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Resistance to space simulating conditions and sporicidal treatments of spores from bacilli of extreme environments origins: implication for Astrobiology

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La Natura è un tempio ove pilastri viventi lasciano sfuggire a tratti confuse parole; l'uomo vi attraversa foreste di simboli, che l'osservano con sguardi familiari.
Come lunghi echi che da lungi si confondono in una tenebrosa e profonda unità, vasta come la notte e il chiarore del giorno, profumi, colori e i suoni si rispondono.
Vi sono profumi freschi come carni di bimbo, dolci come òboi, verdi come prati – altri, corrotti, ricchi e trionfanti,
che posseggono il respiro delle cose infinite, come l'ambra, il muschio, il benzoino e l'incenso;
e cantano i moti dell'anima e dei sensi.

Charles Baudelaire

Sommario

Abstract

Microorganisms able to tolerate environmental extremes, or extremophiles, are ideal candidates to extend our knowledge on the limitations for terrestrial life, including sporicidal treatments, and on their ability to survive under conditions mimicking space environments.

The spore resistance of bacilli isolated from extreme environments, cold (Antarctic soils) and hot (shallow hydrothermal vents of Eolian Islands, Italy), was evaluated towards environmental stressors (wet- and dry-heat, low and high pH values), sporicidal treatments and stresses simulating space-environments (UV-C and X-rays irradiations, desiccations by low pressure, exposition to oxidizing agents and low pressure plasma), as those from the planet surface of Europa (the satellite of Jupiter), Enceladus (the satellite of Saturn) and Mars.

Spores from two thermophilic Eolian strains, *Bacillus horneckiae* SBP3 (SBP3) and *B. licheniformis* T14, and a psychrophilic Antarctic strain, *B. simplex* A43, were frequently the most resistant against simulating space conditions as UV-C, X-ray and heavy ions radiation. Despite belonging to the same species, spores from strains *Bacillus horneckiae* SBP3 and *Bacillus horneckiae* DSM 23175^{T} responded differently to stresses, which may reflect the adaptation to harsh environmental conditions of the Eolian thermophilic strain. Additional comparisons were made with reference strains used in Astrobiology studies, isolated from spacecraft assembly facilities (*B. horneckiae*, *B. nealsonii* and *B. pumilus* SAFR032), and the biodosimetry strain and space microbiology model organisms *B. subtilis* 168.

Spores of *B. horneckiae* SBP3 were able to survive stressing conditions (exposition to heat, acid and alkaline stresses, UV-C, X-rays, heavy ions, low pressure plasma, desiccation and hydrogen peroxide), often at higher degree than those of *B. subtilis*. The results obtained by Raman spectroscopy showed that the damages induced by heat stress to spores are related to the denaturation of proteins, rather than to those of nucleic acids. This finding suggests, that the mechanisms involved to contrast the heat stress are probably related to the thermostability of small-acid soluble proteins in spore-core.

The resistance of spores to terrestrial environmental stressors could also be determinant in resisting to space stressors (as UV-C and X-rays radiations, exposure at temperature 95 and 130°C and desiccation stress). Moreover, exopolysaccharides structure and composition, involved in the biofilm formation, could be also considered as a mechanism to protect spores under stress conditions.

As bacterial multi-resistant forms, the studied spores are expected to possess novel, unexplored applicative potentials in different contexts, such as new bio-indicators for safety and security in human activities, as well as in Astrobiology, in space exploration missions and “Planetary Protection” programs.

1. Introduction

The presence of liquid water is considered a necessary condition for terrestrial and extra-terrestrial life, since it is a solvent for life as we know it, and also it is important either as a reactant or product in most metabolic processes (Rothschild and Mancinelli, 2001; Marion et al., 2003). However, the absence of water in liquid form is not the only limitation for life in the Universe. The bioavailability of water greatly depends on physical and chemical parameters, such as temperature and salinity. Moreover, other limiting life factors, such as UV irradiation (especially the UV-C) and the cosmic radiations, should be taken into consideration due to their destructive effects on DNA.

