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Original Research

Culturomics remains a highly valuable methodology to obtain rare microbial diversity with putative biotechnological potential from two Portuguese salterns

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Abstract

Background: The high salt concentration is the major factor limiting microbial growth at salterns, along with solar radiation, temperature, and pH. These environmental factors play key roles in the acquisition of unique genetic adaptations for the survival of microorganisms in salterns, which can result in the production of interesting secondary metabolites. The main goal of the present work was to isolate and compare the culturable microbiota from two geographically distant salterns in Portugal and access their biotechnological potential. **Methods**: Culturomics approaches using different culture media were applied for microbial isolation. All isolates were identified either by 16S rRNA or ITS genes sequencing, and their biotechonological potential was assessed by PCR. **Results**: Overall, 154 microbial isolates were recovered that were phylogenetically assigned to 45 taxa from 9 different phyla. From these, 26 isolates may represent putative new taxa. The predominant genera obtained were *Penicillium* (41 isolates, 26.6%), *Streptomyces* (13 isolates, 8.4%) and *Sinomicrobium* (11 isolates, 7.1%). Moreover, the polyketide synthase I gene was present in 64 isolates, the nonribosomal peptide synthethase gene in 16 isolates, and both genes in 23 isolates. **Conclusions**: This study adds up valuable knowledge on the culturable microbiota of Portuguese salterns and on its potential for production of secondary metabolites. In the long run, this study provides a widely diverse microbial collection for future works. **Data public repository**: All DNA sequences were deposited in the GenBank database at National Centre for Biotechnology Information (NCBI) web platform under accession numbers OK169439-OK169485, OK216020-OK216124, OK287059 and OK326927.

Keywords: salterns; microbial isolation; microbial diversity; culturomics; bioactive potential; molecular screening; polyketide synthase I; nonribosomal peptide synthethase

1. Introduction

Portugal has an extensive coastline harbouring a wide range of different environments, including solar salterns. In Portugal, the production of salt in traditional salterns, especially in the North, takes place in the summer season. During salt production, saltern waters are hypersaline environments characterized by high concentrations of NaCl, UV radiation, temperature and pH. These factors are determinant in biodiversity modulation [1], narrowing the microbial community to well adapted halophilic (extremophiles) or halotolerant microorganisms. The microbial diversity of hypersaline environments has been targeted by the scientific community along the last decade [1–7]. The abundance of microorganisms from different phyla was acknowledged on this type of extreme environment, comprising members of Euryarchaeota, Planctomycetota, Bacteroidota, Rhodothermota (previously included in Bacteroidota [8]), Pseudomonadota, Actinomycetota and Cyanobacteria [2,3], Bacillota [7,9], Gemmatimonadota [6] and Eukaryota as microalgae [10] and Ascomycota [11,12].

The ability of microorganisms to produce natural products (NPs) with relevant biotechnological value is well recognised [13] and NPs obtained from extremophiles have proved their biotechnological value in a wide range of fields [14]. As extremophiles are often exposed to sudden and repeated fluctuations derived from global changes, such as temperature and water availability, they require a great physiological adaptability at different cellular levels, namely in their biological membranes, proteins and extracellular metabolites [14]. Due to these metabolic adaptations, the unexploited microbiota of salterns presents a high potential for novel NP discovery with applications in important biotechnological fields, such as medicine, pharmaceutics, cosmetics, agriculture, and the food industry [15–18].

One of the currently applied molecular methodologies to assess the potential of microorganisms to produce bioactive molecules consists in Polymerase Chain Reaction (PCR) protocols. Genes of nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) are

commonly targeted as a preliminary screen of bioactive properties, since they are well known to be responsible for the production of enzymatic complexes that are involved in the synthesis of NPs. Specifically, NRPSs are extensively associated with the production of structurally diverse peptides presenting a wide range of activities, like antibacterial, antitumour, cytostatic or immunosuppressive [19], while PKSs are engaged in the assembly of distinct NPs – the polyketides. This class of NPs includes many biotechnologically important compounds such as antibiotics, antiparasitics, anticancer, immunomodulators, antifungals and anticholesteremics [20]. In fact, the annual sales of medicines derived from polyketides had already reached 20 billion US dollars [21].

The microbial diversity of Portuguese salterns has been poorly studied. Few studies were obtained by culture independent approaches [22,23], and a few more through culture dependent methods [24–26]. The present study targeted the culturable microbial diversity, in late summer of 2018, of two Portuguese salterns, Aveiro and Olhão salterns. These have different geographical locations: Aveiro is situated in the North, being influenced by the Atlantic Ocean, while the Olhão saltern is in the South, and is exposed to the influence of not only the Atlantic Ocean but also the Mediterranean Sea. Average temperature and pluviosity levels per year are also different at both locations: 15.6 °C and 1064 mm in Aveiro and 18.2 °C and 482 mm in Olhão [27]. The main goal of the present study was to assess the microbial diversity of both salterns, during salt production season, through a broad range of microbial isolation and subsequent phylogenetic analysis. The present study also aimed at isolates biotechnological prospection by molecular screening for NRPS and PKS-I genes. This work provides additional insights about the microbiota of Portuguese salterns and about its potential for NPs production. Moreover, this study contributes with a wide and diverse microbial collection for further prospections.

2. Material and methods

2.1 Sampling and microbial isolation

Fresh and wet salt, sediment from under the salt and water, all sampled directly from salt production ponds, were collected from Aveiro saltern (40º38'50" N, 8º39'46" W) in September 2018 and from Olhão (37°1'35" N, 7°51'59" W) in August 2018, during salt production season. All samples were used for microbial isolation, aiming at obtaining members from different phyla, applying, therefore, different methodologies represented in Fig. 1 and specified below.

2.1.1 Planctomycetota

Media M600, M607 [28] and M607SW (this study) (**Supplementary Table 1**), supplemented with 200 μg/mL ampicillin (Batch: 1R001084; AppliChem, Darmstadt, Germany), 1 mg/mL streptomycin (Batch: 6H015348; AppliChem) and 50 μg/mL cycloheximide (Batch: 4C016643; AppliChem/ Panreac, Darmstadt, Germany), were used to target the isolation of Planctomycetota phylum members. The antibiotic supplementation was used because Planctomycetota known members present resistance to these drugs [28], and cycloheximide presents antifungal activity.

Salt samples were dissolved in autoclave-sterilized seawater (SW) until saturation and serially diluted until 10^{-3} in sterile SW. All SW used in this study was collected from Estação de Zoologia Marítima "Dr. Augusto Nobre" at Molhe beach, Porto, Portugal (N: 41°9'49.981"; W: 8°41'11.820"). Both, initial suspension and dilutions, were individually used as inoculum in the different isolation media. The wet salt samples, at the bottom, accumulated a brown residual liquid, which was directly plated in the isolation media and serially diluted in sterile SW until 10^{-3}, which were also individually plated in the isolation media.

Concerning sediment samples, 1 g of each saltern sediment was individually suspended in 1 mL of sterile SW by vigorously vortexing. From this suspension, serial dilutions until 10^{-3} were made. Both, initial suspension and dilutions, were individually used as inoculum in the different isolation media. The saltern water samples were directly plated in the isolation media and serially diluted in sterile SW until 10^{-3}, which were also plated in the isolation media.

