidase-negative and catalase-positive, and showed a slightly psychrophilic, neutrophilic and slightly halophilic metabolism. The phylogenetic analyses revealed that strain M10.9X^T was closely related to *Sagittula stellata* E-37^T and *Sagittula marina* F028-2^T. The genomic G+C content of strain M10.9X^T was 65.2mol%. The average nucleotide identity and digital DNA–DNA hybridization values were 76.6 and 20.9%, respectively, confirming its adscription to a new species within the genus *Sagittula*. The major cellular fatty acids were $C_{18:1}$ *ω7c*/$C_{18:1}$ *ω6c* and $C_{16:0}$. The polar lipids consisted of phosphatidylglycerol, phosphatidylethanolamine, an unidentified aminolipid, an unidentified glycolipid, an unidentified phospholipid and an unidentified lipid. According to the resuts of a polyphasic study, strain M10.9X^T represents a novel species of the genus *Sagittula* for which the name *Sagittula salina* sp. nov. (type strain M10.9X^T=DSM 112301^T=CECT 30307^T) is proposed.

The genus *Sagittula* was first described by González *et al.* in 1997 and reclassified within the *Rhodobacteraceae* family by Lee *et al.* in 2012 [1, 2]. At the time of writing, the genus *Sagittula* is composed of only two species: *Sagittula stellata* and *Sagittula marina*, both isolated from marine environments and promising strains with bioremediation capacities [1–3]. In the present research, we describe the polyphasic characterization of strain M10.9X^T, which was isolated from the inner sediment of an aluminium can during the study on the microbial diversity of marine waste. Anthropic residue distributed worldwide represent a major environmental problem and constitute new ecological niches which may harbour potential new microbial species.

Strain M10.9X^T was isolated from the inner sediment of a can collected from Malva-rosa beach, on the western Mediterranean Coast (València, Spain; 39° 27′ 48.3″ N 0° 19′ 07.6″ E), during a study of the microbial communities associated with marine waste residues [4]. The sediment was resuspended in PBS (1×, pH 7.4) and 50 µl was then spread on marine agar (MA; Laboratorios Conda S.A. Ref: 1059). The plates were incubated at 18 °C for a week. Strain isolation was carried out by restreaking on fresh media until pure cultures were obtained. Cell suspensions in marine broth (MB; Laboratorios Conda S.A. Ref: 1217) supplemented with 15% glycerol (v/v) were cryopreserved at −80 °C. A polyphasic approach was followed in order to determine the taxonomic status of strain M10.9X^T. After isolation, analysis of the 16S rRNA gene sequence in EzBioCloud revealed that *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T were closely related to strain M10.9X^T. Therefore, these strains were selected as comparative strains. Unless otherwise specified, the reference strains *S. stellata* DSM 11524^T and *S. marina* DSM 102235^T, from the DSMZ (German Collection of Microorganisms and Cell Cultures, Leibniz Institute, Braunschweig, Germany), and strain M10.9X^T were grown simultaneously on MA medium at 30 °C.

The phenotypic characterization of strain M10.9X^T was carried out after a week of growth at 30 °C. The Gram-staining test was performed with KOH 3% (w/v), recording viscosity as a positive result for Gram-negative bacteria [5, 6]. In order to test oxidase activity, a commercial oxidase test stick for microbiology (PanReac AppliChem) was used following the manufacturer's instructions. Hydrogen peroxide 30% (v/v) was used to test catalase activity, by recording bubble formation as a positive result

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Keywords: *Sagittula*; marine waste; new species; Rhodobacteraceae; Alphaproteobacteria.
Abbreviations: ANIb, average nucleotide identity; dDDH, digital DNA–DNA hybridization; MA, marine agar; MB, marine broth; ML, maximum-likelihood; NJ, neighbour-joining.
The 16S rRNA gene sequence of strain M10.9X^T has been deposited in DDBJ/ENA/GenBank under the accession number MW785249. The genomic sequence of strain M10.9X^T has been deposited under the DDBJ/ENA/GenBank accession number JAGISH000000000.
Two supplementary tables and one supplementary figure are available with the online version of this article.
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Appendix B
Supplementary Tables and Figures

Chapter I - Supplementary Information

Supplementary Tables

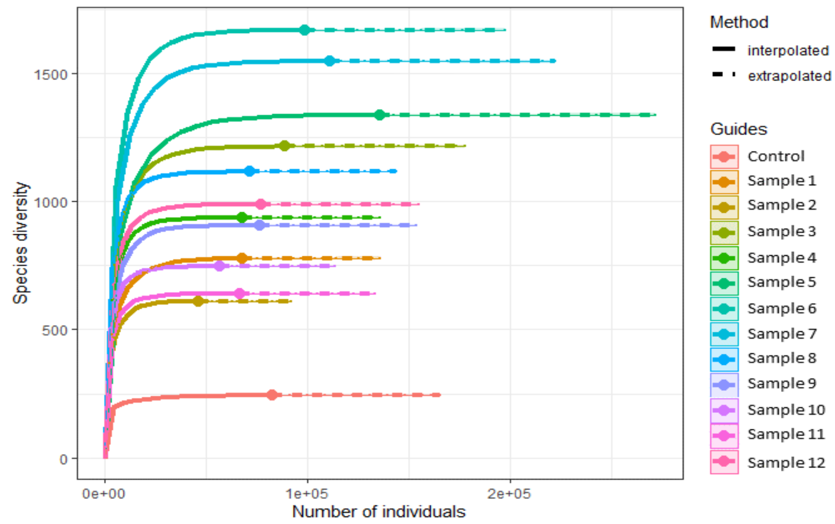
Supplementary Table 1. Identification of wasted chewing gum aerophilic isolates.

Isolate	Similarity (%)	Identification
Sample1- No1	98.45	*Curtobacterium herbarum*
Sample1- No2	98.60	*Pantoea vagans*
Sample1- No3	99.86	*Microbacterium arborescens*
Sample1- No4	99.62	*Pseudomonas oryzihabitans*
Sample1- No5	99.16	*Paenibacillus illinoisensis*
Sample1- No6	99.07	*Microbacterium aerolatum*
Sample2- No1	99.28	*Arthrobacter tumbae*
Sample2- No2	99.69	*Serinicoccus sediminis*
Sample2- No3	99.50	*Arthrobacter ruber*
Sample2- No4	98.77	*Sphingomonas insulae*
Sample2- No5	99.12	*Serinicoccus profundi*
Sample2- No6	99.58	*Arthrobacter agilis*
Sample2- No7	97.91	*Aureimonas phyllosphaerae*
Sample3- No1	99.75	*Bacillus altitudinis*
Sample3- No17	98.84	*Agrococcus jenensis*
Sample3- No18	99.31	*Williamisia marianensis*

