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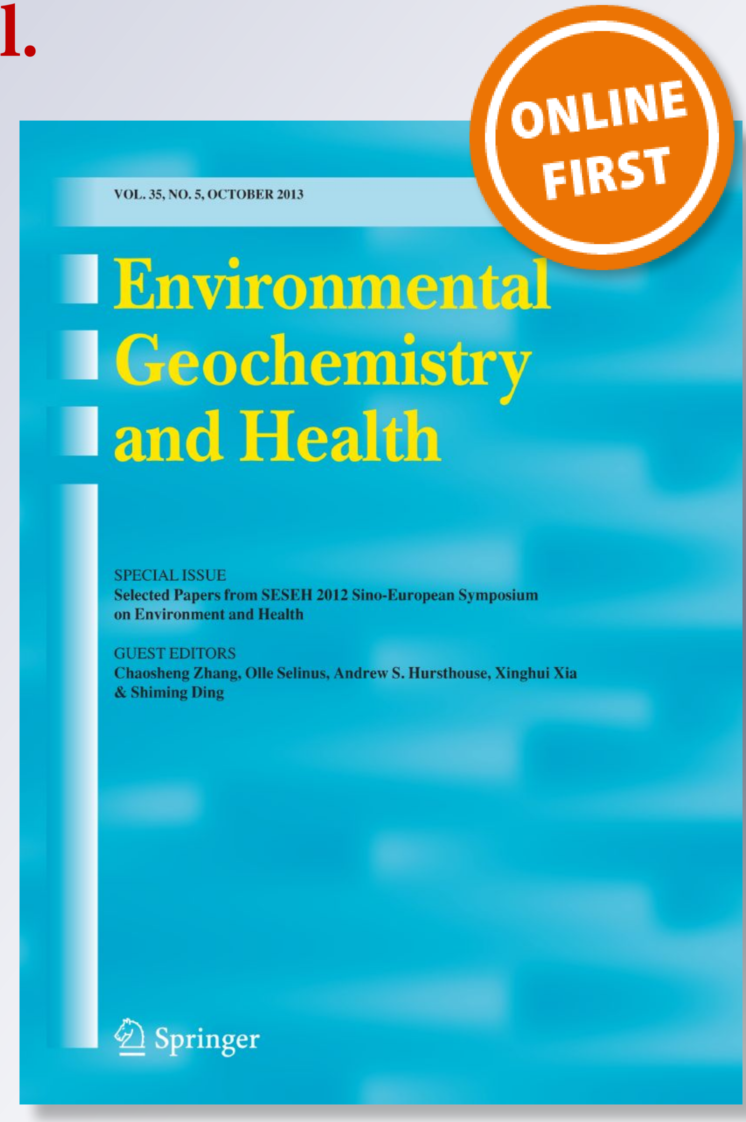
Environmental Geochemistry and Health
Official Journal of the Society for Environmental Geochemistry and Health

ISSN 0269-4042

Environ Geochem Health
DOI 10.1007/s10653-019-00473-6



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https://doi.org/10.1007/s10653-019-00473-6

ORIGINAL PAPER

Chemical signature and antimicrobial activity of Central Portuguese Natural Mineral Waters against selected skin pathogens

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Received: 2 February 2019 / Accepted: 9 November 2019

Abstract The common therapeutic indications of Portuguese Natural Mineral Waters (NMWs) are primarily for respiratory, rheumatic and musculoskeletal systems. However, these NMWs have been increasingly sought for dermatologic purposes. Opposing to what is observed in the major European Thermal Centres, there are few scientific evidences supporting the use of Portuguese NMWs for clinical applications. The aim of this study was to characterize the antimicrobial profile of individual NMWs from the central region of Portugal and correlate the results with their physicochemical characterization. An extensive multivariate analysis (principal component analysis) was also performed to further investigate this possible correlation. Six collection strains representing skin microbiota, namely *Staphylococcus aureus*, *Escherichia coli*, *Corynebacterium amycolatum, Candida albicans, Staphylococcus epidermidis* and *Cutibacterium acnes,* were analysed, and their antimicrobial profile was determined using Clinical and Laboratory Standards Institute M07-A10, M45-A2, M11-A6 and M27-A3 microdilution methods. Different NMWs presented different antimicrobial profiles against the strains used; the physicochemical composition of

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10653-019-00473-6) contains supplementary material, which is available to authorized users.

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NMWs seemed to be correlated with the different susceptibility profiles. *Cutibacterium acnes* showed a particularly high susceptibility to all NMWs belonging sulphurous/bicarbonated/sodic ionic profile, exhibiting microbial reductions up to 65%. However, due to the complex physicochemical composition of each water an overall conclusion regarding the effect of a specific ion on the growth of different microorganisms is yet to be known.

Keywords Natural Mineral Waters · Physicochemical properties · Antimicrobial activity · Skin microbiota

Introduction

Natural Mineral Waters (NMWs) can be defined as deep circulating waters of specific bacteriological profile, with physicochemical characteristics that are stable at its origin within the range of natural fluctuations, and possess some therapeutic properties or health beneficial effects (Cantista 2008).

The curative properties of these waters were widely recognized long before modern medical treatments were developed. Presently, balneotherapy (also called crenobalneotherapy) is considered an alternative treatment for several diseases in many countries (Hercogova et al. 2002). The term balneotherapy stands for a set of methods and practices (bathing, drinking, inhalation, etc.) that use medical mineral waters, medical peloids and/or natural gasses for therapeutic purposes in Health Resort Medicine Centres (Gomes et al. 2013).

In the Portuguese territory, there are about 50 Thermal Centres, all located in the north and central regions (Cantista 2008). The search for these Thermal Centres to treat different afflictions has increased since 2017 and approximately 13 million Euros have been invested in these health facilities by their users for recreational or therapeutic purposes in Portugal (Direção Geral de Energia e Geologia 2017).

The main therapeutic indications for each Thermal Centre include different afflictions ranging from neuromuscular, gastrointestinal, dermic, articular and respiratory diseases. These are attributed to different NMWs and their physicochemical characteristics (Gomes et al. 2010; Rebelo et al. 2015).

According to the European Legislation (2009/54/EC Directive), physical and chemical characterizations are used to make a classification of the different mineral waters, based on the analysis of their main parameters (Quattrini et al. 2016). NMWs can be classified in different manners, and, simplistically, they can be divided according to their therapeutic actions or physicochemical characteristics.

Regarding physicochemical characteristics, NMWs are classified according to different parameters such as osmotic pressure, temperature, chemical composition and mineralization (Araujo et al. 2017). In Portugal, two main physicochemical classifications are adopted based on total mineralization and chemical composition (Cantista 2008).

For total mineralization, the Institute of Hydrology of Lisbon proposes the following classification: (1) hyposaline waters: total mineralization below 200 mg/L; (2) poorly mineralized water: total mineralization between 200 and 1000 mg/L; (3) mesosaline waters: total mineralization between 1000 and 2000 mg/L; (4) hypersaline waters: total mineralization above 2000 mg/L (Cantista 2008), whereas the official site of 'Termas de Portugal' and the Portuguese Directorate General for Energy and Geology (DGEG) use the main chemical components of each water to group NMWs by similar ionic profiles (as referred in 'Methods'—'Thermal Centres recruitment' section).

Besides physicochemical classification, NMWs can be divided based on their therapeutic indications. The common therapeutic indications of Portuguese NMWs are primarily for respiratory, rheumatic and musculoskeletal systems (Table 1), and this can be justified by the presence of dominant components, namely sulphur and bicarbonate. However, the benefits to the digestive, endocrine–metabolic diseases and nephrourinary systems have also been described (Araujo et al. 2017). Likewise, four NMWs from the central region are known for their therapeutic actions against skin conditions.