Agar media were used for direct isolation, while broth versions of the media were used for enrichments. Specifically, enrichments were carried out in 24-well plates containing 900 μL of broth isolation media per well. Enrichments were carried out by using not only the raw samples, as in the case of the water and the brown residual liquid, but also the initial suspensions of salt and sediment. In all cases, 100 μL of each sample were individually used as initial inoculum and then serially diluted in the corresponding broth medium until 10^{-3}. All broth conditions for each sample concentration were carried out in triplicate. The inoculated 24-well plates were incubated at room temperature (RT) at 120 rpm, while inoculated agar media plates were incubated at 26 °C. When visible growth was observed in the wells, 100 μL of each culture suspension were transferred to the corresponding agar medium and incubated at 26 °C until axenic cultures were achieved.

2.1.2 Actinomycetota

Agar media regularly used for Actinomycetota isolation were selected, namely R2A-Agar (BD Difco™, Maryland, USA), Starch-Casein-Nitrate agar (SCN; adapted from Küster and Williams [29]), M3 [30] and a modified Nutrient-Poor Sediment extract agar (NPS; adapted from Jensen *et al.* [31]) (**Supplementary Table 1**). All agar media were supplemented with 50 mg/L cycloheximide (Batch: 9I011709; Sigma-Aldrich, Missouri, United States), 50 mg/L nalidixic acid (Batch: 8D012744; Ap-

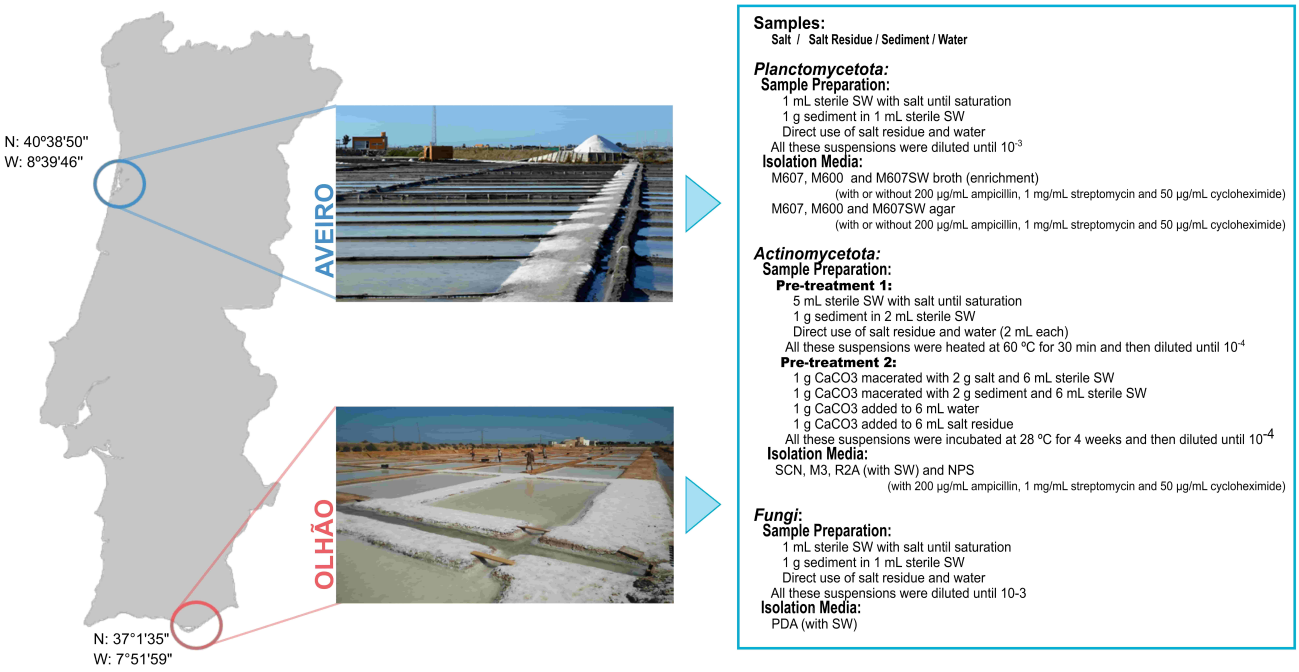

Fig. 1. Representation of the sampling locations along with the overall culturomics methodologies applied.

pliChem) and 50 mg/L nystatin (Batch: 9J012880; Sigma-Aldrich).

Salt samples were dissolved in 5 mL of sterile SW until saturation, while 1 g of each sediment sample was suspended in 2 mL of sterile SW by shaking at 200 rpm for 30 min and by vortexing at maximum speed for 5 min. Two mL of the salt brown residual liquid and of the water samples were directly used as inoculum. All these samples were exposed to a heat pre-treatment at 60 °C for 30 min to select for Actinomycetota [32–34]. Pre-heated samples were serially diluted until 10^{-4} in sterile SW, and all samples, either diluted or not, were individually plated on the above-mentioned isolation agar media (**Supplementary Table 1**).

Incubation of samples with calcium carbonate ($CaCO_3$) was previously reported as a strategy that promotes the selection of Actinomycetota in isolation events [35]. So, this strategy was applied in all saltern samples. Specifically, (i) 1 g of sterile $CaCO_3$ was macerated with 2 g of each salt sample and 6 mL of sterile SW were added to help on the maceration process; (ii) 1 g of sterile $CaCO_3$ was added to 6 mL of each salt brown residual liquid sample; (iii) 1 g of sterile $CaCO_3$ was macerated with 2 g of each sediment sample and 6 mL of sterile SW were added to help on the maceration procedure; and (iv) 1 g of sterile $CaCO_3$ was added to 6 mL of each saltern water sample. All these suspensions with $CaCO_3$ were incubated at 28 °C for 5 weeks and then were serially diluted until 10^{-4} in sterile SW. All the pre-treated samples, either diluted or not, were individually plated in the isolation agar media (**Supplementary Table 1**) and incubated at 28 °C.

2.1.3 Fungi

Potato Dextrose Agar (PDA; BD Difco™) is widely used for Fungi cultivation and was the medium selected for the present study, which was prepared with SW. All samples, namely salt, salt brown residual liquid, sediment and water, as well as the serial dilutions of these samples were prepared as described above for isolation of Planctomycetota. All these suspensions, either diluted or not, were individually plated in PDA.

In all methodologies previously described, bacterial colonies with different morphologies were restreaked until the achievement of axenic cultures. All pure isolates were cryopreserved in 24% glycerol at –80 °C.

2.2 Microbial DNA extration

Each axenic isolate was cultivated in the corresponding broth medium of isolation and these cultures were used for DNA extraction. For isolates obtained in PDA medium, NZY Plant/Fungi gDNA Isolation kit (NZYTech, Lisbon, Portugal) was used, according to the manufacturer's instructions. In the case of the isolates obtained in the remaining media, DNA was extracted using the E.Z.N.A. Bacterial DNA kit (Omega Bio-Tek, Norcross, Georgia, USA), according to the manufacturer's instructions. DNA quantification was done by using the μDropTM platform (Thermo Fisher Scientific, Massachusetts, USA) and DNA quality was checked through a 30 min electrophoresis at 100 V in a 0.8% agarose gel with 1X Tris-acetate-EDTA (TAE) buffer (Bio-Rad, Hercules, California, USA) and stained with GreenSafe Premium (NZYTech).

2.3 Microbial identification

Bacterial DNA was PCR-amplified with the universal primers 27F and 1492R [36], while Fungi DNA was PCR-amplified by using ITS1 and ITS4 primers [37].