The main objective of Astrobiology is to investigate the origin, evolution and distribution of life in the Universe and to search forms of life able to survive under extra-terrestrial conditions (Horneck et al., 2010). As a consequence, astrobiological research requires the study of a variety of environments under different conditions, as those of hot, cold, acidic or alkaline terrestrial environments, as analogous to those of the main astrobiological targets, as Mars, Europa (satellite of Jupiter) and Enceladus (satellite of Saturn).

Microorganisms able to tolerate environmental extremes, or extremophiles, are ideal candidates to extend our knowledge on the limitations for terrestrial life due to their ability to survive also to conditions that mimicking space environments. Therefore, the studies on these microorganisms in space environments aim to understand the influence of gravity or radiations at cellular or subcellular levels, and the likelihood of interplanetary transport of microorganisms via meteorites. On the other hand, some applications perspectives are oriented in the use of microorganisms in bioregenerative life support systems, the monitoring, characterization, and control of spacecraft microflora, and associated microbial crew health concerns. Indeed, the control of microorganisms especially bacteria on the spacecraft pose high demands on the microbiological research community and set up the procedures to avoid contamination from terrestrial bacteria of other celestial bodies. In this field the research aims to

validate the decontamination procedures against the most resistant form of microbes as bacterial spores.

1.1 Antarctic soil at Edmonson point

Edmonson Point (Northern Victoria Land, Antarctica) is located at the foot of Mount Melbourne, the active volcano close to the Mario Zucchelli Station (MZS) (Fig. 1).

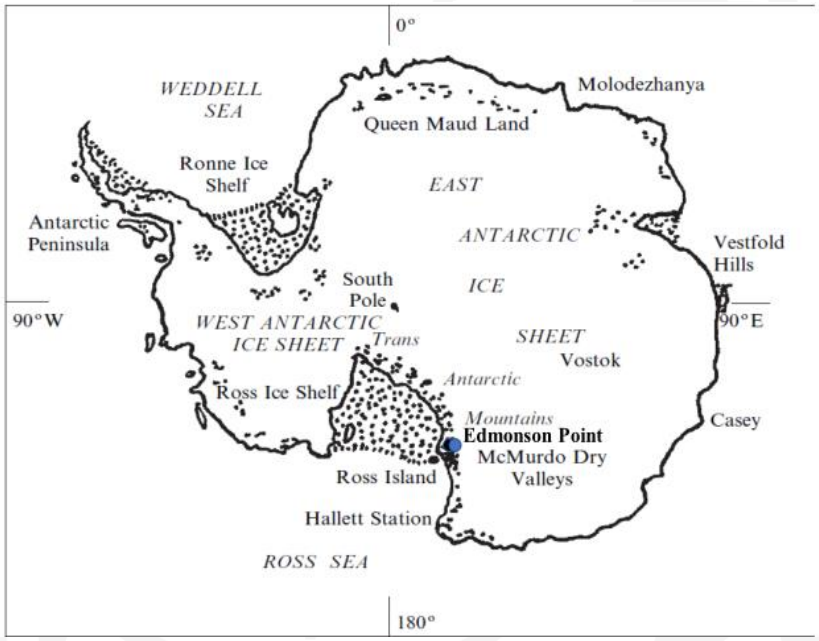


Fig.1 Location map with areas of ice-free ground (from Campbell and Claridge, 2006)

This area is characterized by ground water that flows through the surface-active layer, allowing the development of mosses in patches (Papale et al., 2018). The lithosphere in this site is mainly composed by basalts with slightly low contents of Al, Fe, and Ca (Cannone et al., 2008). Therefore, the black color of the basalts induces the melt earlier of the snow patches and the volcanic ash outcropping absorbed large amount of solar radiations (Cannone and Guglielmin, 2009). The ice surface is strongly influenced by seasonally fluctuations in temperature, and also by more radiations exposure than deeper layers (Steven et al., 2006).

Although different form of life including mosses, lichens, yeasts and algae were recognized, the psychrophilic prokaryotes dominate microbial communities in this

cold environment, where they play a relevant role in the functioning of the Antarctic ecosystem (Hinzman et al., 2003; Papale et al., 2018; Steven et al., 2009).