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Table 1 Therapeutic applications (grey cells) of NMWs from the central region of Portugal, adapted from Director General of Health (Direção-Geral da Saúde 1989)

Thermal Centres	Endocrine metabolic system	Circulatory system	Blood	Urinary system	Digestive system	Respiratory system	Skin	Rheumatic and musculoskeletal conditions
Águas Penamacor						▨		▨
Alcafache	▨					▨		▨
Fonte Santa de Almeida						▨		▨
Caldas da Rainha						▨		▨
Sangemil						▨		▨
Cró						▨	▨	▨
Carvalhal					▨	▨	▨	▨
Curia	▨	▨		▨				▨
Felgueiras						▨		▨
Ladeira de Envendos					▨	▨	▨	▨
Longroiva						▨		▨
Luso		▨		▨		▨		▨
Manteigas						▨		▨
Monfortinho	▨	▨		▨	▨	▨	▨	▨
Monte Real					▨	▨		▨
São Pedro do Sul	▨					▨		▨
Unhais da Serra		▨			▨	▨		▨
Vale de Mó			▨		▨			

The dermatologic therapeutic indication of NMWs is also well established in different countries. In fact, the French cosmetic industry introduced NMWs due to their biological effects, suggesting its application in various skin conditions (Nunes and Tamura 2012). These beneficial effects have been attributed to the presence of sulphur, silica and different cations, such as sodium, calcium and potassium in the waters' chemical composition (Matz et al. 2003; Araujo et al. 2017).

The skin plays the role of principal barrier against microbial invasion due to its constant interaction with the external environment (Petkovšek et al. 2009). Skin is also colonized by a diverse population of microorganisms that play a fundamental role in the control of skin physiology, including skin immunity and inflammatory processes (Ridaura et al. 2018).

Staphylococci and other gram-positive bacteria such as *Corynebacterium* species are common bacterial colonizers of the skin and mucous membranes of humans, typically above the waist (Cogen et al. 2008). In fact, *S. epidermidis* is the most frequently isolated species from human epithelia and typically has a benign relationship with its host and can even exert a probiotic function by preventing colonization of more pathogenic bacteria such as *S. aureus* (Otto 2009; Abu-Ghazaleh 2016).

Studies have also shown that innocuous colonists such as gram-positive *C. amycolatum* can have a highly context-specific effect on the skin immune system (Belmares et al. 2007). Nevertheless, these opportunistic bacteria can be responsible for some hospital infections, particularly in immunocompromised patients (Zalas et al. 2004).

Contrarily to *S. epidermidis*, *S. aureus* is a colonizer also found in skin and nostrils of healthy subjects, but is the almost-universal cause of furuncles, carbuncles and skin abscesses and is the most commonly identified agent responsible for skin and soft tissue infections (Achermann et al. 2014). In addition, *S. aureus* overgrowth, as part of the alteration of skin microbiota, is a relevant factor for epithelial barrier disturbance or immune dysfunction present in atopic dermatitis (Meylan et al. 2017). *Staphylococcus aureus* can also be implicated in the aetiology of severe infections like pneumonia, meningitis, endocarditis, toxic shock syndrome, septicaemia and others (McCaig et al. 2006; dos Santos et al. 2007).

Typically, microorganisms that colonize the skin, particularly below the waist, can be both gram-positive and gram-negative species (Ki and Rotstein 2008). Enteric species, such as *Enterobacteriaceae*, gravitate and colonize this area of the skin and, although the most common causative agents are aerobic streptococci, several reports associating the enterobacterium *Escherichia coli* with skin and soft tissue infections have been published (Achermann et al. 2014). *Escherichia coli* was found to be the causative agent of cellulitis, necrotizing fasciitis, skin infections after surgical interventions, infections in burn injuries, among others (Petkovšek et al. 2009). It has also been increasingly associated with severe skin infections in immunocompromised patients, such as those associated with diabetes (Ko et al. 2018).

Cutibacterium acnes (formerly known as *Propionibacterium acnes*) is a gram-positive, facultative, anaerobic rod that is a major colonizer and inhabitant of the human skin along with *Staphylococcus*, *Corynebacterium* and *Streptococcus* species (Achermann et al. 2014). This species is mostly found in sebaceous sites and therefore is frequently associated with acne vulgaris (Achermann et al. 2014). This chronic skin disease affects the pilosebaceous unit, and there are several factors contributing to its development that range from inflammation caused by inflammatory mediators; alteration of the keratinization process (which leads to comedone development); increased sebum production under androgen control and finally follicular colonization by *C. acnes* (Brown and Shalita 1998). The anaerobic and lipid-rich conditions within the pilosebaceous unit provide an optimal microenvironment for *C. acnes* growth (Cogen et al. 2008).

Besides bacteria, also fungi are part of the commensal skin microbiota with various species having the ability to become pathogenic (Underhill and Iliev 2014). *Candida albicans* is the *Candida* species most often responsible for symptomatic skin infections, which prefers occluded regions of the skin, subjected to friction, where humidity and CO_2 accumulate. Common symptoms of *Candida* skin infections include thickening of the skin, hyperkeratosis and erythema (Kühbacher et al. 2017). These infections are prompted by several physical and immunological factors that cause the shift from a normal skin colonizer to an opportunist pathogen.

The major skin conditions that are frequently treated with balneotherapy with a high rate of success are psoriasis and atopic dermatitis. Other conditions include acne vulgaris, contact dermatitis, eczema, mycosis fungoides, seborrhoeic dermatitis, among others (Matz et al. 2003). Since the aetiological factor of most of these dermatological conditions comprises microbial colonization and even infection, it becomes vital to understand the effect that NMWs have in the microorganisms related to these skin affections.

Nevertheless, despite the high number of skin conditions with therapeutic indications for balneotherapy, the efficacy of the therapy in different skin diseases and the mechanisms underlying the bioactive effects of NMWs are only partly understood (Lee et al. 2012).

Although some advances on the effect of Portuguese NMWs on skin conditions have been made, the majority of the studies with both Portuguese and foreign waters focused on their anti-inflammatory, antioxidant and regenerative properties, with some of the mechanisms underlining such activities already hypothesized (Hercogova et al. 2002; Ferreira et al. 2010; Richard et al. 2010; Faga et al. 2012; Braga et al. 2013; Nicoletti et al. 2017). However, the effect of these waters on skin commensal and pathogenic microorganisms, responsible for a different number of skin conditions and infections, has not been reported. Therefore, the goal of this study was to describe the physicochemical characteristics of each Natural Mineral Water (NMW) addressing the most important parameters using a comprehensive statistical analysis and to analyse the antimicrobial profile of each individual NMW in order to correlate with the physicochemical characteristics. This study also contributed to elucidate the potential of NMWs to modulate the growth of microorganisms involved in dermal affections.

Methods

Thermal Centres recruitment and water collection

All 18 active Thermal Centres belonging to the central region of Portugal (Fig. 1) were contacted and asked

Fig. 1 Graphical representation of the Thermal Centres located in the central region of Portugal (marked in dark grey). Adapted from Portuguese Directorate General for Energy and Geology

for their consent to participate in the study. The 16 centres, who accepted, received appropriate flasks (500 mL; VWR collection) for each type of parameters to be measured.

NMWs were collected from the spring/borehole of each Thermal Centre, after purging and ensuring all appropriate water collection procedures (APHA 2005). One sample [consisting of six water flasks] was collected in each Thermal Centre for physicochemical parametric analysis.

The collected water samples were placed in thermal boxes with frozen accumulators and transported to the laboratory under the recommended conditions (5 ± 3 °C) and refrigerated in the dark until use.

To ensure the confidentiality of the Thermal Centres involved, each centre was uniquely labelled.