For the isolates which strain designation starts with "C.", the 16S rRNA gene primers, 27F and 1492R, were used and the PCR mixture consisted of 1 × Qiagen Multiplex PCR Master Mix (Qiagen, California, USA), 0.1 μM each primer and 25 ng of DNA template, in a final volume of 10 μL. The PCR reaction was carried out on the Veriti® Thermal Cycler (Applied Biosystems, Massachusetts, USA) using the following PCR program: initial denaturation for 5 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min, and a final extension of 10 min at 72 °C. PCR products were visualized through electrophoresis in a 0.8% agarose gel with 1 × TAE buffer, which was stained with SYBR Safe (Thermo Fisher Scientific). Purification and sequencing of these amplicons was carried at i3S (Porto, Portugal).

For the remaining isolates, including Fungi, the PCR mixture (25 μL) consisted of 1 × NZYTaq 2 × Green Master Mix (NZYTech), 0.1 μM each primer and 25 ng of DNA template. PCR program was carried out in a MyCycler™ Thermo Cycler (Bio-Rad) and, for 16S rRNA gene amplification, consisted in an initial denaturation step of 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 56 °C (annealing temperature) and 1.5 min at 72 °C, and a final extension of 10 min at 72 °C. For amplification of the ITS region, the PCR program was the same as previously described, with exception that an annealing temperature of 55 °C was applied. PCR products were visualized through electrophoresis in a 0.8% agarose gel with 1 × TAE buffer, which was stained with GreenSafe Premium. These amplicons were purified using GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, United Kingdom) and sequenced at GATC Biotech (Constance, Germany).

Sequence analyses were carried out using Geneious Prime (Biomatters Ltd, Auckland, New Zealand). Curated sequences were matched in the EzBioCloud [38] database to determine their closest relatives. Phylogenetic trees were built using MEGA 7 (Pennsylvania State University, Pennsylvania, USA) software [39]. Briefly, all sequences, either from the isolates in this study and from the closest related strains determined on EzBioCloud, were grouped by phyla. Separately, phyla-grouped sequences were aligned using the ClustalW algorithm with a Gap Open Penality of 15.00 and a Gap Extension Penalty of 6.66. The resulting multiple alignments for each phylum were used to construct phylogenetic trees, by applying the Maximum Likelihood statistical method, the phylogeny test based on the Bootstrap method considering 1000 replicates, and the Tamura-Nei substitution model. Different strains were used as outgroup depending on the phyla.

All sequences obtained on this study were submitted to the GenBank database [40] at National Centre for Biotechnology Information (NCBI) web platform under the following accession numbers: OK169439-OK169485, OK216020-OK216124, OK287059 and OK326927.

2.4 Diversity, richness, dominance and distribution of salterns microbial communities

PAST 3.22 (University of Oslo, Oslo, Norway) [41] software was used to calculate the Fisher's α, Margalef's and Simpson's indexes, which allow the characterization of the microbial community in terms of diversity, richness, and evenness, respectively.

A sample rarefaction curve was estimated in PAST 3.22 by using the Mao's Tau index [42]. Similarities among samples were calculated on PAST 3.22 by using the Sørensen coefficient and Bray-Curtis index. All results were computed taking into account a 95% confidence and 1000 iterations as bootstrap value.

2.5 PCR screening of the bioactive potential of saltern isolates

The putative potential of all isolates obtained in this study to produce NPs was assessed through PCR. Briefly, isolates DNA was screened for the presence of NRPS and PKS-I genes. NRPS genes were amplified using primers MTF2 [5′- GCNGG(C/T)GG(C/T)GCNTA(C/T)GTNCC-3′ (AGGAYVP, core motif I)] and MTR [5′-CCNCG(AGT)AT(TC)TTNAC(T/C)TG-3′ (QVKIRG, core motif V)] [43], while β-ketosynthase (KS) domain fragments within the Type I polyketide synthase genes (PKS-I) was amplified using primers MDPQQRf (5′-RTRGAYCCNCAGCAICG-3′) and HGTGTr (5′-VGTNCCNGTGCCRTG-3′) [44]. PCR reactions of 25 μL containing 1 × NZYTaq 2 × Green Master Mix, 0.8 μM of each primer and 25 ng DNA template were prepared. The PCR protocol was carried out in a MyCycler™ Thermo Cycler and consisted of an initial denaturation at 95 °C for 5 min, 11 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, with the annealing temperature being reduced 2 °C every cycle; followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 40 °C for 30 s and extension at 72 °C for 1 min, and a final extension of 10 min at 72 °C [45]. Amplicons were visualized in a 1.2% agarose gel prepared with 1 × TAE buffer and stained with GreenSafe Premium. NRPS and PKS amplicons have an expected size of approximately 1000 bp [43] and 700 bp [44], respectively. The Planctomycete UC49.1 was used as positive control for amplification of both genes.

3. Results and discussion

3.1 Microbial isolation and identification

Aiming at a broader range of microbial isolation, 6 media with different nutritional compositions along with 2

pre-formulated media (**Supplementary Table 1**) were assayed. A medium selective for fungi, PDA, allowed the isolation of 45 fungal strains. The most effective media for bacteria were M600 (32 strains), R2A (32 strains) and M607 (31 strains). The remaining media used were less rich in nutrients and apparently less successful (14 strains altogether in 4 different media). Ultimately, the media used proved to be effective in the isolation of a highly heterogenous microbiota from salterns.

The microbial isolation screening carried out for samples from Aveiro and Olhão salterns allowed the isolation of 154 strains, of which 47 were affiliated with Fungi and 107 with Bacteria. Particularly, 26 fungal and 75 bacterial isolates were obtained from Aveiro, and 21 fungal and 32 bacterial isolates from Olhão. All these isolates have been putatively identified based on the ITS and 16S rRNA genes for Fungi and Bacteria, respectively. The isolates relatedness was examined by building phylogenetic trees (**Supplementary Fig. 1**).

Despite the high abundance of salterns in Portugal, their microbiota has been poorly explored. The existent research reports comprises (i) the description of new strains detected by metagenomics approaches of Tavira saltern (Algarve salt flats) microbiota [46], (ii) metagenomics studies of Algarve salt flats [22,23,47], (iii) characterization of novel isolated species from Tavira saltern [26] and from Tejo salt flats [48], (iv) an archaeal isolate genome announcement from Figueira da Foz salt flats [49] and (v) extensive isolation approaches targeting Aveiro saltern and a saltern from Olhão (Algarve salt flats) focusing on the overall bacterial microbiota [24]. In this last study, the direct comparison between the microbiota of both salterns was not possible since the nature of samples, the methodology applied and the time of sampling were different between both salterns. None of these studies have targeted the fungal community, most likely because the prevalence of fungi in marine and aquatic environments, including hypersaline ones, was overlooked for many years [50–52]. In the last decades, Fungi members have been reported in every aquatic environment explored, including marine and hypersaline ones [50–52]. The first description of fungi in salterns was reported by Gunde-Cimerman and collaborators [53], which aroused a fascinating interest in the scientific community [12,54,55]. Since this, saltern fungi have been studied worldwide, however the Portuguese salterns mycobiota remains unknown. In this study, the microbial diversity was assessed at genera (Fig. 2) and phyla (Fig. 3) levels. The fungal isolates obtained were phylogenetically related with the phylum Ascomycota (Fig. 3), with the exception of isolate AW17 that was affiliated to the genus *Sporobolomyces* (Fig. 2), specifically to the species *Sporobolomyces ruberrimus*, a yeast within the phylum Basidiomycota (Fig. 3).

Colour scheme representation by phylum: grey for Ascomycota, black for Basidiomycota, blue for Pseudomonadota, orange for Actinomycetota, purple for Bacillota, brown for Bacteroidota, green for Planctomycetota, yellow for Rhodothermota and red for Gemmatimonadota. Full filled with colour represent isolates common to both salterns and filled with pattern represents exclusivity to a specific saltern. (*) represents isolates identified only to the family level.