Adaptative mechanisms of Antarctic Bacteria thriving at low temperatures principally involve membrane modifications and enzymatic activities (Shivaji and Prakash, 2010; Singh et al., 2014). At low temperatures the membrane fluidity depends on the fatty acid composition wherein the unsaturated fatty acids, short chained and branched fatty acids are synthesized in response to low temperature (Chintalapati et al., 2004). Further, the cold inducible desaturases that convert saturated fatty acids to unsaturated fatty acids are also responsible for adaptation of Antarctic bacteria. In addition, the role of cis-trans isomerase has been reported as implicated in the maintaining the membrane fluidity in Antarctic bacteria (Kiran et al., 2005). As reported for *Pseudomonas syringae*, at low temperature the enzymatic phosphorylation of lipopolysaccharides allows to modulate the permeability of the outer membrane (Gerday et al., 2000; Metpally and Reddy 2009).

The pigmentation is a common phenomenon in Antarctic bacteria. Pigments influence the bacteria growth under freeze–thaw conditions, and therefore they are suggested to be involved into the stress adaptation to low temperatures (Jaganadham et al., 2000; Mata-Gómez et al., 2014). Carotenoid pigments can give rigidifying effects, in order to regulate the fluidity and stabilize the cell membrane (Gruszecki et al 2005).

New insights in cold adaptation mechanisms of psychrophiles will be provided in the next future by comparative genome analyses of specific genes, as well as by the comparative evaluation of genes expression under the exposure to a combination of different stresses (high salt, high and low pH) (Shivaji -et al., 2017).

1.2 Shallow hydrothermal vents of Eolian Islands

Marine hydrothermal systems (shallow and deep-sea vents) are considered extreme environments, since they are characterized by high temperature and unusual conditions (high concentrations of H_2S, hydrocarbons, heavy metals) for most organisms (Caccamo et al., 2000; Maugeri et al., 2009, 2010; Gugliandolo et al., 2012). The vents area off the Eolian Islands (Italy) presents sites easy to reach to isolate novel

thermophiles and to provide new insights into microbial diversity (Caccamo et al., 2000; Gugliandolo et al., 2012; Spano` et al., 2013). Since these sites have parallels to primordial conditions, microorganisms adapted to live in extreme conditions could help to gain new knowledge for understanding the environmental constraints for life, with broader implications to the field of Astrobiology, which investigates the possibility that life exists beyond Earth. Among these sites the shallow submarine hydrothermal vents off the Eolian Islands of Vulcano and Panarea have been known to be active, since historical times, although only recently have scientific studies reported the presence of deep-located emissions (up to 800 m depth) off the eastern coast of Vulcano Island (Maugeri et al., 2010).

1.2.1 Shallow vents off Vulcano Island

Vulcano Island is located in the south of Eolian volcanic complex, near the Tindari-Letojanni fault a structure cuts the northeastern sector of Vulcanello and the southwestern lava domes of Lipari (Favalli et al., 2005). The island is characterized by the still active "La Fossa" cone, developed during the last 6 km inside the "Caldera della Fossa", affected by fumarolic activity (Revil et al., 2008).

Tab 1 Analytical data of hydrothermal fluids from selected sites of the Eolian Islands. Vulcano Island (Italy). Gas concentration in mLgas L^{-1} seawater (Maugeri et al., 2010)

Site	Depth (m)	T°C	pH	CO_2	H_2S	CH_4	N_2	O_2
Vulcano Is.								
Levante Harbour	6	32–38	4.4–6.3	nd	bdl-8.4	nd	nd	bdl-5.7
Levante Harbour	2	35	6.11	1010	1.8	nd	nd	0.60
Levante Harbour	0.8	60	5.75	992	5.1	nd	nd	0.20
La Roja	3	49	6.03	582	0.01	0.004	7.24	0.75
P. Conigliara	15	45	6.09	1492	bdl	0.001	6.32	0.24
Reference site	Sea level	15	8.10	0.24	bdl	bdl	9.60	4.80

The chemical characteristics of the Vulcano shallow vents (Table 1) were similar to those reported for hydrothermal solutions from deep-sea oceanic vents, showing that similar processes occur at hydrothermal vents independent of their location (Caracausi, et al., 2005; Italiano and Nuccio 1991). Elemental sulphur, varying in colour from white to yellow to orange, is a common mineral found in sediments around the vents.