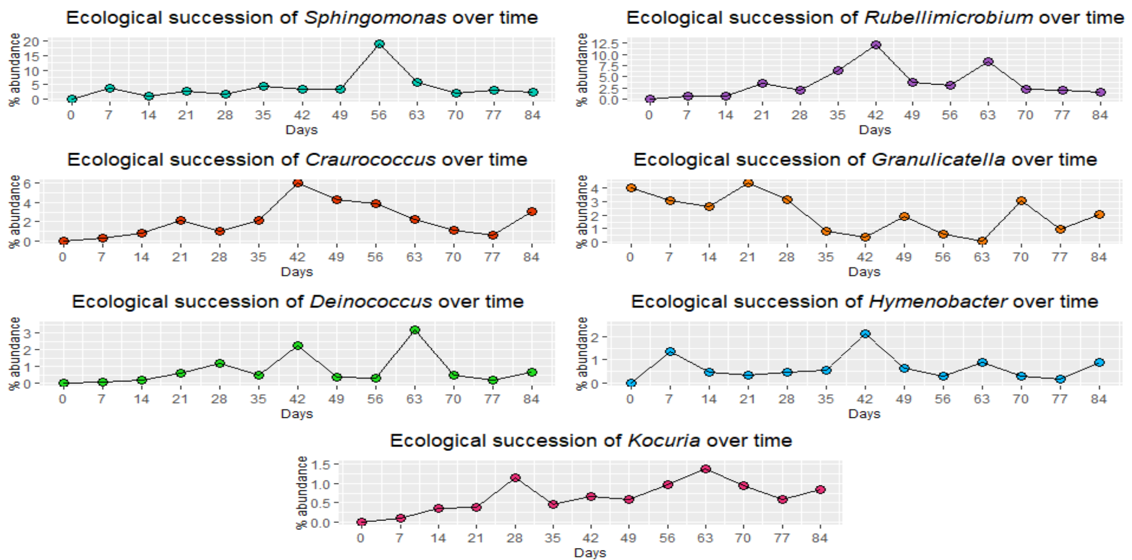
Supplementary Table 2. Identification of wasted chewing gum Microaerophilic isolates.

Isolate	Similarity (%)	Identification
Microaerophile- No1	99.51	*Arthrobacter ruber*
Microaerophile- No2	99.18	*Cellulosimicrobium cellulans*
Microaerophile- No3	98.90	*Sphingomonas insulae*
Microaerophile- No4	99.59	*Terribacillus goriensis*
Microaerophile- No5	99.54	*Bacillus simplex*

Supplementary Figures



Supplementary Figure 1. Rarefaction curves at OTU level were plotted in order to assed the depth of the 16S sequencing process and observed the species diversity in each sample.



Supplementary Figure 2. Ecological succession of most abundant environmental genera in a dynamic study

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Chapter II - Supplementary Information

Supplementary Tables

Supplementary Table 1. Physical characters of table salt samples and ingredients. All salts lacked added iodine. Atlantic salt, Ibiza salt, and Supermarket salt originated from marine environments. Atlantic salt has high natural minerals and trace elements. Ibiza salt contains more than 80 essential minerals and trace elements, such as magnesium, selenium, and fluorine. Himalaya pink salt extracted from the foothill of Himalaya alpine, where the layers of sediments from an ancient ocean formed during the past 200 million years. Himalayan black salt is unrefined mineral rock salt extracted from the Himalayan Mountain range with less NaCl Content. They are rich in iron and hydrogen sulfide, respectively. Hawaiian black salt contains up to 2 % activated carbonate. Viking salt is supplemented with smoked flavor, black pepper, turmeric, dextrose, and dried onion**.**

Name of Salt	Color	Particle Size (mm)	Origin / Packaging	Total salt Content (%)	Sodium Content	Package Size (g)
Atlantic salt	White	1.0-2.0	Spain	-	-	125
Ibiza salt	White	0.5-1.0	Spain	90.5	-	150
Himalaya pink salt	Pink	1.0-2.0	Pakistan	-	-	250
Black salt	Black	1.5-4.0	Hawaii	98.97	39.59 g	100
Viking salt	Yellow	0.1-0.5	France	-	25.49 mg	100
Himalaya black salt	Pinkish Gray	1.0-5-0	Pakistan	-	-	180
Supermarket salt	White	0.5-3.0	Spain	< 90	-	125

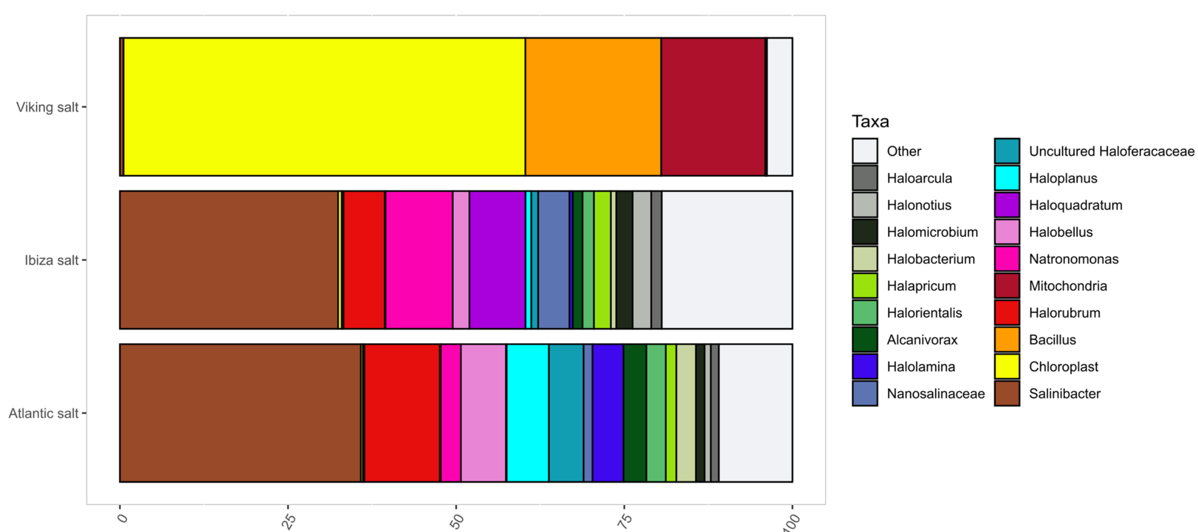
Supplementary table 2. Identification of bacteria and archaea from different table salts based on genus and species level, and source of isolation.