Overall information of the environmental prevalence of strains phylogenetically closely related with the isolates obtained with the present study is compiled in Table 1 (Ref. [1,5,9,11,24,26,53,54,56–107]), where presence or absence in salterns environments was highlighted.

Within Fungi, members of genus *Penicillium* were the only ones detected in both salterns and the most abundant ones (Figs. 2 and 3; **Supplementary Table 2**).

The Pseudomonadota genera *Rhodovibrio* and *Microbulbifer* were the only ones common to both saltern (Fig. 2). The similarity score of all 4 *Microbulbifer* isolates with *M. halophilus* (<98.50% in the 16S rRNA gene) revealed a distant relatedness, suggesting that these isolates can be members of a novel taxon (Fig. 4, Ref. [39]). Two other species of Pseudomonadota were detected as singletons in Olhão saltern, which were closely affiliated with *Salinicola zeshunii* and *Aureimonas glaciistagni*. Neither of these two species have been associated specifically with microbiota of salterns (Table 1). Furthermore, one Pseudomonadota isolate was affiliated with strains of *Brevundimonas* not yet related with halophilic environments (Table 1), but instead associated with humanized habitats, namely washing machines [108] and space laboratory [109]. Additionally, the *Roseibacterium* isolate AW9 obtained on this study showed a low similarity (97.53% in the 16S rRNA gene) with the closest related species, *R. elongatum*, which might be indicative of a novel species (Fig. 4). Also from Aveiro saltern, 4 strains of phylum Pseudomonadota phylogenetically related to uncultured members of *Aquichromatiaceae* were isolated, but showing low levels of similarity (98.08 up to 98.30% in the 16S rRNA gene), so highlighting the novelty of these strains (Fig. 4).

Within the phylum Bacillota, *Mesobacillus* and *Rossellomorea* were the only two Bacillota genera that were detect at both Aveiro and Olhão salterns. Despite the geographical distance, the 2 strains (AW18 and OW14) closely affiliated with the novel established genus *Rossellomorea* [110] showed a perfect similarity between their 16S rRNA gene sequences and a close phylogenetic relationship with the isolate *Rossellomorea* sp. es.034 (PDIY01000001). Therefore, these strains are strong candidates for the description of a novel species (Fig. 4). Moreover, 2 isolates (AW3 and AW5) showed a similarity of up to 98.66% in the 16S rRNA gene with *Bacillus sinesaloumensis*, and other 2 isolates (OSBR114 and OW16) showed similarity scores of up to 98.4% in the 16S rRNA gene with *Metabacillus litoralis*. All of them may be representatives of novel taxa (Fig. 4).

Table 1. Environmental occurrence of the species isolated in this study from the two Portuguese salterns, Aveiro and Olhão, taking into consideration their phylogenetic rank.

Kingdom	Phylum	Class	Order	Family	Closest related microorganism	Isolates from this study	Environments reported
Fungi	Ascomycota (**Supplementary Fig. 1a**)	*Dothideomycetes*	*Pleosporales*	*Pleosporaceae*	*Alternaria alternata*	ASBR4	**Salterns [56]**
			Dothideales	*Dothioraceae*	*Aureobasidium pullulans*	ASED7	**Salterns [53,57,58]**
					Cladosporium herbarum	AW2	**Salterns [54,59]**
			Capnodiales	*Davidiellaceae*	*Cladosporium perangustum*	AS110	Marine [60]
					Cladosporium phaenocomae	AS102	Plants [61], deteriorated wood [62]
		Eurotiomycetes	*Eurotiales*	*Trichocomaceae*	*Penicillium brevicompactum*	ASED17, ASED27, ASED28, ASBR9, AS103, AS104, AS108, AS111, OSBR109, OS1, OS3, OS4, OS6, OS7, OS8, OW12	**Salterns [54]**
					Penicillium brocae	ASED21, ASED25, ASED29, OSBR107, OSBR117, OS2, OS5, OS10, OW1, OW2, OW3, OW8, OW17	Marine [63]
					Penicillium chrysogenum	ASED16, ASED18, ASED19, ASBR1, ASBR2, ASBR5, AW1, AW11, OSBR108, OW5	**Salterns [11,54,64]**
					Penicillium dierckxii	ASED8	Hypersaline [65]
					Penicillium sp.	OSBR118	Plants (NCBI: EF694632)
	Basidiomycota (**Supplementary Fig. 1b**)	*Microbotryomycetes*	*Sporidiobolales*	*Incertae sedis*	*Sporobolomyces ruberrimus*	AW17	Psychrophilic [66–68]
Bacteria	Actinomycetota (**Supplementary Fig. 1c**)	*Actinobacteria*	*Micrococcales*	*Dermabacteraceae*	*Brachybacterium paraconglomeratum*	C.OS8	Hypersaline [69,70]
				Brevibacteriaceae	*Brevibacterium sediminis*	C.AS1, C.AS9	**Salterns [24]**
				Microbacteriaceae	*Microbacterium aerolatum*	C.ASBR1, C.OS5	Air [71]
					Microbacterium amylolyticum	C.AS10, C.AW5	**Salterns [24]**
					Microbacterium ginsengiterrae	C.AW1	Soil [72]

Table 1. Continued.

Kingdom	Phylum	Class	Order	Family	Closest related microorganism	Isolates from this study	Environments reported
				Micrococcaceae	*Micrococcus luteus*	C.OS4	**Salterns [73]**
					Rothia kristinae	OSBR105, OSBR106	Human commensal and opportunistic pathogen [74]
			Mycobacteriales	*Gordoniaceae*	*Gordonia oryzae*	AW6	Plants [75]
				Tsukamurellaceae	*Tsukamurella tyrosinosolvens*	OW4	Human commensal and opportunistic pathogen [76]
			Propionibacteriales	*Nocardioidaceae*	*Nocardioides salarius*	OSBR100	Marine [77]
			Streptomycetales	*Streptomycetaceae*	*Streptomyces intermedius*	C.AS7, C.ASBR4, C.AW3	Members of this genus have been associated with Salterns [9,78–80]
					Streptomyces rubrogriseus	C.AS5, C.ASBR6, C.ASBR8, C.ASBR9, C.ASBR10, C.ASBR11, C.ASED8, C.OS6, C.OS7, C.OSBR1	Members of this genus have been associated with Salterns [9,78–80]
			Streptosporangiales	*Nocardiopsaceae*	*Nocardiopsis lucentensis*	C.ASBR7, C.ASED1, C.ASED2, C.ASED3	**Salterns [79,81]**
					Nocardiopsis prasina	C.ASED4, C.AW6	Members of this genus have been associated with Salterns [9,78–80]
		Acidimicrobiia	*Acidimicrobiales*	*Iamiaceae*	Uncultured bacterium	OSBR104	Air [82]
	Bacteroidota (**Supplementary Fig. 1d**)	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Psychroflexus tropicus*	AW7	Hypersaline [83]
					Sinomicrobium oceani	C.AS2, C.AS3, C.AS4, C.AS6, C.AS8, C.ASED5, C.ASED7, C.ASED9, C.OS3, C.OS9	Members of this genus have been associated with Salterns [1]
Kingdom	Phylum	Class	Order	Family	Closest related microorganism	Isolates from this study	Environments reported
Bacteria	Bacillota (**Supplementary Fig. 1e**)	*Bacilli*	*Bacillales*	*Bacillaceae*	*Alkalihalobacillus hwajinpoensis*	ASED10, ASED13	**Salterns [24,73,84]**
					Bacillus safensis	AW4	**Salterns [85]**
					Bacillus sinesaloumensis	AW3, AW5	Human commensal [86]
					Cytobacillus luteolus	OSBR113	**Salterns [24,84]**
					Mesobacillus sp.	AW16, OSBR115	**Salterns [24]**
					Metabacillus litoralis	OSBR101, OSBR112, OSBR114, OSBR116, OW6	**Salterns [84]**

Table 1. Continued.