From the thermal springs of Vulcano Island, new thermophilic and hyper-thermophilic microorganisms belonging to the Bacteria and *Bacillus* genus, and of interest in both pure and applied research have been isolated (Table 2).

Tab 2 *Bacillus* spp. collected from shallow hydrothermal vents of Vulcano Island

Strain	Reference
Bacillus aeolius	Gugliandolo *et al* . 2003
B. licheniformis B3-15	Maugeri *et al*. 2002
Geobacillus sp 1bw	Maugeri *et al*. 2002
Geobacillus sp 5-2	Maugeri *et al*. 2002
Geobacillus sp 10-1	Maugeri *et al*. 2002
G. vulcani	Caccamo *et al* . 2000

The bacteria that inhabited these sites showed specific nutritional pathways and they are adapted to stress conditions of the high temperature habitat (Gugliandolo and Maugeri, 1998; Maugeri et al., 2001). Previous studies have been characterized the microbial community composed by different autotrophic (phototrophic and chemo-lithotrophic) bacterial populations which show a wide range of temperature tolerance.

1.2.2 Panarea Island

The Panarea Island represents a small emerged volcanic system formed, as the other islands, about 180–200 ka ago. on a large platform at a depth of about 100 m (Favalli et al., 2005). The most active submarine hydrothermal system of the Eolian islands is

located in different vents off Panarea Island (Italiano and Nuccio 1991). Together with several islets (Dattilo, Bottaro, Lisca Bianca and Lisca Nera), to the east of the main island, Panarea forms a small archipelago that emerges from a nearly circular submarine platform with a shelf break at a water depth of 130 meters. This surface represents the flat summit of a submarine stratovolcano that rises more than 1200 meters above the surrounding seafloor and has a basal diameter of 20kilometers.

Since, November 2002 there was increasing interest toward the area around the group of islets due to the great exhalative event. Despite the numerous and detailed information existing on the geomorphologic features of the Panarea Volcanic

Complex, the investigations on this submarine hydrothermal system are still ongoing with the aim of better constraining the hydrothermal system (Esposito et al 2018). The sites near the islets cited above are characterized by unusual and unique combination of physical chemical characteristics resumed in Tabble3.

Tab.3 Physico-chemical characteristic of Panarea vents (from Gugliandolo et al 2012)

Site	Depth (m)	Temperature (°C)	pH	Conductivity (mS cm^{-1})
Black point	23.0	130	3.30	46.20
Bottaro	8.0	55	5.42	42.90
Campo 7	21.3	60	4.92	49.20