Genus	Species level	Source of Isolation
Acinetobacter	*A. vivianii*	Ibiza salt
Alkalihalobacillus	*A. berkeleyi*	Supermarket salt
	A. hwajinpoensis	Ibiza salt and Supermarket salt
Aquibacillus	*A. koreensis*	Supermarket salt
Bacillus	*ABCF_s*	Atlantic salt, Himalayan pink salt, and supermarket salt
	B. aequororis	Atlantic salt
	B. aidingensis	Himalayan pink salt
	B. altitudinis	Viking salt
	B. aryabhattai	Himalayan pink salt
	B. cabrialesii	Viking salt
	B. circulans	Himalayan pink salt
	B. endophyticus	Ibiza salt
	B. filamentosus	Ibiza salt and Viking salt
	B. flexus	Ibiza salt
	B. infantis	Atlantic salt
	B. licheniformis	Himalayan pink salt and Viking salt
	B. luteolus	Black salt
	B. megaterium	Ibiza salt and Himalayan pink salt
	B. onubensis	Ibiza salt
	B. oryzaecorticis	Atlantic salt
	B. paralicheniformis	Himalayan pink salt and Viking salt
	B. pumilus	Viking salt
	B. salidurans	Supermarket salt
	B. sonorensis	Himalayan pink salt
	B. subtilis	Viking salt
	B. tequilensis	Atlantic salt, Himalayan pink salt, and Viking salt
	B. thaonhiensis	Himalayan pink salt
	B. timonensis	Viking salt
	B. velezensis	Viking salt

	B. zanthoxyli	Atlantic salt and Himalayan pink salt
	B. zhangzhouensis	Himalayan pink salt and Viking salt
	Brevibacterium frigoritolerans	Ibiza salt
Brevundimonas	*B. diminuta*	Supermarket salt
Cytobacillus	*C. firmus*	Atlantic salt
	C. oceanisediminis	Ibiza salt and Himalayan pink salt
Dermacoccus	*D. profundi*	Himalayan pink salt
Fictibacillus	*F. nanhaiensis*	Atlantic salt
Gracilibacillus	*G. dipsosauri*	Himalayan pink salt
	G. salitolerans	Himalayan pink salt
EU817569_g	*EU817569_s*	Supermarket salt
Haloarcula	*H. hispanica*	Atlantic salt
	H. marismortui	Atlantic salt
Halobacillus	*H. alkaliphilus*	Atlantic salt
	H. dabanensis	Himalayan pink salt
	H. hunanensis	Himalayan pink salt
	H. litoralis	Atlantic salt and Himalayan pink salt
	H. sediminis	Black salt
	H. trueperi	Atlantic salt
Halobacterium	*H. hubeiense*	Atlantic salt and Himalayan pink salt
Halorubrum	*H. salinum*	Ibiza salt
	H. sodomense	Ibiza salt
	H. xinjiangense	Ibiza salt
Lentibacillus	*L. jeotgali*	Himalayan pink salt
	L. juripiscarius	Ibiza salt
	L. lacisalsi	Himalayan pink salt
	L. salarius	Himalayan pink salt
	L. salicampi	Ibiza salt and Himalayan pink salt
Mesobacillus	*M. subterraneus*	Atlantic salt
Metabacillus	*M. endolithicus*	Ibiza salt
	M. halosaccharovorans	Atlantic salt, Ibiza salt, and Supermarket salt
Micrococcus	*M. luteus*	Atlantic salt
Oceanobacillus	*O. kimchii*	Ibiza salt and Viking salt
	O. picturae	Himalayan pink salt
Peribacillus	*P. simplex*	Ibiza salt
Piscibacillus	*P. halophilus*	Himalayan pink salt
	P. salipiscarius	Himalayan pink salt
Pontibacillus	*P. salipaludis*	Atlantic salt
	P. yanchengensis	Atlantic salt
Salibacterium	*S. halotolerans*	Himalayan pink salt
	S. nitratireducens	Atlantic salt
Salinibacter	*S. ruber*	Ibiza salt
Staphylococcus	*S. epidermidis*	Black salt
Terribacillus	*T. halophilus*	Viking salt
Thalassobacillus	*T. cyri*	Atlantic salt, Ibiza salt, Himalayan pink salt, and supermarket salt
	T. devorans	Ibiza salt, Himalayan pink salt, and supermarket salt
	T. hwangdonensis	Atlantic salt and Himalayan pink salt
Virgibacillus	*V. byunsanensis*	Supermarket salt
	V. dakarensis	Himalayan pink salt
	V. kapii	Himalayan pink salt

Supplementary Figures

Supplementary Figure 1. Six table salts samples from different manufacturers are analyzed in this study.



Supplementary Figure 2. Relative abundance of the microbial diversity from Atlantic, Ibiza, and Viking salt by using bacterial designed primers. These three table salts were richer in bacteria in comparison with the previous NGS analysis.

Chapter III - Supplementary Information

Supplementary Tables

Supplementary table 1. Bacterial strains isolated from the biofilm formed on the ice machine drain pipe isolated at 4, 10, and 25 ºC with the top closest neighbour (EzBioCloud; https://www.ezbiocloud.net). CA (Columbia Blood Agar), BHI (Blood Heart Infusion), LB (Lysogenic Broth), YM (Yeast Mold), TSA (Tryptic Soy Agar), R2A (Reasoner's 2A agar), Efm2, MA (Marine Agar).

Strain	Closest type strain	Similarity (%)	Isolation Media	4ºC	10ºC	25ºC	Accession Number
IM-2b	*Acidovorax temperans* CCUG 11779^{T}	99.40	R2A, BHI	-	-	+	OL634869
IM-27	*Prolinoborus fasciculus* CIP 103579^{T}	100.00	R2A, TSA, CA	-	-	+	OL634876
IM10-5	*Bacillus toyonensis* BCT-7112^{T}	100.00	LB, YM	-	+	+	OL634885
IM-16b	*Chryseobacterium hispalense* DSM 25574^{T}	100.00	TSA, CA	-	-	+	OL634875
IM-12	*Delftia acidovorans* 2167^{T}	100.00	TSA, YM	-	-	+	OL634872
IM-15	*Flavobacterium quisquiliarum* EA-12^{T}	98.97	R2A, TSA, CA, LB, YM	+	-	+	OL634873
IM-32a	*Hydrogenophaga palleronii* NBRC 102513^{T}	99.90	TSA	-	-	+	OL634878
IM-41	*Methylobacterium marchantiae* JT1^{T}	99.90	YM	-	-	+	OL634880
IM-16a	*Nocardia asteroides* NBRC 15531^{T}	99.12	CBI, BHI	-	-	+	OL634874
IM-31	*Nocardia rhizosphaerihabitans* KLBMP S0039^{T}	99.27	TSA, LB, CA	-	-	+	OL634877
IM10-21	*Peribacillus simplex* NBRC 15720^{T}	100.00	LB	-	+	-	OL634886