Physicochemical analysis

Samples were analysed for odour, aspect, colour, deposit, osmolality and pH in triplicate at room temperature (± 25 °C).

Odour, aspect, colour and deposit were determined using internal methods based on Standard Methods for the Examination of Water and Wastewater (SMEWW) (APHA 2005). The pH measurement was taken based on SMEWW using an electrode (Thermo Fisher) which was periodically calibrated with three certified buffer solutions of pH 4.01, 7.00 and 10.01 (APHA 2005). NMWs were also tested for their osmolality based on SMEWW using OSMOMAT 3000 (Gonotec), which was calibrated before each measurement with two certified standard NaCl solutions and a blank of distilled water (APHA 2005).

All other physicochemical analysis were carried out in independent laboratories accredited by the NP EN ISO/IEC 17025 norm and posteriorly sent to our laboratory in separated analytical sheets corresponding to each Thermal Centre.

A microbiological quality control test was also performed for each sample as described in Portuguese Pharmacopeia 9.0, to ensure the absence of microorganisms (INFARMED—Instituto Nacional da Farmácia e do Medicamento 2009). NMWs samples whose microbiological quality results showed colony-forming units (CFU) were filtered through a 0.2-µm pore filter (VWR collection). To ensure sterility of all samples, an additional sterility control was included in the microbial activity assays (as referred in 'Antimicrobial activity' section).

NMWs were grouped by their ionic profile, according to the classification by Portuguese Directorate General for Energy and Geology (DGEG) as follows: sulphurous/bicarbonate/sodic—9 NMWs; sulphurous/bicarbonate/fluoridated/sodic—1 NMW; bicarbonate/sodic—1 NMW; chlorinated/sodic—2 NMWs; bicarbonate/magnesium—1 NMWs; sulphated/calcic—1 NMW and sulphurous/chlorinated/sodic—1 NMW.

Principal component analysis

The role of dominant components in NMW involved in the study was carefully assessed, and putative correlations of the NMW to specific antimicrobial activity were investigated by multivariate analysis.

The 22 analytical variables used for statistical purposes were gathered from three different groups, namely physicochemical constants and non-dissociated substances, anions (mg/L) and cations (mg/L). From a total of 27 initial variables, only 22 variables were considered, excluding the ones with two or more 'NR—Data not reported' on the analytical sheet provided by the Thermal Centres. The variables excluded were temperature emergency, total sulphurization, total sulphur, hydrogen sulphide and bisulphide.

Principal component analysis (PCA) was performed to access the most important physicochemical parameters reducing the dimensionality of the original data matrix retaining the maximum amount of variability (Dennis Child 2006; Tabachnick and Fidell 2007).

This analysis enables us to find the main sources of variability and to establish the relation between varieties and compounds.

From the many extraction techniques available, the relational structure between the variables was analysed by exploratory factor analysis on the correlation matrix, with factor extraction by the principal component method followed by a varimax rotation with Kaiser Normalization. The common factors retained were those with an eigenvalue greater than 1, in line with the percentage of variance retained.

PCA analysis of the physicochemical parameters was preformed using IBM SPSS Statistics (Version 25.0 for Windows, Armonk, NY: IBM Corporation).

Strains

Six collection strains, four from American-Type Culture Collection—*S. aureus* (ATCC 6538), *E. coli* (ATCC 8739), *C. amycolatum* (ATCC 49368) and *C. albicans* (ATCC 10231)—and two from Deutsche Sammlung von Mikroorganismen und Zellkulturen—*S. epidermidis* (DSM 28764) and *C. acnes* (DSM 1897), were used in the study.

These microorganisms were selected as representative of skin microbiome in both healthy and pathological conditions. The role of the selected microorganisms as colonists or pathogens to the human host is presented in Table 2.

In order to assess culture purity and viability, microorganisms were subcultured twice on tryptic soy agar (TSA, VWR Chemicals) for aerobic bacteria,

Table 2 Microorganisms included in the study and their relationship with the human host and associated pathogenicity. Adapted from (Cogen et al. 2008)

Organism	Skin colonization	Pathogenicity
Staphylococcus epidermidis	Common	Opportunistic pathogen
Staphylococcus aureus	Unusual	Usually pathogenic
Propionibacterium acnes	Common (sebaceous sites)	Opportunistic pathogen
Corynebacterium spp.	Common	Opportunistic pathogen
Escherichia coli	Unusual (typically below the waist)	Usually pathogenic
Candida albicans	Common	Opportunistic pathogen

Sabouraud dextrose agar (SDA, VWR Chemicals) for *C. albicans* and Brucella agar (Frilabo) supplemented with haemin (Sigma), vitamin K (Sigma) and 5% defibrinated sheep blood for anaerobic bacteria.

Antimicrobial activity

The antimicrobial activity against the specific strains was evaluated based on the Clinical and Laboratory Standards Institute M07-A10, M45-A2, M11-A6 and M27-A3 microdilution methods (CLSI 2004; CLSI 2008; CLSI 2010; CLSI 2015). Briefly, a 0.5 MacFarland suspension was prepared from bacterial and yeast cultures using sterile phosphate buffer solution [1.37 M NaCl (Fisher Chemical); 27 mM KCl (ChemLab); 100 mM Na_2HPO_4 (Fisher Chemical); 20 mM KH_2PO_4 (ChemLab)]; and the proper dilutions were made with different culture media. Specifically, Mueller–Hinton broth (VWR Chemicals) was used for aerobic bacteria, Mueller–Hinton broth supplemented with 5% defibrinated horse blood was used for *C. amycolatum*, RPMI (Biowest) for *C. albicans* and Brucella broth with haemin, vitamin K and 5% sheep blood for anaerobic bacteria. The microbial suspensions were exposed to different NMWs for 24, 48 or 72 h at 37 °C for the aerobic bacteria, *E. coli*, *S. aureus* and *S. epidermidis*; for *C. amycolatum* and the fungus, *C. albicans*; and for the anaerobic bacteria, *C. acnes,* respectively, upon a 50% dilution. For the cultivation of *C. acnes,* an anaerobic environment was generated using Anaerocult® A (Merck). Growth rate was determined by absorbance measurement at 600 nm using a benchmark microplate reader (Bio-rad). Appropriate controls were included using MilliQ-type water for positive control, and a negative (sterility) control including NMWs and growth medium to confirm the absence of microbial growth in each water. A growth control, composed with culture media and microbial culture used, was also included to analyse the normal growth of the different strains, in each assay.

Antimicrobial assays were carried out in quadruplicate in three independent growth cultures, and all values were expressed as percentages of control (MilliQ-type water).

An unpaired t test was performed to compare each NMW with the control. All analyses were conducted using GraphPad Prism (version 7.03 for Windows, GraphPad Software, La Jolla California USA), and $p < 0.05$ was accepted as denoting significance.

Results

Physicochemical characterization

In order to divide the NMWs used, the classification described by the Institute of Hydrology of Lisbon was followed. The majority of the NMWs in the study (9 of 16) belong the 'Poorly mineralized' group, with mineralization values ranging from 200–1000 mg/L (Table 3). Moreover, 5 of the 16 NMWs belong to the 'Hyposaline' group, which was further differentiated in: (a) NMWs with total mineralization up to about 50 mg/L, pH < 6, hardness < 1 and very high percentage of silica ($> 30\%$), or (b) NMWs with total mineralization > 100 mg/L, pH > 6, hardness > 1 and lower percentage of silica (Cantista 2008). The remaining NMWs belong to the 'Hypersaline' group with mineralization values higher than 2000 mg/L. This classification was in accordance with the one presented in the official DGEG site regarding total mineralization.

Additionally, a distribution of NMWs according to their ionic profile, previously classified by DGEG, was also applied (Table 4). The majority of the NMWs were in the 'sulphurous/bicarbonate/sodic' group.