Kingdom	Phylum	Class	Order	Family	Closest related microorganism	Isolates from this study	Environments reported
					Rossellomorea sp.	AW18, OW14	**Salterns [24]**
					Thalassobacillus cyri	OSBR110, OSBR111	Hypersaline [87]; Members of this genus have been associated with Salterns [73]
				Paenibacillaceae	*Paenibacillus pabuli*	ASED15, ASED30	Members of this genus have been associated with Salterns [84]
				Planococcaceae	*Paenisporosarcina quisquiliarum*	OS9	Soil [88]; Members of this genus have been associated with Salterns [84]
	Gemmatimonadota (**Supplementary Fig. 1f**)	*Longimicrobia*	Uncultured	Uncultured	Uncultured bacterium	AW12	Members of this phylum have been associated with Salterns [73,89]
	Planctomycetota (**Supplementary Fig. 1g**)	*Planctomycetia*	*Planctomycetales*	*Planctomycetaceae*	*Alienimonas californiensis*	ASED1	Algae [90]
					Maioricimonas rarisocia	ASED14	Marine [91]
			Pirellulales	*Pirellulaceae*	*Rhodopirellula pilleata*	ASED26	Marine [92]
					Rhodopirellula rubra	ASBR8	Algae [93]
		Phycisphaerae	*Phycisphaerales*	*Phycisphaeraceae*	Uncultured bacterium	ASED31	Members of this class have been associated with Salterns [26]
	Pseudomonadota (**Supplementary Fig. 1h**)	Alphaproteobacteria	*Hyphomicrobiales*	*Aurantimonadaceae*	*Aureimonas glaciistagni*	OW13	Members of this genus have been associated with Salterns [84]
			Caulobacterales	*Caulobacteraceae*	*Brevundimonas* sp.	ASED5	Members of this genus have been associated with Salterns [94]
			Rhodospirillales	*Rhodovibrionaceae*	*Rhodovibrio sodomensis*	OW16, OW18, ASBR14, ASBR15, ASBR16, AS100, AS106, AS114, AW21, AW23, OW9	**Salterns [95]**
			Rhodobacterales	*Rhodobacteraceae*	*Roseibacterium elongatum*	AW9	Hypersaline [5]
			Hyphomicrobiales	*Stappiaceae*	*Stappia stellulata*	ASED24	Marine [96,97]

Table 1. Continued.

Kingdom	Phylum	Class	Order	Family	Closest related microorganism	Isolates from this study	Environments reported
		Gammaproteobacteria	*Oceanospirillales*	*Alcanivoracaceae*	*Alcanivorax dieselolei*	C.AW4	Members of this genus have been associated with Salterns [24]
					Alcanivorax sp.	C.ASED6	Members of this genus have been associated with Salterns [24]
			Alteromonadales	*Alteromonadaceae*	*Marinobacter confluentis*	AW10	Marine [98]
					Marinobacter sp.	ASED12	Marine [99]
			Oceanospirillales	*Halomonadaceae*	*Halomonas fontilapidosi*	AW8, AW13	**Salterns [24,100,101]**
					Halomonas ventosae	ASED11	**Salterns [100,101]**
					Salinicola zeshunii	OW15	Members of this genus have been associated with Salterns [102–104]
			Lysobacterales	*Lysobacteraceae*	*Luteimonas padinae*	ASBR3, ASBR7, ASBR10	**Salterns [105]**
			Cellvibrionales	*Microbulbiferaceae*	*Microbulbifer halophilus*	C.ASBR5, C.OW1, C.OW2	Members of this genus have been associated with Salterns [106]
			Chromatiales	*Aquichromatiaceae*	Uncultured bacterium	ASBR12, AW14, AW19, AW20	Members of this genus have been associated with marine habitat [107]
	Rhodothermota (**Supplementary Fig. 1i**)	*Balneolia*	*Balneolales*	*Balneolaceae*	Uncultured bacterium	ASBR13, AS101, OSBR103	Members of this family have been associated with Salterns [1,24]

Those associated with saltern environment are highlighted in bold.

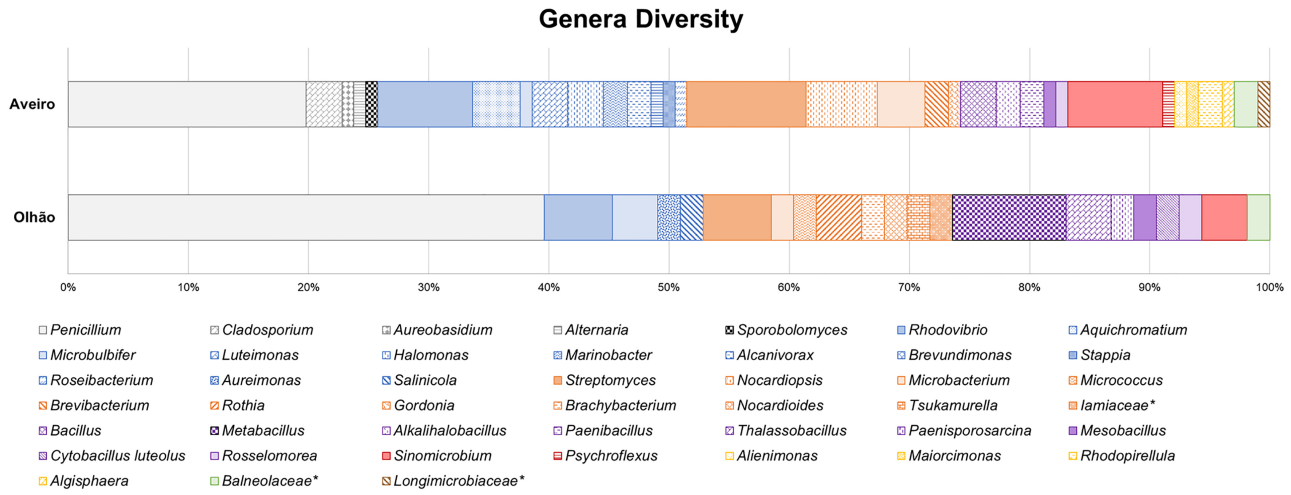

Fig. 2. Microbial diversity of the isolates obtained from Aveiro and Olhão salterns represented by genera.

Within Actinomycetota, 3 isolates were phylogenetically closely related with known opportunistic human pathogens (*Rothia* and *Tsukamurella*), that were obtained from Olhão saltern [74]. The presence of these species in Olhão saltern microbiota may be related with human activity, not only through salt exploitation but also through touristic related activities happening in Olhão salterns, like saltern baths (Table 1). Additionally, one isolate affiliated with *Gordonia oryzae* was obtained. This specie was recently described [75] and was not associated with extreme environments until this study. Furthermore, the Olhão saltern isolate OSBR104 was affiliated with the Actinomycetota family *Iamiaceae*, but the closest related strain described was *Aquihabitans daechungensis* [111] with 92.37% similarity in the 16S rRNA gene. Therefore, OSBR104 may represent a novel taxon (Fig. 4). Additionally, isolates related with *Microbacterium amylolyticum* (C.AS10 and C.AW5), *Microbacteium ginsengiterrae* (C.AW1), *Micrococcus luteus* (C.OS4) and *Nocardioides salarius* (OSBR100) showed low similarities with the mentioned strains (**Supplementary Table 2**) and may represent new taxa (Fig. 4).