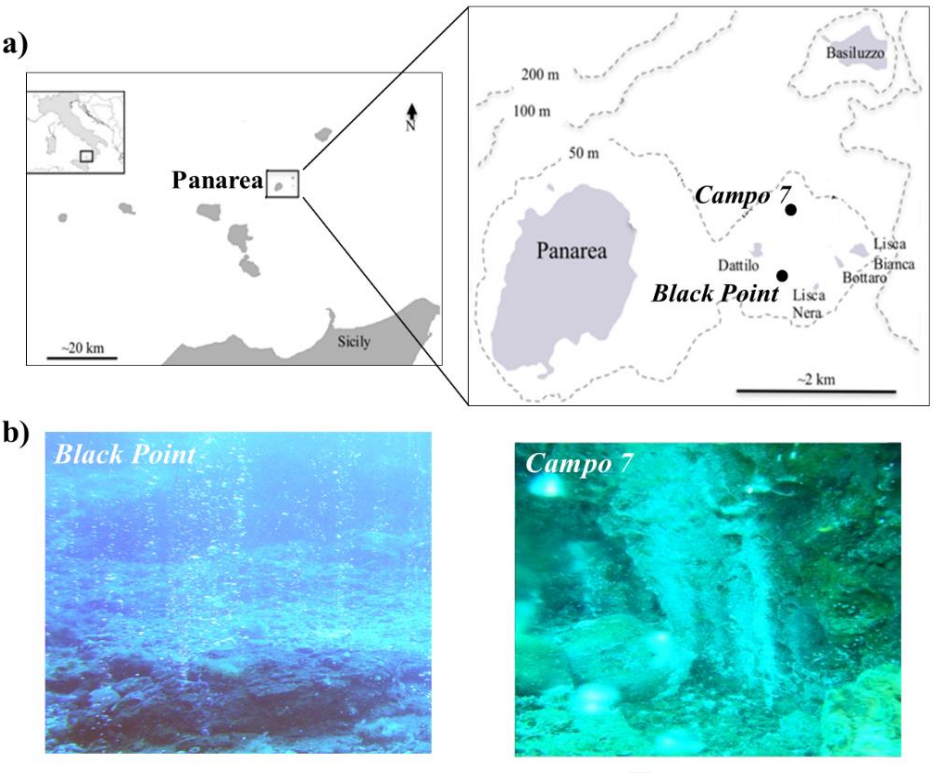


Fig. 2 Sampling sites' location and vents emissions at Black Point (latitude 38°38'23''N–longitude 15°06'28''E) and Campo 7 (latitude 38°37'59''N–longitude 15°06'59''E) respectively, located off the Panarea Island (Eolian Islands, Italy)

The Panarea volcanic complex is the most tectonically active of the Eolian Archipelago and shallow hydrothermal vents constitute excellent natural field for investigating the effects of high CO_2 and temperature, and low pH on marine microorganisms. These sites evoke primordial conditions and at the same time represent model to study the effects of the acidification and temperature increasing.

1.3 Habitability of celestial bodies

The search for evidence of extraterrestrial life is a key motivation for astrobiology. Because the liquid water is the *sine qua non* of life on Earth, and arguably any life in our Solar the first guiding principle in this search has been ‘‘follow the water.’’ The information from spacecraft exploration indicated that liquid water is present now, or was in the past, on many worlds in our Solar System (McKay et al., 2014).

Europa is a Jupiter satellite with 1,300 km in diameter and therefore smaller than the Earth’s Moon (Chela-Flores et al., 2015) it is characterized by the presence of an ocean (Kivelson, 2000; Chela-Flores et al., 2015), cracked surface, indicating the presence of tectonic processes, and by the warmer material existing underneath and a cold crust in surface (Harada, 2006). In addition, the lack of impact craters on the surface would imply active geology (Greenberg, 2002). Due to this internal heating it is believed to the presence of liquid water on the moon and the hydrothermal vents on the ocean floor of Europa (Greenberg, 2010) that could play a part in sustaining life on the moon (Chela Flores et al.,2015)

Moreover, the spectroscopic data performed by the Galileo spacecraft and indicated the possible presence of hydrated salts (Vance et al., 2016; Zolotov and Shock, 2001). These data could be representative of both primordial and present ocean of Europa with liquid water characterized by a pH 10-12, which may then be diluted to a pH 8-9 (Zolotov, 2004). The ocean of Europa may also be salty, and could contain NaCl (Prieto-Ballesteros et al., 2011). The presence of a briny/alkaline ocean on Europa provides another possible habitat for life beyond the Earth (Marion et al., 2003). The mass in solid particles is explicable only if they are frozen droplets of liquid water

(Porco et al., 2006). Their salinity, comparable to that of Earth's oceans, strongly suggests a water reservoir in contact with a rocky core (Postberg et al., 2011). The correspondence between individual jets and small-scale hot spots points to condensation of vapor and liquid as the source of this thermal emission (Porco et al., 2017). Enceladus' subsurface sea was likely produced geothermally, by tidally induced flexure, during a much earlier epoch of higher orbital eccentricity (Behounkova´ et al., 2012). Geothermal flows on Earth often contain redox pairs, such as H_2 and CO_2, that can be the basis for biological processes (Chapelle et al., 2002); by analogy, this has been suggested for Enceladus (McKay et al., 2014). Both H_2 and CO_2 are present in the plume (Waite et al., 2009).

The Cassini orbiter's Visual and Infrared Mapping Spectrometer revealed a surface, predominantly of water ice, with simple organics and CO_2 in association with fractures in the south pole (Brown, 2006). Cassini Composite Infrared Spectrometer identified that the southern polar region is anomalously warm (Spencer et al., 2006). Cassini's Ultra-Violet Imaging Spectrograph confirmed the existence of water vapor plumes in the southern polar region (Hansen, 2006), with the Imaging Science Subsystem actually captured the eruption of icy plumes from 'tiger stripes on the surface (Porco et al., 2006). The occurrence of plumes and the tiger striped trenches in the southern hemisphere (which have higher temperature than the surrounding surface), suggest that the satellite is geologically active and warm (Porco et al., 2006).