Table 3 Mineralization profile of the codified Portuguese NMWs from the Central Region of Portugal

Hyposaline (< 200 mg/L)		Poorly mineralized (200–1000 mg/L)	Hypersaline (> 2000 mg/L)
Silicated	Non-silicated		
8, 7, 16	9, 15	1–6, 12–14	10, 11

Table 4 Classification of the codified Portuguese NMWs involved in the study, grouped by their main ionic profile

Sulphurous/bicarbonate/sodic	Sulphurous/bicarbonate/fluoridated/sodic	Bicarbonate/sodic	Bicarbonate/magnesium	Chlorinated/sodic	Sulphated/calcic	Sulphurous/chlorinated/sodic
1–6, 12–14	15	7	9	8, 16	10	11

Table 5 Measured physicochemical parameters and microbiological control of sulphurous/bicarbonate/sodic NMWs

NMWs code	1	2	3	4	5	6	12	13	14
Ionic profile	SBS	SBS	SBS	SBS	SBS	SBS	SBS	SBS	SBS
Organoleptic features									
Odour	SS	S	S	S	S	S	S	S	SS
Deposit	Null	Null	Null	Null	Null	Null	Null	Null	Null
Aspect	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
Colour	WC	WC	WC	WC	WC	WC	WC	WC	WC
Physicochemical constants and non-dissociated substances									
pH (at 25 °C)	8.7	8.1	8.6	8.9	8.0	9.0	8.3	7.7	8.3
Osmolality (mosmol/kg)	17	26	25	18	28	13	7	12	14
Microbiological control									
Bacterial (CFU/mL)	0	0	0	0	0	0	0	0	0
Fungal (CFU/mL)	0	0	0	0	0	0	0	18	0

SBS sulphurous/bicarbonate/sodic, *SS* sulphur (low intensity), *S* sulphur, *WO* without odour, *WC* without colour. *CFU* colony-forming units

The complete physicochemical fingerprint of each NMW was determined by compiling parameters measured in our laboratory (Table 5 and 6) and information disclosed in the analytical sheets of each Thermal Centre (Tables 7 and 8).

Principal component analysis

From the 27 variables belonging to the physicochemical constants and non-dissociated substances, anions (mg/L) and cations (mg/L) groups, 22 variables were considered for multifactorial analysis.

Total fraction for the physicochemical parameters enrolled in this study is represented in Fig. 2.

Applying factorial analysis to the normalized relative amounts of the 22 analytical variables, four factors were extracted explaining 85.345% of the total variance of initial data set (Tables S1—Supplementary data). Discriminated factor weights of each item in the 4 retained factors, eigenvalues and % of variance explained, after a PCA, with factor extraction by the principal component method followed by a varimax rotation, are presented in Table S2 (Supplementary data).

Factor plot was also produced as presented in Fig S1 (Supplementary data). However, since there were more than three factors, they appear represented in hyperspace; therefore, our factor plot was not useful

Table 6 Measured physicochemical parameters and microbiological control of sulphurous/bicarbonate/fluoridated/sodic, bicarbonate/sodic, chlorinated/sodic, bicarbonate/magnesium, sulphated/calcic and sulphurous/chlorinated/sodic NMWs

NMWs code	7	9	10	11	15	8	16
Ionic profile	BS	BM	SC	SCS	SBFS	CS	CS
Organoleptic features							
Odour	WO	WO	WO	WO	S	WO	WO
Deposit	Null	Present[a]	Null	Null	Null	Null	Null
Aspect	Clear	Clear	Clear	Clear	Clear	Clear	Clear
Colour	WC	WC	WC	WC	WC	WC	WC
Physicochemical constants and non-dissociated substances							
pH (at 25 °C)	5.6	6.0	7.4	7.3	9.4	4.3	5.4
Osmolality (mosmol/kg)	36	13	21	69	4	18	5
Microbiological control							
Bacterial (CFU/mL)	2	0	0	0	11	0	0
Fungal (CFU/mL)	4	0	0	0	64	0	0

BS bicarbonate/sodic, *BM* bicarbonate/magnesium, *CS* chlorinated/sodic, *SC* sulphated/calcic, *SCS* sulphurous/chlorinated/sodic, *SBFS* sulphurous/bicarbonate/fluoridated/sodic, *SS* sulphur (low intensity), *S* sulphur, *WO* without odour, *WC* without colour, *CFU* colony-forming units

[a] Small orange 'tears' in the container

for interpretation. Despite, as the components loadings are presented, it can be noted that dimension 1 is much more relevant than dimension 2, 3 or 4. Then, the model was ran again with three dimensions where the analysis with three components explained 78.221% of total variance. However, the third factor had an internal consistency unsatisfactory of only 0.027 (data not shown).

Therefore, it was decided to run the model again with two dimensions/factors.

Figure 3 presents the components loadings after applying the model with only two dimensions/factors. The analysis with 2 components explained only 69.432% of total variance but both factors has a high internal consistency of 0.699 and 0.910, respectively.

The first factor has high factorial weights of the variables: sodium, fluoride, potassium, silica, chloride, pH, lithium, ammonium and carbonate.

Nitrates variable has a negative association with this factor, while as no association with second factor.

The second factor encompassed high factorial weights of the variables: total mineralization, conductivity, dry residue, sulphate, calcium and magnesium.

The total alkalinity and bicarbonate variables saturate on both factors reflecting the fact that these variables can be explained simultaneously by the two factors, not contributing to their orthogonality. For these reason, one might consider deleting them from the analysis or considering a non-orthogonal solution.

The separation of the different categories from the Principal Component 1–Principal Component 2 scatter point plot (Fig. 3) is presented in Table S3 (Supplementary data). The eigenvalues, percentage of variance explained and Communalities using only two components are showed in Table S4 (Supplementary data).

Figure 4 presents the separation of NMW under study based on the PCA with two components/factors. The detailed association of the different NMW in the two principal components is represented in Table S5 (Supplementary data).

When analysing Fig. 4, it can be observed a clear separation of the NMW in two clusters. All NMWs belonging to the SBS ionic profile are grouped together, thus corroborating their classification. This cluster of SBS NMW was positively influenced by component 1.

All other NMWs belonging to the remaining ionic profiles appeared grouped together with a high positive influence from the component 2.

Antimicrobial activity

Regarding the antimicrobial profile of NMWs, different microorganisms presented different susceptibility

Table 7 Detailed physicochemical composition of the codified NMWs from sulphurous/bicarbonate/sodic ionic profile, according to the analytical report obtained from the participating Thermal Centres