Both classes of the phylum Planctomycetota, namely *Planctomycetia* and *Phycispharae* were isolated in this study. *Planctomycetia* isolates obtained were affiliated with genera *Alienomonas* (1 isolate), *Rhodopirellula* (2 isolates) and *Maioricimonas* (1 isolate) (Fig. 2). Four out of the 5 *Planctomycetia* isolates showed only up to 97.95% similarity in the 16S rRNA gene with the closest related described species (Fig. 4; **Supplementary Table 2**). These data are indicative of novel *Planctomycetia* taxa (Fig. 4). Within the less known Planctomycetota class *Phycispharae*, only one isolate (ASED31) was obtained, for which the species *Algisphaera agarilytica* was the most related one but in a far distant level (88.8% similarity in the 16S rRNA gene), being putatively indicative of a new family within *Phycispharae* (Fig. 4). Curiously, all planctomycetal isolates obtained in the present study were obtained from Aveiro saltern.

Within Rhodothermota, the family *Balneolaceae* was the only one recovered with 3 isolates (ASBR13, AS101 and OSBR103), that due to the phylogenetic distance, could not be related with any known Rhodothermota genus (Fig. 4).

Inside the phylum Gemmatimonadota, only one isolate (AW12) phylogenetically placed within the family *Longimicrobiaceae* was retrieved (Fig. 3). However, the 16S rRNA gene distant relatedness with the closest described species, represented by 85.82% similarity with *Longimicrobium terrae*, may be indicative of a novel family (Fig. 4).

3.2 Diversity, richness, dominance and distribution of the obtained isolates

The most frequent fungal genus was *Penicillium*, since 26.6% of the overall microbial isolates were classified within this genus (**Supplementary Table 2**). The most common bacterial genera retrieved from both salterns were the Actinomycetotal *Streptomyces* (8.4%) and the Pseudomonadota *Rhodovibrio* (7.1%), followed by the genus *Sinomicrobium* (6.5%) that is a member of phylum Bacteroidota (**Supplementary Table 2**).

Regarding overall phyla and genera, the isolated microorganisms associated with Aveiro saltern showed a higher diversity (Fisher's α index) and richness (Margalef's index), as well as a lower dominance (Simpson's index) (Table 2). By running separated analyses of the genera diversity within the fungal kingdom and the genera diversity within the bacterial domain, one difference about these indexes was observed when compared to the overall analysis. Particularly, the diversity of bacterial genera was higher in Olhão saltern population (Table 2).

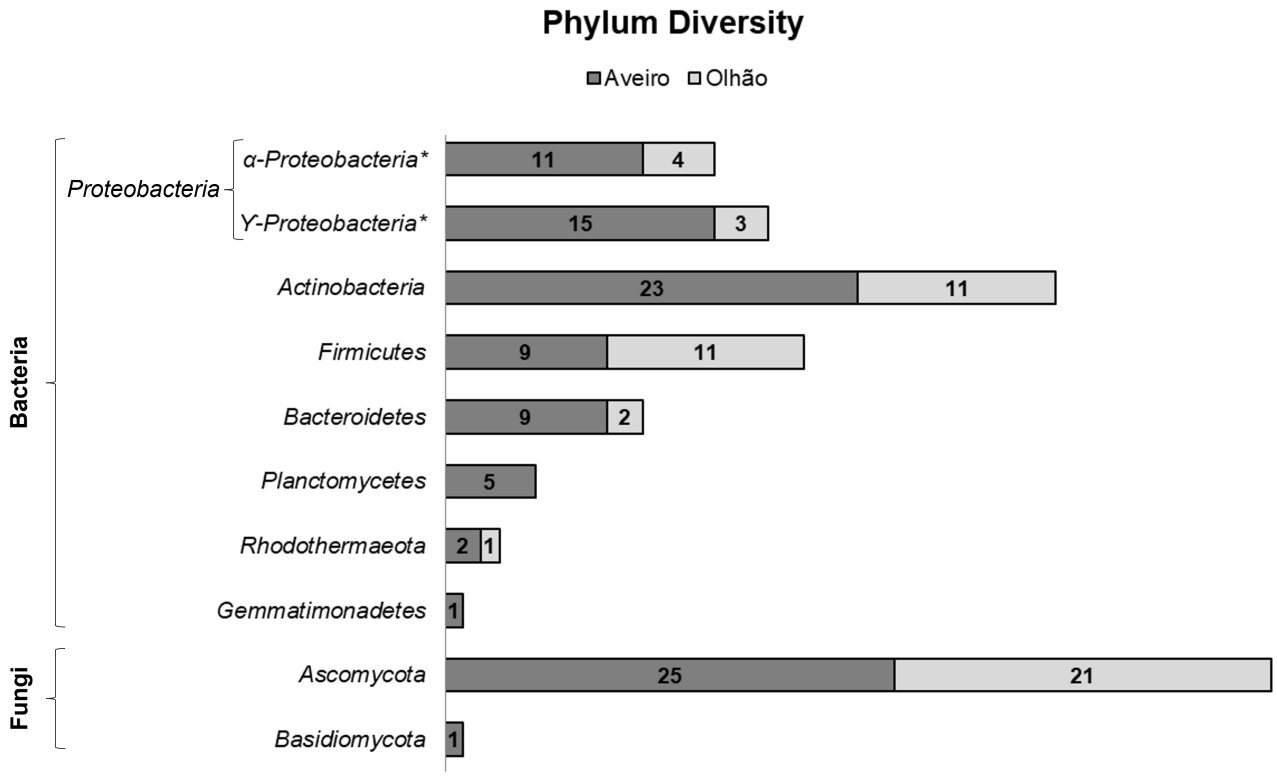

Fig. 3. Overall microbial biodiversity, phyla-ranked, of the isolates obtained from Aveiro and Olhão salterns, belonging to two microbial kingdoms.

Table 2. Diversity, richness and dominance indexes of microbial communities isolated from Aveiro and Olhão salterns.

	Fisher's α		Margalef's		Simpson's	
	Aveiro	Olhão	Aveiro	Olhão	Aveiro	Olhão
General by phylum	2.389 (2.04–2.389)	1.74 (1.74–1.74)	1.733 (1.517–1.733)	1.259 (1.259–1.259)	0.1983 (0.1756–0.2365)	0.2624 (0.2218–0.3471)
General by genus	17.06 (12.82–17.06)	12.85 (6.969–12.85)	6.934 (5.85–6.934)	5.037 (3.526–5.037)	0.07597 (0.05715–0.1071)	0.1826 (0.1136–0.3001)
Fungi	1.841 (0.8763–1.841)	0.2185 (0.2185–0.2185)	1.228 (0.6139–1.228)	0	0.6095 (0.4172–0.855)	1
Ascomycota	1.344 (0.5116–1.344)	0.2185	0.932 (0.3107–0.932)	0	0.6576 (0.4752–0.8528)	1
Basidiomycota	0	–	0	–	1	–
Bacteria	16.21 (11.32–16.21)	22.82 (9.49–22.82)	6.254 (5.096–6.254)	5.482 (3.751–5.482)	0.06453 (0.05209–0.08656)	0.07031 (0.06641–0.1309)
Bacillota	4.632 (1.576–4.632)	5.403 (1.359–5.403)	1.82 (0.9102–1.82)	2.085 (0.8341–2.085)	0.2346 (0.2099–0.5062)	0.2727 (0.1901–0.5702)
Pseudomonadota	5.949 (3.143–5.949)	3.878 (0.9354–3.878)	2.762 (1.842–2.762)	1.542 (0.5139–1.542)	0.1627 (0.1302–0.2899)	0.3061 (0.2653–0.7551)
Bacteroidota	0.7972	0.7959	0.4551	0	0.8025 (0.5556–0.8025)	1
Actinomycetota	1.968 (1.399–1.986)	13.19 (2.261–13.19)	1.276 (0.9568–1.276)	2.919 (1.251–2.919)	0.2968 (0.2401–0.4669)	0.157 (0.1405–0.3554)
Planctomycetota	9.284 (1.235–9.284)	–	1.864 (0.6213–1.864)	–	0.28 (0.28–0.52)	–
Rhodothermota	0.7959	0	0	0	1	1
Gemmatimonadota	0	–	0	–	1	–