IM4-26	*Pseudomonas lactis* DSM 29167^{T}	100.00	R2A, TSA, CA, YM, BHI, LB	+	+	+	OL634883
IM-9	*Prolinoborus fasciculus* CIP 103579^{T}	100.00	YM	-	-	+	OL634871
IM-38	*Rhodococcus cerastii* C5^{T}	99.70	TSA	-	-	+	OL634879
IM-42	*Rhodococcus fascians* C5^{T}	99.53	MA	-	-	+	OL634881
IM4-31	*Sphingomonas aerolata* NW12^{T}	99.63	R2A, TSA, CA, LB	+	+	+	OL634884
IM4-19	*Sphingomonas faeni* MA-olki^{T}	99.91	R2A, LB	+	-	-	OL634882
IM4-X	*Sphingomonas faeni* MA-olki^{T}	98.96	R2A, LB	+	-	-	OL638313

Supplementary table 2. Fungal strains isolated from the biofilm, their accession number on NCBI, and similarities to the closest type strains (BLAST, NCBI rRNA/ITS databases, 16S ribosomal RNA sequences or Internal Transcribed spacer region (ITS), limit to sequences from type material; https://blast.ncbi.nlm.nih.gov/Blast.cgi). CA (Columbia Blood Agar), BHI (Blood Heart Infusion), LB (Lysogenic Broth), YM (Yeast Mold), TSA (Tryptic Soy Agar), R2A (Reasoner's 2A agar), Efm2, MA (Marine Agar).

Isolated fungal strains	The closest type strain	Similarity (%)	Isolation Media	4ºC	10ºC	25ºC	Accession Number
IM-6a	*Aspergillus austroafricanus*	99.68	YM	-	-	+	OL634947
IM10-13	*Briansuttonomyces eucalypti*	99.33	BHI, YM, MA	-	+	+	OL634952
IM4-10	*Cadophora luteo-olivacea*	99.32	R2A, TSA, CA, BHI, YM, Efm2	+	+	+	OL634949
IM4-11	*Filobasidium magnum*	99.74	LB	+	-	-	OL634950
IM-17b	*Neomicrosphaeropsis italica*	99.30	TSA	-	-	+	OL634948
IM-5	*Penicillium citrinum*	99.68	YM	-	-	+	OL634946
IM10-8	*Vishniacozyma victoriae*	99.30	CA, YM	+	+	-	OL634951

Supplementary table 3. Strains isolated from the biofilm formed on the ice machine drain pipe and their similarities to the closest environmental strains/clones (BLAST, NCBI standard nucleotide collection (nr/nt); https://blast.ncbi.nlm.nih.gov/Blast.cgi). The isolation source of the environmental strains/clones indicates in the table. NA (not available).

Isolated strain	The closest environmental strains/clones (Accession Number)	Similarity (%)	Environmental strain/clone (Isolation source)
IM-2b	*Acidovorax delafieldii* strain 179 (EU730925.1) Uncultured bacterium clone d_65_AG_Prop_242 (JN090788.1) Uncultured bacterium clone ncd362e12c1 (HM313242.1)	100.00 99.88 99.88	Water Purifiers in Gunsan Area Aerobic granules cultivated on propionate in a laboratory sequencing batch reactor Skin, antecubital fossa
IM-27	*Acinetobacter lwoffii* strain FDAARGOS_557 (CP054803.1) *Acinetobacter lwoffii* strain FDAARGOS_551 (CP054822.1) *Acinetobacter* sp. strain H111 (MH671625.1)	100 100 100	Clinical isolate Clinical isolate Pine tree
IM10-5	*Bacillus toyonensis* strain WS1-2 (MT605501.1) *Bacillus thuringiensis* strain DT-3 (MT588729.1)	100.00 100.00	NA NA
IM-16b	*Bacterium* strain BS1114 (MK824302.1) *Bacterium* strain BS1096 (MK824284.1) Uncultured bacterium clone A15 (MG744647.1) *Chryseobacterium hispalense* strain FPBSKK1 (KU605691.1)	100.00 100.00 100.00	Tomato rhizosphere Gut content of a freshwater fish species Sweet pepper
IM-12	*Delftia* sp. S17 (HE662648.1) *Delftia acidovorans* strain DS (MN173541.1) Uncultured bacterium clone LS-TB-4 (MH052675.1)	100.00 99.91 99.91	Slope soil from south gate of Nanjing Agricultural University Mud from Dead Sea resort in Jordan Rhizosphere soil
IM-15	*Flavobacterium quisquiliarum* strain ME2 (MN326710.1) Uncultured bacterium clone nbw1182e06c1 (GQ079897.1) *Flavobacterium* sp. PVR09 (KP072754.1)	99.38 99.38 99.38	Ginseng soil The human skin Plant
IM-32a	Uncultured beta proteobacterium clone O-5-26 (KF827217.1) Uncultured bacterium (FR853427.1) *Hydrogenophaga* sp. Gsoil 1545 (AB271047.1)	100 100 100	Biofilm from fresh water NA Soil of a ginseng field
IM-41	*Methylobacterium* sp. strain A3K057 (MN989081.1) *Methylobacterium* marchantiae strain R100B/2 (KR811206.1) *Methylobacterium* sp. PK29_S1 (JF274801.1)	100.00 100.00 100.00	Compound leaves of Fraxinus excelsior Freshwater Lake Radok, East Antarctica Olive-mill wastewater

IM-16a	*Nocardia* sp. JSM 147630 (KR817792.1) *Nocardia* sp. JSM 147631 (KR817793.1)	100.00 100.00	Forest soil in Hunan, China
IM-31	*Nocardia neocaledoniensis* strain NRK66 (MK841332.1) *Nocardia asteroides* strain APN00049 (KC262094.1) *Nocardia neocaledoniensis* strain DSM 44717 (NR_118204.1)	99.79 99.79 99.79	Bronchoalveolar lavage (BAL) Sputum NA
IM10-21	*Bacillus* sp. strain HBUM207125 (MT598008.1) *Peribacillus simplex* strain ILQ109 (MN826155.1) *Peribacillus simplex* strain CsMF-18 (MT415154.1)	100.00 100.00 100.00	NA Rhizosphere of Chenopodium quinoa Stalactite
IM4-26	*Pseudomonas gessardii* strain ST3SE (MN069032.1) *Pseudomonas libanensis* strain FA67 (MT271892.1) *Pseudomonas* sp. strain 3JPC (MT084587.1)	100.00 100.00 100.00	Soil Pharmaceutical wastewater Garlic
IM-9	*Acinetobacter lwoffii* strain FDAARGOS_557 (CP054803.1) *Acinetobacter lwoffii* strain FDAARGOS_551 (CP054822.1) *Prolinoborus* sp. strain B9 (MT576572.1)	100.00 100.00 100.00	Clinical isolate Clinical isolate Skin lesions of *Salmo trutta fario*
IM-38	*Rhodococcus* sp. 2Dben2 (JX177726.1) *Rhodococcus* sp. strain 140616R8 (MK036524.1) *Rhodococcus* sp. I_GA_K_5_3 (FJ267568.1)	99.80 99.80 99.90	Baltic Sea surface water Freshwater RAS systems Kitchen air
IM-42	*Rhodococcus* sp. PDD-41b-2 (JF706542.1) *Rhodococcus* sp. strain Raf1(MT012177.1) *Rhodococcus* sp. TPH-S8 (KP091903.1)	99.91 99.81 99.81	Cloud water NA Total petroleum hydrocarbon contaminated soil
IM4-31	*Sphingomonas* sp. strain C2-1(MT279969.1) *Sphingomonas* sp. strain A3K041 (MN989146.1) *Sphingomonas* sp. strain ICMP 22266 (MH392690.1)	100.00 100.00 100.00	A simulated drinking water distribution system Compound leaves of Fraxinus excelsior Water
IM4-19	*Sphingomonas* sp. strain D3P057 (MN989175.1) *Sphingomonas* sp. strain PDD-32b-57 (MN686253.1) *Sphingomonas* sp. G16 (JQ977017.1)	99.91 99.91 99.91	Compound leaves of Fraxinus excelsior NA Bulk soil
IM4-X*	Uncultured bacterium clone EDW07B005_19 (HM066598.1) Uncultured bacterium clone 9_B09 (FN421797.1) Uncultured bacterium clone 4_E01 (FN421750.1)	99.56 99.19 99.12	A karst aquifer, Texas state well Phyllosphere of soybean Phyllosphere of soybean