NMWs code	1	2	3	4	5	6	12	13	14
Ionic profile	SBS	SBS	SBS	SBS	SBS	SBS	SBS	SBS	SBS
Organoleptic features									
Odour	WO	SS	S	S	S	S	S	S	SS
Deposit	Null	Null	Null	Null	Null	Null	Null	Null	Null
Aspect	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
Colour	WC	WC	WC	WC	WC	WC	WC	WC	WC
Physicochemical constants and non-dissociated substances									
Temperature emergency (°C)	NR	NR	46.6	47.1	NR	NR	NR	46.5	34.6
pH at 20 °C	8.4	7.9	8.6	8.9	8.5	8.6	7.9	8.0	8.3
Conductivity at 20 °C (μS/cm)	303	421	544	383	538	367	404	506	466
Total sulphurization (mL I_2 0.01 N/L)	13	16	43	24	12.4	25	4.0	20	6.0
Total sulphur (mmol/L)	NR	0.22	0.33	NR	0.1	0.30	0.06	0.13	0.19
Hydrogen sulphide (mg H_2S/L)	< 0.5*	< 0.5*	< 0.5*	< 0.5*	2	< 0.04*	0.17	< 0.5*	< 0.5*
Total alkalinity (mg $CaCO_3$/L)	75.5	125	151	114	186	120	130	151	127
Total hardness (mg $CaCO_3$/L)	10	12	6.1	6.5	12	7.3	10.4	13	14
Silica (mg SiO_2/L)	55	43	60	63	33	81	55	87	52
Dry residue at 180 °C (mg/L)	226	295	383	297	87	292	282	379	322
Total mineralization (mg/L)	268	369	449	343	485	385	373	467	407
Anions (mg/L)									
Bicarbonate (HCO_3^-)	82.9	148	151	101	210	140	160	176	152
Carbonate (CO_3^{2-})	< 2*	< 2*	7	9.8	3	2.7	< 1*	< 2*	< 2*
Chloride (Cl^-)	26	32	45	27	51	26	30	54	51
Fluoride (F^-)	16	15	24	21	11	18	16	16	17
Bisulphide (HS^-)	2.2	2.7	7.1	4.0	2.0	2.9	< 0.1*	3.3	1.0
Nitrates (NO_3^-)	< 0.3*	< 0.3*	< 0.3*	< 0.3*	< 0.1*	< 0.3*	1.6	< 0.3*	< 0.3*
Nitrites (NO_2^-)	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.01*	0.05	< 0.01*	< 0.01*
Silicate ($H_3SiO_4^-$)	3.4	< 1	10	16	33	9.1	1.3	2.7	2.4
Sulphate (SO_4^{2-})	7.9	21	12	4.1	5.5	10	1.9	5.3	15
Cations (mg/L)									
Ammonium (NH_4^+)	0.08	0.05	0.70	0.36	0.19	0.36	0.072	0.43	0.12
Calcium (Ca^{2+})	3.9	4.1	2.5	2.6	4.1	2.9	4.1	4.8	5.3
Lithium (Li^+)	0.3	0.65	0.72	0.33	0.31	0.719	0.875	1.5	1.0
Magnesium (Mg^{2+})	0.15	0.35	< 0.1*	< 0.1*	0.35	0.013	0.047	0.18	0.11
Potassium (K^+)	2.0	2.8	4.4	2.8	1.8	3.3	2.3	4.4	2.4
Sodium (Na^+)	67	99	125	91	130	88	97	111	108
Iron (Fe^{2+})	< 0.01*	< 0.01*	< 0.01*	< 0.01*	< 0.025*	< 0.002*	< 0.01*	< 0.01*	< 0.01*

SBS sulphurous/bicarbonate/sodic, *SS* sulphur (low intensity), *S* sulphur, *WO* without odour, *WC* without colour, *NR* data not reported on the analytical sheet

*Result value below the method's quantification limit

Table 8 Detailed physicochemical composition of the codified NMWs from sulphurous/bicarbonate/fluoridated/sodic, bicarbonate/sodic, chlorinated/sodic, bicarbonate/magnesium, sulphated/calcic and sulphurous/chlorinated/sodic ionic profiles, according to the analytical report obtained from the participating Thermal Centres

NMWs code	7	9	10	11	15	8	16
Ionic profile	BS	BM	SC	SCS	SBFS	CS	CS
Organoleptic features							
Odour	WO	WO	SS	NR	S	NR	WO
Deposit	Null	Null	Null	NR	Null	NR	Null
Aspect	Clear	Clear	Clear	NR	Clear	NR	Clear
Colour	WC	WC	WC	NR	WC	NR	WC
Physicochemical constants and non-dissociated substances							
Temperature emergency (°C)	28.6	NR	19.3	34.8	NR	NR	27
pH at 20 °C	5.6	6.3	7.2	6.9	9.5	4.6	5.2
Conductivity at 20 °C (μS/cm)	< 74*	217	2200	3948	206	41.8	48.1
Total Sulphurization (mL I_2 0.01 N/L)	NR	NR	NR	57.06	7.9	NR	NR
Total Sulphur (mmol/L)	NR	NR	NR	NR	0.18	NR	NR
Hydrogen sulphide (mg H_2S/L)	NR	NR	NR	NR	< 0.5*	NR	< 0.04*
Total alkalinity (mg $CaCO_3$/L)	< 20*	72.1	210	NR	60.5	< 0.8*	3.5
Total hardness (mg $CaCO_3$/L)	9	80	1700	NR	7.7	NR	7
Silica (mg SiO_2/L)	23.7	8	9.8	18	33	10.1	10
Dry residue at 180 °C (mg/L)	42	118	2200	NR	162	27	35
Total mineralization (mg/L)	57.2	169	2130.8	2989.6	191	26.9	36
Anions (mg/L)							
Bicarbonate (HCO_3^-)	9.86	87.8	250	314.6	51.3	< 1*	4.3
Carbonate (CO_3^{2-})	0	< 2*	< 3*	NR	2.5	< 2*	< 1*
Chloride (Cl^-)	< 15*	19	27	1006.8	6.9	7.3	9.9
Fluoride (F^-)	0.04	0.2	0.79	1.46	9.8	< 0.1*	< 0.1*
Bisulphide (HS^-)	NR	NR	NR	4.1	1.3	NR	NR
Nitrates (NO_3^-)	< 5*	< 0.3*	0.15	0.31	< 0.3*	1.5	1.6
Nitrites (NO_2^-)	< 0.04*	< 0.01*	< 0.01*	NR	< 0.01*	< 0.01*	< 0.01*
Silicate ($H_3SiO_4^-$)	37.5	< 1*	< 2*	NR	23	< 1*	< 1*
Sulphate (SO_4^{2-})	1.33	9.6	1200	NR	13	2.2	1.2
Cations (mg/L)							
Ammonium (NH_4^+)	< 0.05*	< 0.05*	0.05	0.35	0.11	< 0.05*	< 0.05*
Calcium (Ca^{2+})	1.32	4.2	560	270.2	3.1	0.44	0.66
Lithium (Li^+)	0.0045	< 0.1*	0.026	0.04	0.16	< 0.1*	< 0.1*
Magnesium (Mg^{2+})	1.48	17	60	57.0	< 0.1*	0.72	1.3
Potassium (K^+)	0.69	1.4	2.0	5.0	0.9	0.39	0.37
Sodium (Na^+)	2.61	13	21	660.1	46	4.2	6.3
Iron (Fe^{2+})	0.005	9	< 0.025*	NR	< 0.01*	< 0.03*	< 0.01*

BS bicarbonate/sodic, *BM* bicarbonate/magnesium, *CS* chlorinated/sodic, *SC* sulphated/calcic, *SCS* sulphurous/chlorinated/sodic, *SBFS* sulphurous/bicarbonate/fluoridated/sodic, *SS* sulphur (low intensity), *S* sulphur, *WO* without odour, *WC* without colour, NR, data not reported on the analytical sheet