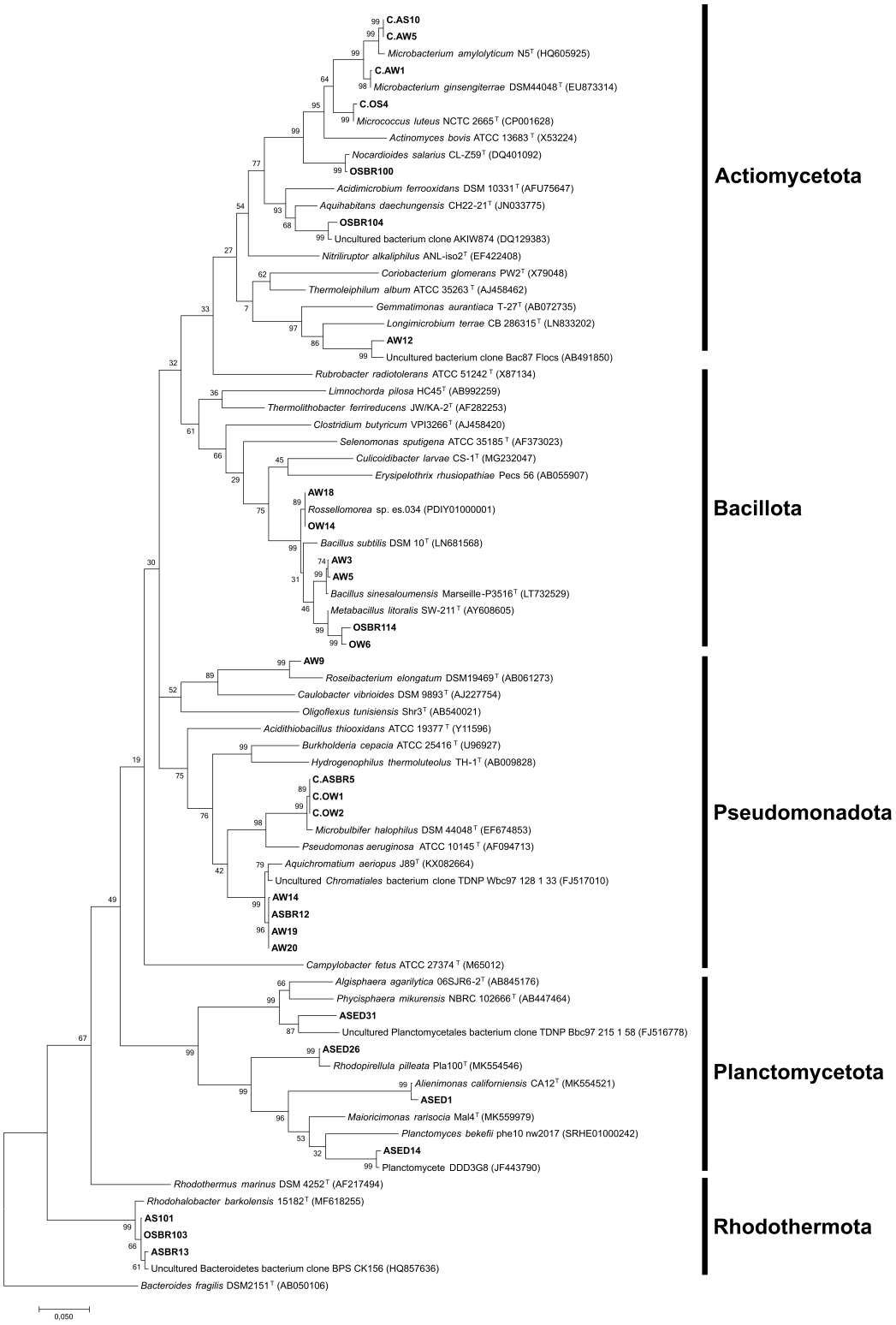

Fig. 4. Phylogenetic framing of the putative novel taxa isolated from the two Portuguese Salterns. Maximum-Likelihood (ML) phylogenetic trees constructed with MEGA 7 software [39], using 16S rRNA gene sequences of the saltern isolates and of the phylogenetically closest sequences obtained in the EzBioCloud webserver. Bootstrap values were calculated based on 1000 replications and the numbers at each branch represent the bootstrap support in percentage for each cluster. The tree was constructed using representative members of each class within the phyla detected; *Bacteroides fragilis* (AB050106) was used as outgroup; Bar, 0.050 substitutions per nucleotide position.

In Aveiro, the highest diversity and richness were registered within Planctomycetota and Pseudomonadota, while the lowest value of dominance was observed in Pseudomonadota, highlighting the heterogeneity of the Pseudomonadota isolates community (Table 2). At Olhão saltern, Actinomycetota and Pseudomonadota showed the highest diversity, while the lowest diversity was observed within Ascomycota and Rhodothermota (Table 2). Additionally, in Olhão, the highest values of diversity and richness along with the lowest value of dominance were observed within Actinomycetota (Table 2), demonstrating the high heterogeneity of Actinomycetotal isolates obtained.

Despite the high isolated diversity, the Mao's Tau rarefaction curve presented a continuous rise without achieving an asymptote, confirming that not all microbial diversity was recovered (Fig. 5).

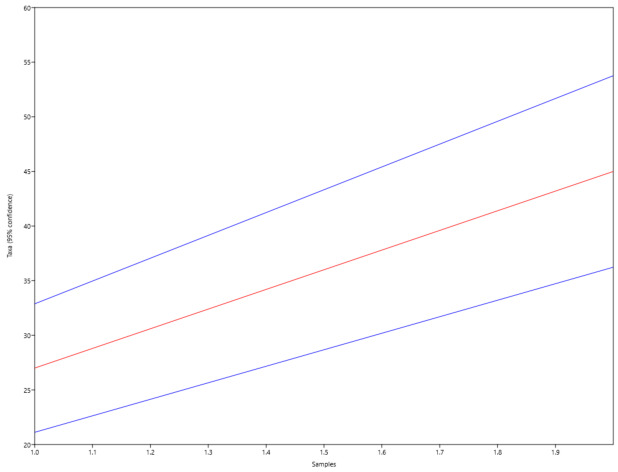

Fig. 5. Mao's Tau rarefaction curve (red) obtained on the genera diversity analysis of microbial isolated communities from Aveiro and Olhão salterns and representing the species accumulation with 95% confidence intervals (in blue).