IM-6a	*Aspergillus tabacinus* strain DUCC5721 (MT582761.1) *Aspergillus versicolor* strain DUCC5711 (MT582751.1) *Aspergillus versicolor* isolate R47 (MT420642.1)	99.68 99.68 99.68	An Asthma patient houses An Asthma patient houses Barber shops in informal settlements
IM10-13	*Phoma herbarum* isolate GR5-3-20-2 (LC515071.1) *Phoma herbarum* strain JN0398 (MK359683.1) *Phoma herbarum* strain IS179 (LC085217.1)	100.00 100.00 100.00	Sediment of Walker glacier, Canadian High Arctic Plant Moss
IM4-10	*Cadophora malorum* isolate IVA-283 (MN833360.1) *Cadophora malorum* strain IS090 (LC085207.1) *Cadophora fastigiata* strain F-30 (MF077223.1)	100.00 100.00 100.00	Former coal-spoil sites in Arctic Moss Deep root plant rhizosphere
IM4-11	*Filobasidium magnum* (MT635292.1) *Filobasidium* sp. isolate FBFY28 (MK186942.1) *Filobasidium magnum* strain TY4 (MK226288.1)	99.74 99.74 99.74	Mesotrophic lake water Plant Wheat
IM-17b	*Phoma herbarum*strain N-20Ps-2-2-1 (KJ191690.1) *Phoma herbarum* strain N-18Sm-2-2-1 (KJ191684.1) *Phoma herbarum* GR5-3-20-2 (LC515071.1)	100.00 100.00 100.00	NA NA Sediment
IM-5	*Penicillium citrinumç* strain DUCC5728 (MT582768.1) *Penicillium citrinum* strain EE104-F2 (MT560285.1) *Cladosporium* sp. isolate BC15-5 (MT383120.1)	99.68 99.68 99.68	An Asthma Patients House Closed Habitat NA
IM10-8	*Vishniacozyma carnescens* strain AD191(MN922485.1) *Tremellomycetes* sp. strain V07 (MF614974.1) *Vishniacozyma carnescens* strain AY805 (MG250423.1)	100.00 100.00 100.00	Endophytic yeasts of durum wheat In-growth mesh bags in barren ground Inner surface of termite's tapetumsamples from Namib Desert

*Possible new species

Supplementary table 4. Samples included in the beta diversity analysis. All samples come from studies in which the microbiome was determined by 16S rRNA V3-4 region sequencing.

Biome	Sample Name	Description	Reference	Run accession
Cold environment	Arctic terrestrial pond	Arctic-Terrestrial Pond in tundra	Kleinteich *et al*., 2017	ERR2204459
	Arctic ice-based pond	Arctic - Ice-based Pond	Kleinteich *et al*., 2017	ERR2204492
	Arctic soil crust	Arctic - Soil crust	Kleinteich *et al*., 2017	ERR2204489
Freshwater	Lake pore water A	France - Lake Grangent pore water	Keshri *et al*., 2018	SRR5749817
	Lake pore water B	France - Lake Aydat pore water	Keshri *et al*., 2018	SRR5749812
	Lake pore water C	France - Lake Pavin pore water	Keshri *et al*., 2018	SRR5749804
	Lake pore water D	Austria - Lake Neusiedl pore water	von Hoyningen-Huene *et al*., 2019	SRR8266734
Tap water	Tap water A	France - Tap water in Paris	Perrin *et al*., 2019	ERR2611918
	Tap water B	France - Tap water in Paris	Perrin *et al*., 2019	ERR2611911
Biofilm on machines	Dishwasher biofilm A	Slovenia - biofilm from dishwasher	Raghupathi *et al*., 2018	SRR3343800
	Dishwasher biofilm B	Denmark - biofilm from dishwasher	Raghupathi *et al*., 2018	SRR3343755
	Air scrubber biofilm C	Belgium - biofilm from air scrubber from a pig housing facility	Van der Heyden *et al*., 2019	SRR7477781
	Air scrubber biofilm D	Belgium - biofilm from air scrubber from a pig housing facility	Van der Heyden *et al*., 2019	SRR7477802
Ice machine	Ice machine biofilm	Sample under review	-	-

Supplementary table 5. Summary statistics of the reconstructed metagenome-assembled genomes (MAGs) and selected by DAS Tool. The completeness and contamination were estimated with CheckM and the number of CDS (protein coding sequence) with Prokka. G + C, guanine-cytosine content. High-quality MAGs in grey (Bowers *et al*., 2017).