*Result value below the method's quantification limit

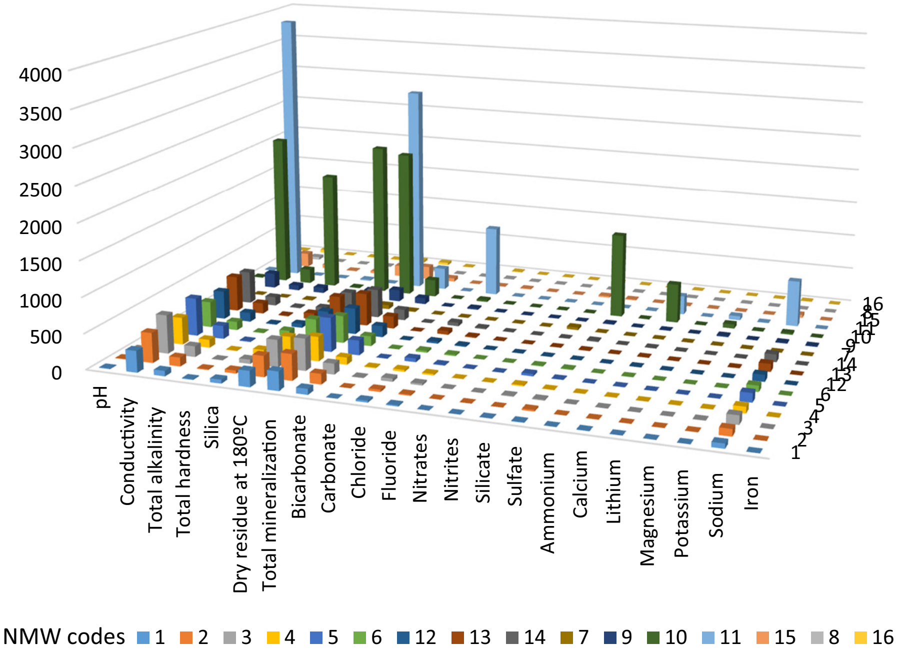

Fig. 2 Total fraction for the physicochemical parameters belonging to the chemical groups studied (physicochemical constants and non-dissociated substances, anions and cations), for all NMWs. All variables with exception of pH and conductivity (µS/cm) are expressed in mg/L

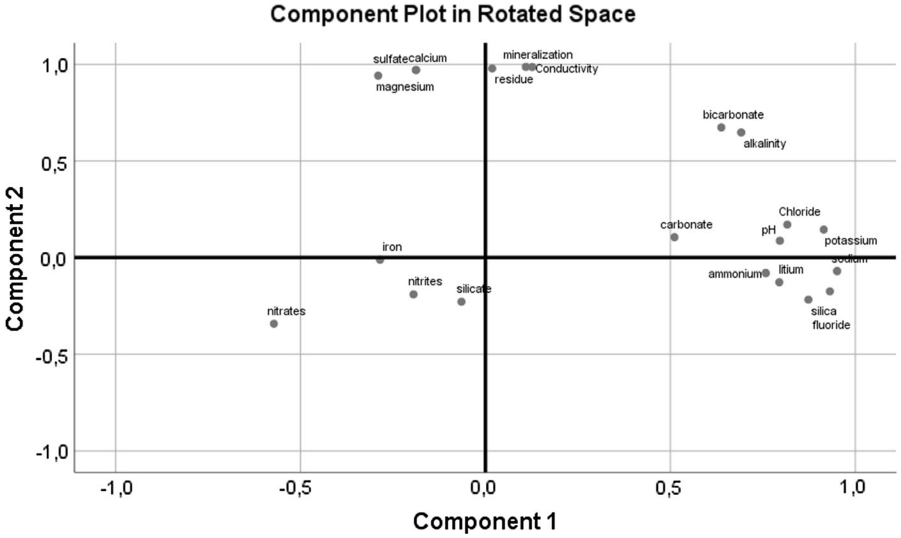

Fig. 3 Factor plot illustrated for two components of the physicochemical parameters with all variables included in absolute value. The PCA retains 69.432% of the variance in the two components

profiles when exposed to NMWs (Fig. 5a–f), as depicted in Table 9.

Regarding the aerobic gram-positive *S. aureus*, an overall increase in growth was observed after exposure to NMWs. Interestingly, all hyposaline mineral waters increased *S. aureus* bacterial growth. Three NMWs belonging to different ionic profiles (NMWs 12 and 13 from sulphurous/bicarbonate/sodic and 10 from sulphated/calcic profiles) decreased the growth of this bacteria thus suggesting that a different mechanism apart from the major chemical elements may be implicated.

Regarding *S. epidermidis*, an overall decrease in bacterial growth was found across different ionic profiles. All NMWs with a sulphuric and/or sodic basis, except NMW 3, caused a 10–35% decrease in bacterial growth. Three of the 16 NMWs studied (NMWs 7 from bicarbonate/sodic, 9 from bicarbonate/magnesium and 11 from sulphurous/chlorinated/sodic profiles) increased bacterial growth by about 30%.

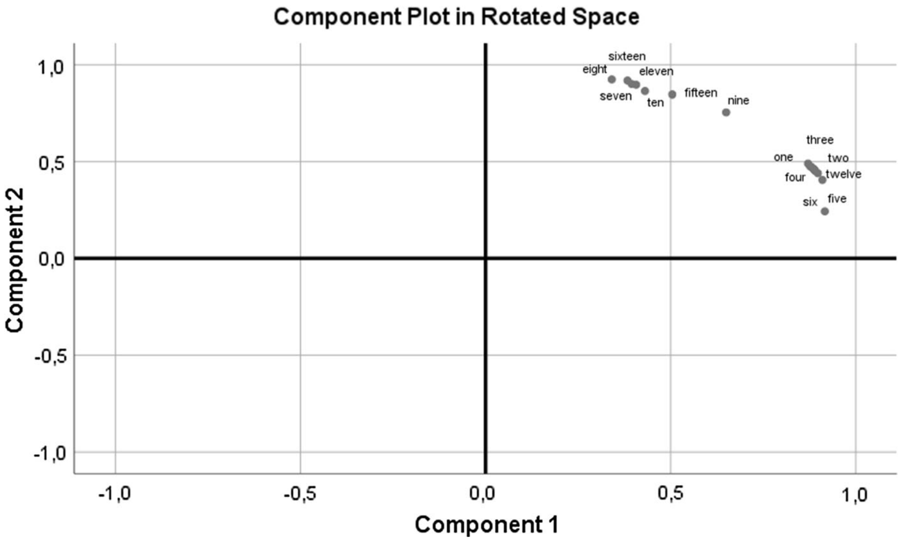

Fig. 4 Factor plot illustrated for two components of the NMW with all variables included in absolute value

Corynebacterium amycolatum presented an interesting susceptibility profile since NMWs with the highest and lowest mineralization values caused an increase in bacterial growth. Despite two sulphurous-based NMWs producing a slight decreased bacterial growth (≈ 25%, NMWs 3 and 4 from sulphurous/bicarbonate/sodic group), no effect was observed with the remaining NMWs belonging to this ionic group.

The anaerobic gram-positive bacteria *C. acnes* was the most susceptible microorganism tested towards NMWs. As we can observe in Fig. 5, all poorly mineralized and hypersaline NMWs decreased bacterial growth by 10% (NMW 11) until a remarkable 65% (NMW 3). Interestingly, none of the hyposaline NMWs induced alterations in bacterial growth.

Regarding the pathogenic bacteria *E. coli*, the NMWs 3, 4 and 5 from the sulphurous/bicarbonate/sodic profile, NMWs 8 and 16 from the chlorinated/sodic group and the sulphated/calcic NMW 10 decreased bacterial growth. Furthermore, six analysed NMWs did not affect bacterial proliferation. However, four NMWs from three distinct ionic and mineralization types (NMWs 1 and 2 from bicarbonate/sodic group and NMWs 7 and 11 belonging to the bicarbonate/sodic and sulphurous/chlorinated/sodic groups, respectively) increased bacterial growth.

Finally, growth of the opportunistic skin pathogen *C. albicans* was not significantly altered in most of the waters tested. Five NMWs were able to impair fungal growth, in particular NMW 10 with an inhibition rate of 45%. Nevertheless, two NMWs belonging to different ionic profiles, bicarbonate/sodic and bicarbonate/magnesium groups, albeit with similar mineralization types, enhanced *C. albicans* growth by approximately 20%.

Discussion

NMWs are complex natural elements composed of a wide range of chemical elements and physicochemical parameters that may underlie their beneficial therapeutic applications and specific physiological activities.