Cassini mission data analyses of the E-rings of Saturn grains has identified sodium chloride and possibly sodium carbonate minerals. These compounds could be provided by the plumes on Enceladus (Spahn, 2006), pH 8-11 and contain 0.2% and 2% (w/v) NaCl and Na_2CO_3 0.02-0.1 M (Zolotov, 2007: Postberg et al., 2009; Postberg, 2011). As occurs on Europa, the presence of a liquid ocean on Enceladus could provide a possible habitat for life.

The surface of Mars has been investigated using both orbital and rover missions equipped with a variety of instruments (Arvidson et al., 2011; Vago et al., 2015). Wet

chemistry analysis of pH, conductivity and redox potential of soils were conducted using the Conductivity Analyzer (MECA), aboard on the Phoenix lander. During the last mission on Mars, the ExoMars rover was also equipped with a Life Marker chip, based on an antibody assay-based life, that would enable a search for specific molecules indicative of life (Martins, 2011). Mars has an atmosphere which is predominantly constituted by carbon dioxide (~95.3%), nitrogen (2.7%), argon (1. 6%) and trace amounts of oxygen (0.13%), water (0.02%) and carbon monoxide (0.07%) (Hansen et al., 2006). Atmospheric pressure, measured at the two Viking lander sites, varied between 6.7mbar and 9.9mbar (Tillman et al., 1993), and temperature varied between -123 °C and +25 °C, with diurnal and seasonal fluctuations (Horneck et al.,

2010). In addition, the atmospheric surface is exposed to solar UV radiation and ionizing radiation (IR) of solar energetic protons and galactic cosmic rays (Dartnell et al., 2007). The effects of radiations, pressure and desiccation on organisms and amino acids could influence the possible habitability on the Martian surface or subsurface (Dartnell et al., 2007).

Water in the form of ice is present on the soil surface and is stored at the poles as ground ice (Feldman et al., 2002; Smith et al., 2009), however the existence of liquid water on Mars could be more exciting for the search for life on Mars. Data from the Phoenix mission has led to the hypothesis that with the presence of deliquescent salts, liquid saline water could be present on Mars. Freeze-thaw cycles can lead to the formation of saline solutions with a freezing point below the summer ground temperatures recorded at the Phoenix lander site (Renno et al., 2009) and the existence of briny, liquid water has also been suggested by the interpretation of darkened soil streaks on equator facing slopes (McEwen et al., 2011). Spectral evidence for liquid saline water has also been reported at the polar region (Renno and Mehta, 2011).

Cold and hot environments, with acid or alkaline-saline conditions, such as those in Antarctica and shallow hydrothermal systems could be considered as analogues of Europa, Enceladus and Mars in our solar system. Similar extraterrestrial condition have been recorded in acidic rivers (Amils et al., 2007: Fernandez Remolar and Knoll,

2008), hydrothermal systems (Hagerty and Newsom, 2003:) and saline environments (Grasby and Londry, 2007) Antarctic dry valleys (Wentworth et al., 2005), Svalbard (Steele et al., 2004; Steele et al., 2007) and the Atacama Desert (Wettergreen et al., 2005) and alkaline or alkaline-saline environments as Lake Magadi (Ehlmann et al., 2011; Dyar et al., 2012).

Despite the surfaces of planets and celestial bodies could evoke the primordial or actually terrestrial environments there are numerous factors that influencing the life in the universe (Table4) the most important are: i) the temperature that were in the range from -90 to 130 °C, ii) the desiccation due to the negative pressure; iii) the galactic cosmic radiation and iv) the ultraviolet radiation.