MAG	Contigs	Genome Length (Mb)	N50	Completeness (%)	Contamination (%)	G + C (%)	CDS	Genome Accession Number
002	69	3.5	122230	99.01	1.97	36.32	3091	GCA_021155285.1
003	110	4.5	72755	74.23	3.56	67.05	4338	GCA_021155305.1
11	206	3.4	24610	89.2	2.19	65.72	3176	GCA_021155265.1
13	273	2.3	10164	85.58	1.52	70.41	2241	GCA_021155185.1
013	52	3.7	153103	99.7	1.44	64.66	3657	GCA_021155205.1
14	685	6.4	12260	91.88	1.71	66.51	6132	GCA_021154995.1
015	67	3.0	98499	99.57	0.05	42.40	2821	GCA_021155245.1
016	53	1.1	872541	100	3.99	40.15	4910	GCA_021155195.1
017	156	6.1	70907	95.73	1.22	67.36	5638	GCA_021155165.1
20	549	2.7	5077	76.96	3.59	55.57	2716	GCA_021155145.1
25	1226	6.1	5241	66.53	4.38	70.72	5667	GCA_021155115.1
030	999	2.8	3558	83.17	3.29	52.66	2959	GCA_021155105.1
31	112	9.4	166518	90.54	3.42	56.44	7046	GCA_021155085.1
38	22	3.4	224598	98.33	0.95	32.19	2881	GCA_021155045.1
039	944	1.7	1922	59.41	2.28	55.77	1798	GCA_021155065.1
40	93	3.5	69131	76.76	0.41	66.71	3169	GCA_021155025.1
50	184	3.6	30378	83.67	1.17	65.64	3412	GCA_021154985.1
52	501	3.8	9404	84.61	1.82	66.02	3537	GCA_021154945.1

Supplementary table 6. Unweighted UniFrac dissimilarity matrix.

	Dishwasher biofilm A	Dishwasher biofilm B	Air scrubber biofilm A	Air scrubber biofilm B	Arctic terrestrial pond	Arctic ice-based pond	Arctic soil crust	Lake pore water A	Lake pore water B	Lake pore water C	Lake pore water D	Tap water A	Tap water B
Dishwasher biofilm B	0.014												
Air scrubber biofilm A	0.160	0.158											
Air scrubber biofilm B	0.143	0.141	0.059										
Arctic terrestrial pond	0.120	0.118	0.171	0.157									
Arctic ice-based pond	0.117	0.116	0.154	0.148	0.076								
Arctic soil crust	0.141	0.139	0.083	0.067	0.155	0.148							
Lake pore water A	0.115	0.113	0.130	0.118	0.115	0.120	0.119						
Lake pore water B	0.108	0.107	0.121	0.119	0.136	0.131	0.123	0.100					
Lake pore water C	0.126	0.125	0.149	0.132	0.139	0.132	0.127	0.110	0.124				
Lake pore water D	0.113	0.112	0.131	0.119	0.127	0.125	0.127	0.101	0.065	0.120			
Tap water A	0.119	0.115	0.115	0.119	0.136	0.125	0.125	0.103	0.121	0.134	0.113		
Tap water B	0.119	0.115	0.116	0.118	0.135	0.125	0.124	0.102	0.121	0.133	0.113	0.003	
Ice machine biofilm	**0.150**	**0.148**	**0.081**	**0.087**	**0.158**	**0.145**	**0.093**	**0.120**	**0.114**	**0.142**	**0.122**	**0.114**	**0.114**

Supplementary table 7. Taxonomic affiliation and novelty of each metagenome-assembled genome (MAG). The taxonomic affiliation of each MAG was assessed with the MiGA which is based on Average Nucleotide and Amino Acid Identity (ANI and AAI). The ANI value with the closest published genome of each MAG was calculated using JspeciesWB. High-quality MAGs in grey (Bowers *et al*., 2017).

MAG	Closest published genome		Closest published genome		
		MiGA result closest specie (p-value)		ANI (%)	AAI (%)
002	Bacteria;Bacteroidetes;Chitinophagia;Chitinophagales;Chitinophagaceae	0.833	*Sediminibacterium salmoneum* NBRC 103935 (GCA_000511175.1)	76.45	74.77
003	Bacteria;Proteobacteria;Betaproteobacteri;Burkholderiales;Comamonadaceae	0.741	*Hydrogenophaga palleronii* NBRC 102513 (GCA_001571225.1)	83.13	79.15
11	Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Sterolibacteriaceae	0.851	*Methyloversatilis discipulorum* FAM1 (GCA 000527135.1)	80.7	72.93
13	Bacteria;Actinobacteria;Actinobacteria; Micrococcales	0.919	*Microcella alkaliphila* AC4r (GCA_004216855.1)	76.6	66.97
013	Bacteria;Proteobacteria; Alphaproteobacteria;Rhodbacterales	0.947	*Tabrizicola piscis* NZ CP034328T (GCA_003940805.1)	74.4	63.99
14	Bacteria;Actinobacteria;Actinobacteria;Corynebacteriales;Mycobacteriaceae; Mycobacterium;Mycobacterium gordonae	0.0206	*Mycobacterium gordonae* DSM	98.32	98.46

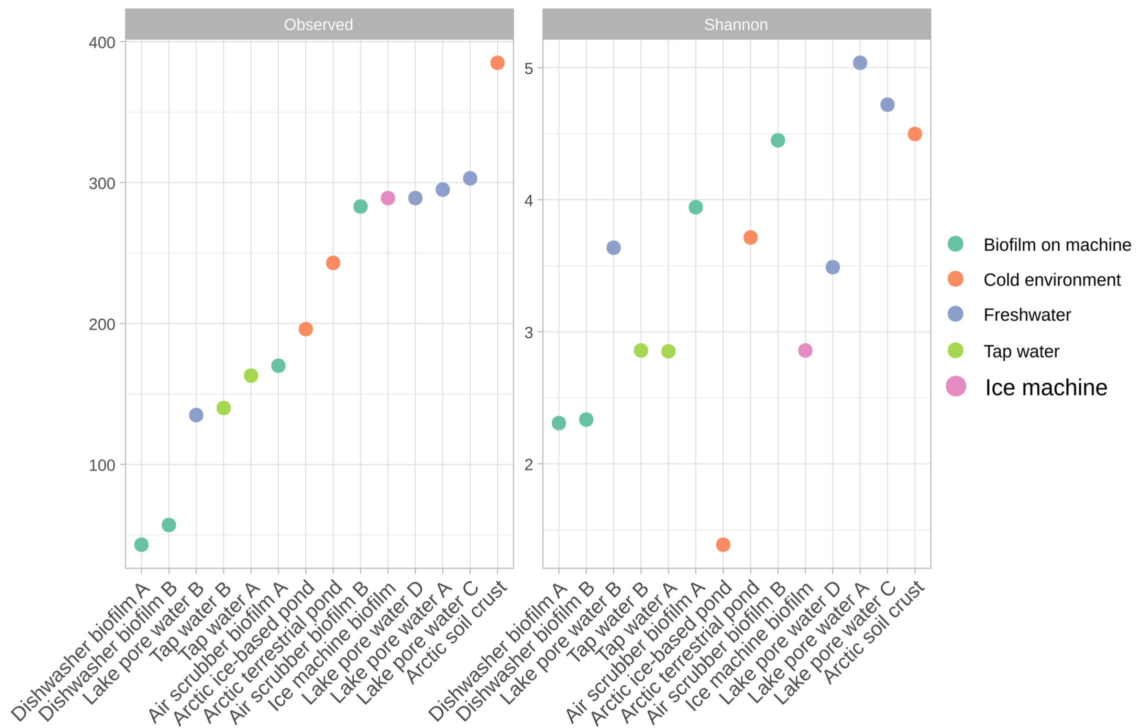
			44160 (GCA_002101675.1)		
015	Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Methylophilaceae	0.867	*Methylotenera versatilis* 301 (GCA_000093025.1)	72.59	69.61
016	Bacteria;Bacteroidetes;Cytophagia;Cytophagales	0.976	*Ohtaekwangia koreensis* DSM 25262 (GCA_900167975.1)	67.47	56.58
017	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae;Pigmentiphaga	0.216	*Pigmentiphaga kullae* K24 (GCA_004216695.1)	90.05	91.21
20	Bacteria;Acidobacteria	0.998	*Acidisarcina polymorpha* SBC82 (GCA_003330725.1)	64.17	43.02
25	Bacteria;Actinobacteria	0.999	*Streptomyces viridosporus* NRRL 2414 (GCA_002078235.1)	65.36	40.52
030	Bacteria;Proteobacteria;Betaproteobacteria	0.987	*Pandoraea sputorum* DSM 21091 (GCA_000814845.2)	67.16	53.12
31	Bacteria;Firmicutes	0.998	*Heliomicrobium gestii* DSM 11169 (GCA_009877435.1)	62.97	41.28
38	Bacteria;Bacteroidetes;Sphingobacteriia	0.993	*Arcticibacter tournemirensis* DSM 23085 (GCA_006716645.1)	65.55	49.2
039	Bacteria;Proteobacteria	0.999	*Geomonas edaphica* Red53 (GCA_004917075.1)	64.19	40.77
40	Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Sterolibacteriaceae;Methyloversatilis	0.142	*Methyloversatilis discipulorum* FAM1 (GCA_000527135.1)	92.91	95.32
50	Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae	0.842	*Hydrogenophaga palleronii* NBRC	81.3	73.91