Until now, only a limited number of studies focused on the antimicrobial activity of NMWs against specific bacteria and fungi that are associated with different skin conditions. As far as we are concerned, this is the first study describing the antibacterial and antifungal activity of Portuguese NMWs.

A study performed by Akiyama and collaborators showed antimicrobial effects of acidic hot-spring water from Japan against *S. aureus* strains isolated from atopic dermatitis patients, mainly due to the acidic pH (pH 2) of the tested waters (Akiyama et al. 2000). Interestingly, in our study, NMWs with more acidic pH (NMWs 8, 16 and 7; pH 4.6–5.6) did not follow the same pattern since a significant increase in bacterial growth of some microorganisms, particularly *S. aureus*, was observed. Other studies have already described that pH itself may not be directly responsible for alterations in microbial growth and other parameters as physicochemical characterization of the NMWs should be considered (Inoue et al. 1999).

Moreover, all NMWs from the hyposaline mineralization group enhanced the growth of *S. aureus*, which may indicate an influence of total mineralization in the growth of these bacteria.

In fact, by the multivariate analysis performed, all hyposaline waters that enhanced *S. aureus* growth are grouped together and are positively influenced by component 2, which includes the variable total mineralization (Figs. 3 and 4).

In addition, a majority of NMWs classified as 'sodic' increased *S. aureus* growth. Its relatively higher resistance to adverse conditions, in addition to its ability to grow in higher concentrations of sodium, may justify the worst antimicrobial profile in comparison with the other bacteria tested (Abu-Ghazaleh 2016).

Relatively to *S. epidermidis*, *C. acnes* and *C. albicans,* an inhibition of their growth was observed by NMWs with a sulphurous/bicarbonated and sodic composition, with *S. epidermidis* and *C. acnes* showing higher susceptibility.

Considering Figs. 3 and 4, all NMWs belonging to the sulphurous/bicarbonate/sodic ionic profile appeared grouped together and are positively influenced by component 1, specifically by the variables chloride, potassium and carbonate. Therefore, these variables may be related to the antimicrobial effect against these specific microorganisms.

Also hydrogen sulphide (H_2S), the active molecule in sulphurous mineral waters, is currently showing potential therapeutic applications (Carbajo and Maraver 2017). H_2S seems to act as a cellular regenerator, having keratoplastic or keratolytic as well as antioxidant, antibacterial and antifungal activities (Carbajo and Maraver 2017). In addition, it has been reported that sulphur may interact with oxygen radicals, producing sulphur and disulphur hydrogen, which may be transformed into pentathionic acid ($H_2S_5O_6$), and this element may be the source of the antibacterial and antifungal activity of sulphurs waters (Matz et al. 2003). It is also known that the sulphuric waters can be effective in improving symptoms of some dermatological conditions (Matz et al. 2003). Specifically, when considering their detergent property along with antimicrobial and keratolytic effects, these sulphuric waters can be used on oily and combination skin to remove excess sebum and to treat mild acne (Nunes and Tamura 2012).

Accordingly, our results with *C. acnes* showed a high susceptibility profile, particularly with NMWs with a sulphurous background, corroborating this hypothesis. Unfortunately, physicochemical parameters related to sulphur (total sulphurization, total sulphur and hydrogen sulphide) were not reported for a large number of NMW and therefore were not included in the mathematical analysis performed, and conclusions could be drawn regarding this chemical element.

Analysing the results from a specific NMW perspective, NMWs 7 and 9 from the bicarbonate/sodic and bicarbonate/magnesium groups produced an overall positive impact on microbial growth since these NMWs induced the growth of the majority of the microorganisms tested, with exception of *C. acnes*. The effect of iron in bacterial growth is well established in the literature as this metal is an essential requirement for bacteria proliferation (Patruta and Hörl 1999; Cross et al. 2015). This could explain the effect of NMW 9, which has a high ferric composition, and produced a significant increase in bacterial and fungal growth in five of the six microorganisms tested. For NMW 7, although showing similar effects, no direct association with its physicochemical content can be drawn.

Relatively to NMW 10, from the sulphated/calcic ionic profile, it seems that this particular NMW reduced microbial growth in all strains tested, apart from *C. amycolatum*.

Although being clustered with all NMWs influenced by component 2 (Figs. 3 and 4) it is the only NMW presenting higher levels of sulphate (Fig. 2), thus possibly explaining the different effect of this NMW relatively to the other NMWs grouped in this cluster.

Finally, NMW 3 had the ability to inhibit bacterial and fungal growth, specifically *E. coli*, *C. amycolatum*, *C. acnes* and *C. albicans* with no effect against *Staphylococci* growth. This may be an interesting result since both *Staphylococci* bacteria belong to normal epithelial microflora with *S. epidermidis* having a probiotic function by preventing colonization of *S. aureus* (Duguid et al. 1992). Therefore, a non-alteration on this bacteria–bacteria relation may be beneficial. Interestingly, NMW 3 has higher levels of sulphur-related parameters (total sulphurization, total

Fig. 5 Graphical representation of microbial growth after exposure to different NMWs at 37 °C during 24 h for aerobic bacteria (**a**–**c**); 48 h for *C. amycolatum* (**d**) and *C. albicans* (**e**); and 72 h for *C. acnes* (anaerobic bacteria) (**f**). All values were expressed as percentage of the control prepared with MilliQ-type water, which was taken as 100% and represented by intermittent black line; *$p < 0.05$. All analyses were conducted using GraphPad Prism (version 7.03 for Windows, GraphPad Software, La Jolla California USA)