Tab4. Data obtained from Biopan 6, September 2007 (Horneck et al., 2010)

Environmental parameters in space enviroments	
Temperature (°C)	-38 to 45
Pressure(Pa)	10^{-6}
UV fluence(J/m^2)	10^{7}
Ionizing radiation havy ions (parts/um^2)	5×10^{-8}

1.3.1 High and low temperatures

High temperature influences the main macromolecules of cells; indeed, it increases the fluidity of membranes, at high temperatures (>70 °C) the α-helices of DNA is subject normally to denaturation and chemical modification and the structure and function of proteins were also affected. To maintain optimal membrane fluidity, the cell must adjust the composition of the membrane including the amount and type (for example, saturated versus unsaturated) of lipids (Nicolaus et al., 2000). Proteins able to cope the high temperatures include increasing ion-pair content, forming higher-order oligomers and decreasing flexibility at room temperature. The DNA of hyperthermophiles, such as *Pyrococcus furiosus*, is known to be more stable *in vivo* than that of mesophiles, such as *Escherichia coli* (Sevcenco et al., 2010). Monovalent and divalent salts enhance the stability of nucleic acids because these salts screen the negative charges

of the phosphate groups, and because KCl and $MgCl_2$ protect the DNA from depurination and hydrolysis (Marguet and Forterre,1998). The G–C pair of nucleic acids is more thermostable than the A–T or A–U pairs because of the additional hydrogen bond (Zhang et al., 2015), therefore DNA from thermophiles usually possesses higher G-C ratio than mesophiles.

At low temperatures, under the zero, with nucleation, water freezes. The resulting ice crystals can rip cell membranes, and solution chemistry stops in the absence of liquid water. Freezing of intracellular water is almost invariably lethal. However similar modification of membrane, enzyme and other strategies allow to survive to these microorganisms (some of these mechanisms are described above)

1.3.2 Desiccation

Desiccation damages the DNA structures, breaking the single and double strands (Fredrickson, 2008; Kottemann et al., 2005). Single strand breaks (SSB) are more common than double strand breaks (DSB) that were considered more lethal type of DNA damage because no complimentary strand is available as a template for DNA repair (Daly, 2009; Kobayashi and Handa, 2001) and due to inhibit DNA replication, transcription and protein synthesis (Kobayashi and Handa, 2001).

To repair the SB damage, a DNA template could be provide by the presence of more than one set of chromosomes per cell (Daly, 2009; Minton and Daly, 1995). Studies on *Deinococcus radiodurans,* a desiccation and radiation resistant bacterium with four chromosomes, have shown that the numbers of DSB are comparable between resistant 'wild type' strains and sensitive 'mutants', a strain which has an inactivated *uvr*A gene, which is involved in DNA repair, for example (Daly et al., 2007; Fredrickson, 2008; Slade, 2011). This suggests that direct DNA protection does not explain the resistance to desiccation.

The resistance to desiccation or radiation identified in organisms as *D. radiodurans* is related to the ability to maintain functional DNA repair mechanisms (Minton, 1996; Daly et al., 2004; Daly et al., 2007; Fredrickson, 2008; Daly, 2009). The dehydration induced the damages on cellular through the protein damages and due to the formation of reactive oxygen species (ROS), such as hydroxyl and peroxyl radicals (Fredrickson,

2008; Kottemann et al., 2005; Daly, 2011). The protein in resistant organisms resulted accumulated minor damages than those possessed by sensitive organisms (Daly et al., 2007; Fredrickson, 2008; Krisko and Radman, 2010).

1.3.2 Galactic cosmic rays: Ionizing radiation (X-rays) and Heavy ions charge (HZE)

Galactic cosmic rays (GCR) are composed by of 85% protons, 14% α-particles (helium nuclei), and about 1% heavier nuclei charged particles that originate from sources beyond our solar system. The distribution of GCR is believed to be isotropic throughout interstellar space. The spectrum of the GCR consists of 98% protons and heavier ions (baryon component) and 2% electrons and positrons (lepton component). The baryon component is composed of 87% protons, 12% helium ions (alpha particles), and the remaining 1% heavy ions of charge 3 from lithium to 92 from uranium. Due to their high abundance, iron ions are highly penetrating, giving them a large potential for radiobiological damage (Horneck, 2010). The ionizing radiations produced hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^-) by the radiolysis of water, and superoxide ions (O^{2-}) are formed in the presence of dissolved oxygen (Repine,1997). Generally, the cytotoxic and mutagenic effects induced by ionizing radiation are thought to be the result of DNA damage caused during the course of irradiation, which includes single-strand breaks (SSB), double-strand breaks (DSB), case modification, abasic sites, and sugar modification (Cadet et al., 2017). The amounts of DNA damage caused by given doses of ionizing radiation for different bacteria are very similar, although the range of ionizing-radiation resistance levels is wide. X-ray sources are commonly used in space radiobiology studies (Dartnell et al., 2010). These sources offer a high irradiation intensity, high penetration, and practicality of experimentation, even though it is taken into account that the ionization and, consequently, some biological effects it causes do not fully reproduce the effects of accelerated particles (Dartnell et al., 2010) Ionizing radiation can damage cellular components though direct deposition of radiation energy into biomolecules and also indirectly by generating reactive oxygen species (ROS).