			102513 (GCA_001571225.1)		
52	Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;Sphingomonas	0.407	*Sphingomonas faeni* MA-olki (GCA_003053745.1)	85.9	92.08

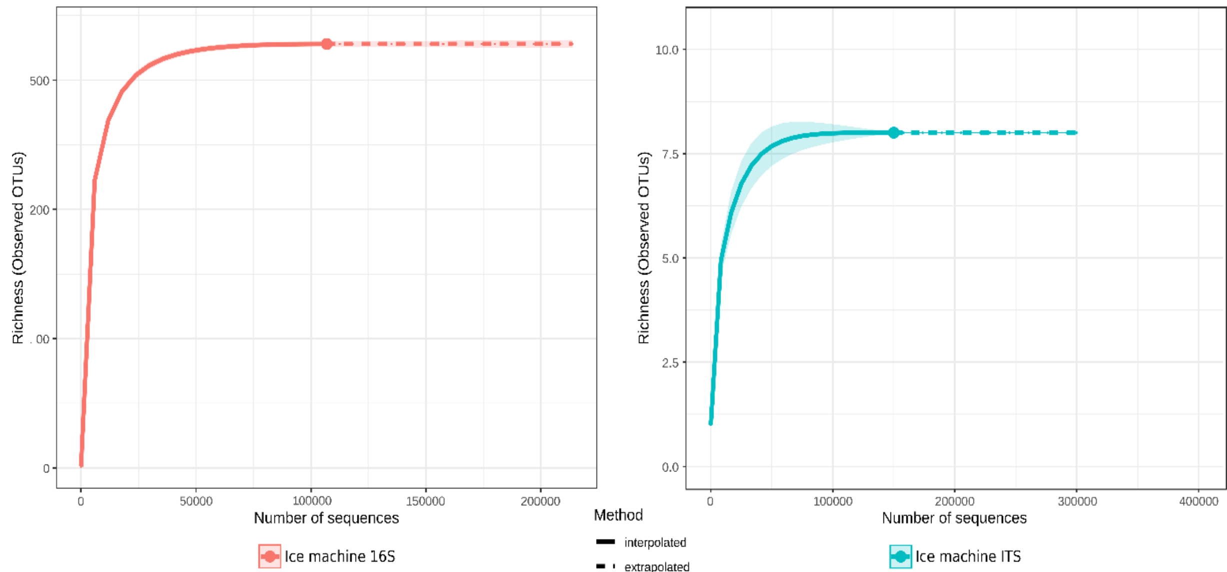
Supplementary table 8. DNA sequences of 5 most abundant ASVs.

ASV	Relative abundance (%)	DNA sequence (5'-3')
28aafbae6847410c759150195aeaa4ef	96.24	AATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCATTGCTTGGTGTTGGGTGTTTGTCTCGCCTTTGCGTGTAGACTCGCCTTAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTACATTTTAACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTT
e3cbd9d4f2411ae392c1169679e9b9d4	2.05	AATGCGATAAGTAGTGTAAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCATTGCTTGGTGTTGGGTGTTTGTCTCGCCTTTGCGTGTAGACTCGCCTTAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTACATTTTAACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTT
95c62bf7b2c516651322dd8be08c4ff6	1.68	AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTATAACCACTCAAGCTCTCGCTTGGTATTGGGGTTCGCGGTTCCGCGGCCCCTAAAATCAGTGGCGGTGCCTGTCGGCTCTACGCGTAGTAATACTCCTCGCGTCTGGGTCCGGTAGGTCTACTTGCCAGCAACCCCCAATTTTTACAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTT
0d741b9c5dc1974201603223bb3c73ec	0.01	CCTTCTCTCCTTCTTTTTCCTTTCCTCCTTCCTTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCATTGCTTGGTGTTGGGTGTTTGTCTCGCCTTTGCGTGTAGACTCGCCTTAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTACATTTTAACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTT
73cde2de5d622cd81a2e5d090782bfe6	0.01	AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGTGTCATGAAACCTCACCCCACTTGGGTTTTTGCCTGAGCGGTGGTGTATTGGGTGTTGCCTTGCCAAAGGCTCGCCTTAAAAACATAAGCACCTTGGATGTAATACGTTTCATCCTTCTGGGTGGCTGATAACCCCACATATTCATGATCTGGCCTCAAATCAGGTAGGGCTACCCGCTGAACTT

Supplementary Figures



Supplementary Figure 1. Alpha diversity of all samples included in beta diversity analysis, with number of observed ASVs (total number of different microorganisms found in the sample) and Shannon index (total number of different microorganisms detected corrected by their relative abundance).



Supplementary Figure 2. Rarefaction curves for the 16S ribosomal RNA and ITS2 analyses in the ice machine biofilm sample.