Concerning the fungal biodiversity, 3 singletons were observed in Aveiro, while none was detected in Olhão. These singletons represent 6.4% of the whole fungal diversity (Supplementary Material). The overall bacterial diversity was comprised by 40 different genera, with 25 singletons, that represent 23.4% of the overall bacterial diversity (**Supplementary Table 2**). These may be rare members of the isolated bacterial communities of salterns. Additionally, from the 28 bacterial genera recovered from Aveiro saltern, 12 occurred as singletons, representing 16.0% of the overall diversity of isolates, while Olhão taxa biodiversity consisted in 20 different bacterial genera, where 13 occurred as singletons, representing 37.1% of the overall diversity of isolates (**Supplementary Table 2**). From all these singletons, only 2 were common to both salterns, namely, the Bacillota genera, *Mesobacillus* and *Rossellomorea* (**Supplementary Table 2**).

The Sørensen and Bray-Curtis indexes were determined between the microbial populations of both salterns as 0.33 and 0.43, respectively. These parameters are measures of the similarity between both microbial populations, therefore highlighting the dissimilarities between them. Taking into account that sampling dates were close and that all the procedures undertaken were the same for all samples, these differences between the two microbial populations studied may be a result of the different geographical locations of the two salterns (Aveiro at the North of Portugal vs. Olhão at the South of Portugal).

3.3 Molecular assessment of NRPS and PKS-I genes

The biotechnological potential of all isolates obtained in this study was screened by the PCR analysis of PKS-I and NRPS genes (Table 3; **Supplementary Table 2**). From the 154 microorganisms isolated in this study, 51 (33.1%) did not present any PKS or NRPS genes and 80 (52%) presented one (64–41.6% PKS positive and 16–10.4% NRPS positive) or both genes (23 bacteria, 14.9%) (Table 3; **Supplementary Table 2**). Overall, this screening revealed that 66.9% of the isolates may present at least one of the genes, revealing their putative biotechnological potential (Table 3; **Supplementary Table 2**). Although microbial ability to produce bioactive metabolites is not strictly linked to PKS and NRPS genes, because many other genes have been associated with bioactive NPs production [112], the genomic presence of these genes already proved to be a good indicator of the production of antimicrobial compounds [113]. Furthermore, NPs derived from these genes, as polyketides (PK), nonribosomal peptides (NRP) or even PK/NRP hybrids, have demonstrated their high economical and pharmacological value [20,114]. These gene clusters were previously detected in ascomycetes and in a high number, which may be associated with the production of several different NRPS- and PKS-derived NPs [115].

The high percentages and widespread distribution of NRPS and PKS genes observed in the phyla Pseudomonadota, Actinomycetota, and Bacillota was already reported by Wang *et al*. [115]. In contrast, both Bacteroidota and Planctomycetota are less explored phyla in what concerns their potential for the production of bioactive molecules, being research fields that are being launched with encouraging results obtained from a preliminary screening of NRPS and PKS genes [116–118]. Concerning Rhodothermota, since this phylum was recently branched out from Bacteroidota [8], no studies targeting their overall genomic potential for production of bioactive NPs are yet published. Regardless of the only 3 representatives obtained, since, up to the present moment, the phylum Rhodothermota has only 13 described species from 4 different families of only 1 class, the presence of at least one of these genes in all isolated members of this phylum pave the way for future deeper studies on this poorly known phylum. As detected in the present work, NRPS and PKS-I gene clusters were previ-

Table 3. Number of isolates, grouped by genera within each phylum that potentially amplified positively the bioactivity-related genes, NRPS e PKS-I, through PCR screening.

Phylum	Genus	Number of Isolates	Only NRPS	Only PKS	Both
Actinomycetota	*Streptomyces*	13	0	9	1
	Nocardiopsis	6	0	6	0
	Micrococcus	1	0	0	1
	Microbacterium	5	1	0	2
	Brevibacterium	2	0	1	0
	Rothia	2	0	1	0
	Gordonia	1	0	1	0
	Brachybacterium	1	0	0	0
	Nocardioides	1	0	0	0
	Tsukamurella	1	0	0	1
	*Iamiaceae**	1	0	0	0
Pseudomonadota	*Rhodovibrio*	11	1	3	1
	Wenzhouxiangella	4	1	2	1
	Microbulbifer	4	0	1	3
	Luteimonas	3	1	0	1
	Halomonas	3	0	3	0
	Marinobacter	2	1	0	0
	Alcanivorax	2	0	0	0
	Brevundimonas	1	1	0	0
	Stappia	1	0	0	1
	Roseibacterium	1	0	0	0
	Aureimonas	1	0	1	0
	Salinicola	1	0	1	0
Bacillota	*Bacillus*	3	3	0	0
	Metabacillus	5	0	0	0
	Alkalihalobacillus	2	0	2	0
	Paenibacillus	2	0	0	2
	Thalassobacillus	2	0	0	1
	Paenisporosarcina	1	1	0	0
	Mesobacillus	2	1	0	0
	Cytobacillus	1	0	0	0
	Rossellomorea	2	2	0	0
Bacteroidota	*Sinomicrobium*	10	0	8	0
	Psychroflexus	1	0	0	1
Planctomycetota	*Alienimonas*	1	0	0	0
	Maiorcimonas	1	1	0	0
	Rhodopirellula	2	0	1	1
	Algisphaera	1	0	1	0
Rhodothermota	*Balneolaceae**	3	0	1	2
Gemmatimonadota	*Longimicrobiaceae**	1	0	0	0
Ascomycota	*Penicillium*	41	1	21	4
	Cladosporium	3	0	1	0
	Aureobasidium	1	1	0	0
	Alternaria	1	0	0	1
Basidiomycota	*Sporobolomyces*	1	0	0	0

ously detected in ascomycetes and in a high number, which may be associated with the production of several different NRPS- and PKS-derived NPs [115]. No NRPS or PKS-I amplicons were detected either in Gemmatimonadota or Basidiomycota (Table 3; **Supplementary Table 2**). In fact, Gressler *et al*. [119] reported that PKSs and NRPSs are not the main contributors for basidiomycetes diversity of NPs. On the other hand, the poorly known phylum Gemmatimonadota was firstly described less than two decades ago and presently only has 6 described species. This scarce number of described species is due to the difficulties in successfully isolating Gemmatimonadota members in laboratory,

despite being widely distributed in the environment [120]. Therefore, most of the research made on its bioactive potential relies on metagenomes, which demonstrated the presence of not only NRPS, PKS and hybrid PKS/NRPS gene clusters [121], but also a widespread and high prevalence of bacteriocin-related biosynthetic gene clusters [122].

Overall, the microbiota isolated from both salterns showed a great biotechnological potential. Curiously, although with lower number of isolates, the phyla that showed higher bioactive potential were the Rhodothermota, Bacteroidota and Planctomycetota, and not the well-known bioactive top producers, Actinomycetota [123].

4. Conclusions

This work provides evidence that salterns remain an understudied extreme environment and that culturomic methods are still an important approach for the study of microbial diversity, since a high number of novel taxa from different phyla was obtained. In fact, although metagenomics is a fundamental approach for novel microbial diversity detection, pure cultures are needed to enable the biological characterization of many species yet unknown. Furthermore, the isolated microbiota from salterns showed a substantial underexplored bioactive potential providing data and biological material that encourages further research works as bioactivity screenings.

In general, the overall microbial diversity obtained has been previously associated with salterns or with other hypersaline environments, however our results also pointed out genera not yet linked to these environments.

Abbreviations

NP, natural product; PKS-I, polyketide synthase I; NRPS, nonribosomal peptide synthethase; SW, seawater; PCR, polymerase chain reaction; NCBI, National Centre for Biotechnology Information; RT, room temperature; SCN, starch-casein-nitrate agar; NPS, nutrient-poor sediment extract agar.

Author contributions

EA, MFC and OML designed the research study. EA performed the research. EA, MFC and OML analyzed the data. EA, MFC and OML wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbe1402011.

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