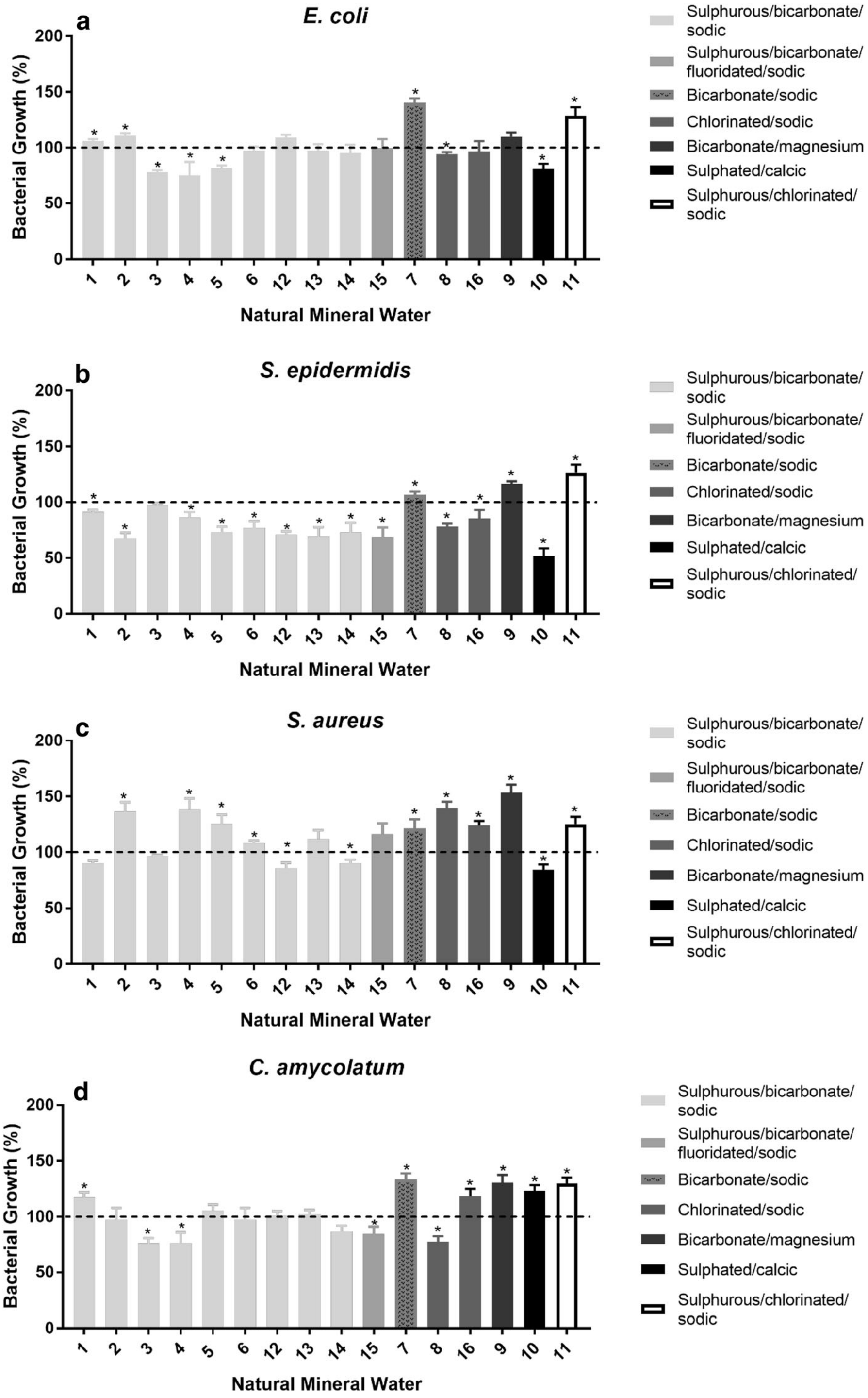

Fig. 5 continued

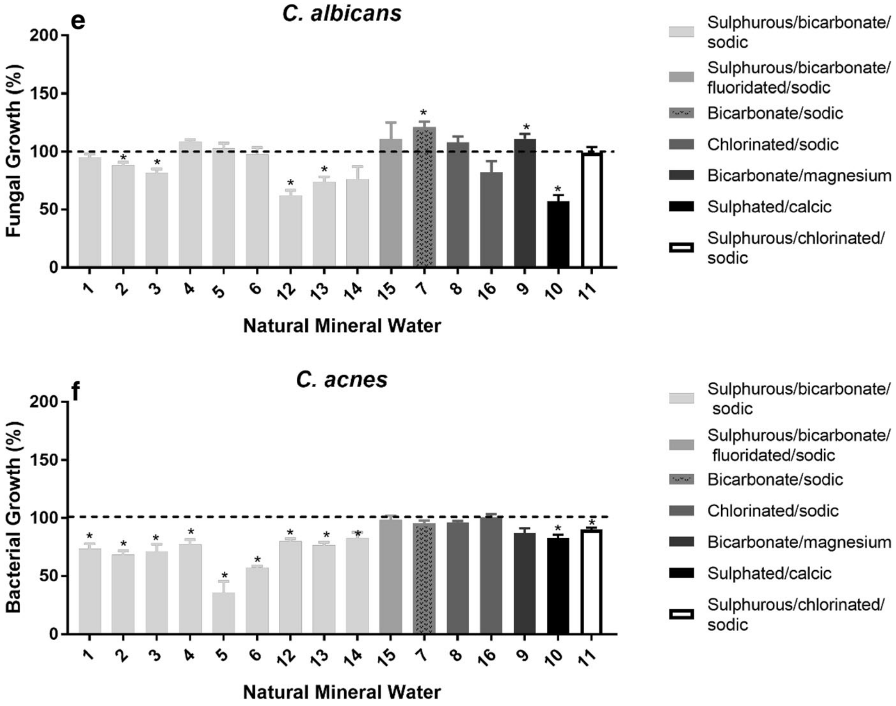

Table 9 Schematic representation of microbial growth changes after exposure to different NMWs

Classification		NMWs	Species tested					
Mineralization	Ionic profile	Code	*S. aur*	*E. coli*	*C. amy*	*S. epi*	*C. acn*	*C. alb*
Poorly mineralized	Sulphurous/bicarbonate/sodic	1	–	↑	↑	↓	↓	–
		2	↑	↑	–	↓	↓	↓
		3	–	↓	↓	–	↓	↓
		4	↑	↓	↓	↓	↓	–
		5	↑	↓	–	↓	↓	–
		6	↑	–	–	↓	↓	–
		12	↓	–	–	↓	↓	↓
		13	–	–	–	↓	↓	↓
		14	↓	–	–	↓	↓	–
Hyposaline	Sulphurous/bicarbonate/fluoridated/sodic	15	↑	–	↓	↓	–	–
	Chlorinated/sodic	8	↑	↓	↓	↓	–	–
		16	↑	↓	↑	↓	–	–
	Bicarbonate/sodic	7	↑	↑	↑	↑	–	↑
	Bicarbonate/magnesium	9	↑	–	↑	↑	–	↑
Hypersaline	Sulphated/calcic	10	↓	↓	↑	↓	↓	↓
	Sulphurous/chlorinated/sodic	11	↑	↑	↑	↑	↓	–

S. aur, Staphylococcus aureus; E. coli, Escherichia coli; C. amy, Corynebacterium amycolatum; S. epi, Staphylococcus epidermidis; C. acn, Cutibacterium acnes; C. alb, Candida albicans

↓ means decreased growth; ↑ means increased growth; – marks no effect on microorganism sulphur and bisulphide) than the other NMWs from the same group. Additionally, NMW 3 has a higher level of fluoride than the other NMWs studied. The antimicrobial activity of fluoride has already been described through its interference with uptake and degradation of polysaccharides by the bacterial cell in addition to reducing the ability of the cell to maintain pH homoeostasis and could play a role, in addition to other intrinsic characteristics of these NMWs (Van Loveren 2001; Nouri and Titley 2003).

Conclusions

Different NMWs presented different antimicrobial profiles against several skin microbiota, with most data supporting a role played by the physicochemical composition. NMWs parameters like pH had no direct influence on microbial growth, suggesting that specific ionic composition could be involved.

In general, all NMWs that grouped together by the principal component analysis, and that were positively influenced by chloride, potassium and carbonate (belonging to SBS ionic profile), have an overall beneficial or null effect on microbial growth, presenting some degree of antimicrobial activity against one or more microrganisms enrolled.

Regarding the second cluster of NMW that grouped together, positively influenced by component 2 (including the variables total mineralization, dry residue and conductivity), it becomes clear that, despite belonging to different ionic classifications, the majority of these NMWs presented an opposite effect on bacterial growth, increasing the microbial growth of the tested microorganisms.

Assessment of correlation between a specific therapeutic indication and the water content in cations and anions was difficult as indicated by other authors (Araujo et al. 2017). A specific effect can be due to the water as a whole (or a combination of elements) rather than to a particular chemical element (Araujo et al. 2017). The same principle may be present in our study regarding the waters' antimicrobial activity.

Even though communicable diseases (bacterial, viral, fungal and parasitic infections) are presented by the Thermal Centres as temporary contraindications to the use of the facilities (Hernández Torres et al. 2006), NMWs exhibited antimicrobial susceptibility profiles, some of them with clinical interest. Therefore, the applications of Portuguese NMWs in a domestic environment, formulated as cosmetics or even medical devices, for treatment of acne or other skin conditions, seem to be an interesting approach to apply the added value of these important endogenous resources.

Acknowledgements The authors would like to acknowledge all Thermal Centres involved in the project and the financial support provided by FEDER funds through the POCI—COMPETE 2020—Operational Programme Competitiveness and Internationalisation in Axis I—Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491) and National Funds by FCT—Foundation for Science and Technology (Project UID/Multi/00709/2013), Provere Termas Centro—Projeto Âncora de Inovação, co-funded by Centro 2020, Portugal 2020 and European Union funds.

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