Chapter IV - Supplementary Information

Supplementary Tables

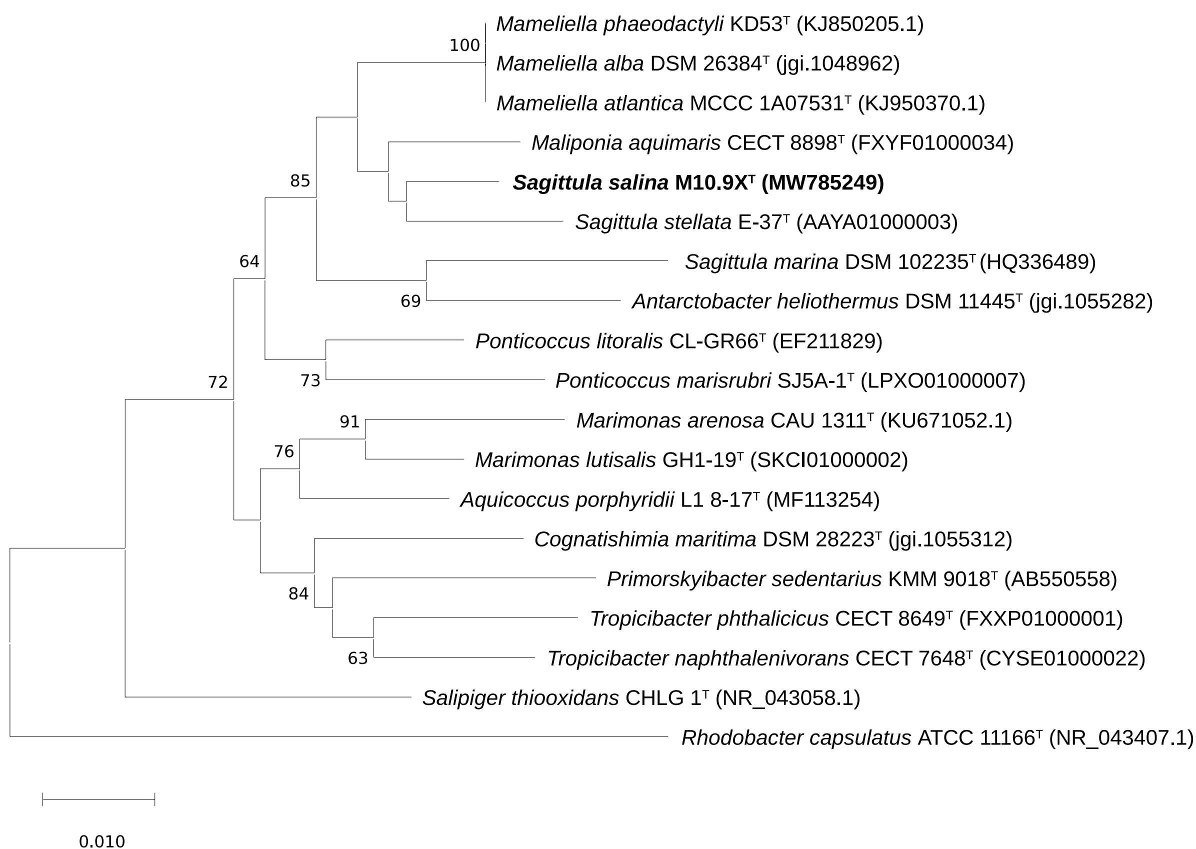
Supplementary Table 1. Comparison of carbon source assimilation of strain M10.9X^T and two reference strains using Gen III Micro Plates. Strains: 1, strain M10.9X^T; 2, *S. marina* DSM 102235^T; 3, *S. stellata* DSM 11524^T. Data for reference strains were analyzed parallelly in the present study. +, positive; -, negative; w, weak reaction. All strains are positive for α-D-glucose, pectin, D-mannose, D-mannitol, D-galacturonic acid, methyl pyruvate, γ-amino-butyric acid, D-maltose, D-fructose, D-arabitol, L-alanine, L-galactonic acid lactone, D-lactic acid methyl ester, D-trehalose, β-methyl-D-glucoside, D-galactose, myo-inositol, L-arginine, D-gluconic acid, L-lactic acid, β-hydroxy-D, L-butyric acid, D-cellobiose, D-salicin, 3-methyl-D-glucoside, glycerol, D-glucuronic acid, gentiobiose, N-acetyl-D-glucosamine, D-fucose, D-glucose-6-PO_4, glucuronamide, α-keto-glutaric acid, sucrose, N-acetyl-β-D-mannosamine, L-fucose, D-fructose-6-PO_4, D-malic acid, propionic acid, D-turanose, L-rhamnose, quinic acid, L-malic acid, inosine, L-serine, D-saccharic acid, bromo-succinic acid, and negative for D-serine.

Characteristic	1	2	3
D-Raffinose	+	-	+
D-Sorbitol	-	+	+
Gelatin	-	+	+
p-Hydroxy-phenylacetic acid	-	+	+
Tween 40	-	+	+
Dextrin	-	+	+
α-D-Lactose	-	+	+
Glycyl-L-Proline	-	+	+
D-Melibiose	-	+	+
α-Hydroxy-butyric acid	+	-	+
L-Aspartic acid	-	+	+
Citric acid	-	+	+
α-Keto-butyric acid	-	-	+
L-Glutamic acid	-	+	+
Acetoacetic acid	-	+	+
L-Histidine	-	+	-
Mucic acid	-	+	+
N-Acetyl-D-galactosamine	-	+	+
D-Aspartic acid	-	+	+
L-Pyroglutamic acid	-	+	+
Acetic acid	-	+	+
Stachyose	-	+	+
N-Acetyl neuraminic acid	-	+	+
Formic acid	-	+	+

Supplementary Table 2. Genomic distance indexes for strain M10.9X^T, compared to other type strains of the genus *Sagittula* and other members of the family *Rhodobacteraceae***.** The digital DNA-DNA Hybridization (dDDH) and the Average Nucleotide Identity (ANIb) value (%) is estimated. Strains: 1, *Sagittula stellata* E-37^T; 2, *Sagittula marina* DSM 102235^T; 3, *Ponticoccus litoralis* DSM 18986^T; 4, *Maliponia aquimaris* CECT 8898^T; 5, *Ponticoccus marisrubri* JCM 19520^T.

	1	2	3	4	5
dDDH (%)					
M10.9X^T	20.9	20.2	20.8	20.8	20.3
ANIb (%)					
M10.9X^T	76.59	75.57	75.5	75.66	74.99

Supplementary Figures



Supplementary Figure 1. Neighbour-Joining (NJ) phylogenetic tree showing the position of strain M10.9X^T among other members of the family *Rhodobacteraceae* based on 16S rRNA gene sequences. The Kimura two-parameter model is applied. Numbers at the nodes indicate bootstrap percentages based on 1000 replicates (values less 50 % are not indicated). Scale bar 0.010 substitutions per nucleotide position. *Rhodobacter capsulatus* ATCC 11166^T (NR_043407.1) was considered as an outgroup.

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