There is an apparent relationship existing between desiccation and radiation tolerance, DSB breaks and the production of reactive specie of oxygen (ROS) are produced by both forms of stress (Fredrickson, 2008).

1.3.3 UV radiation

Ultraviolet radiation (UV) is a component of sunlight that greatly influences living organisms, its effects depending upon the type of organism, the wavelength region and the irradiation dose (intensity x duration) (Fig.3) (Coohill and Sagripanti 2009).

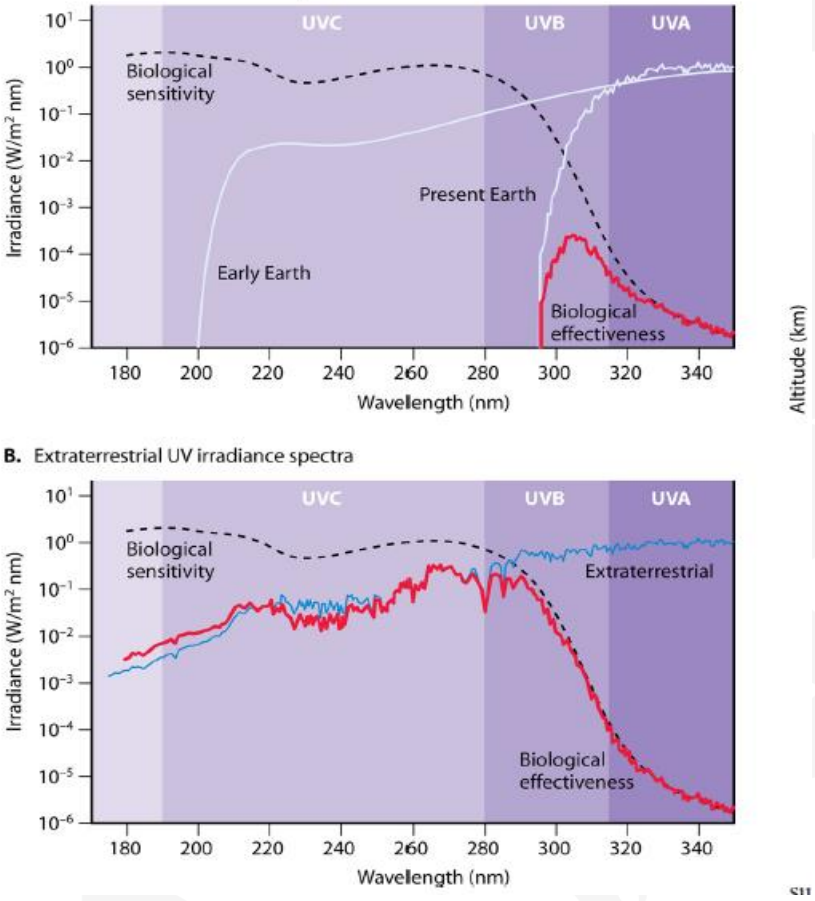


Fig.3. Solar terrestrial (A) and extraterrestrial (B) UV irradiance spectra, action spectra for DNA damage as an example of biological sensitivity (dashed lines), and biological effectiveness spectra (bold red lines) for terrestrial and extraterrestrial conditions. (Horneck et al., 2010)

UV spectrum can be distinguished in the order of potency as UV-C > UV-B > UV-A ranging from 200 to 400 nm. The term UV-A is used to indicate the region from 320

to 400 nm; these radiations are among the most abundant types that reach the Earth's surface and are known for physiological effects on organisms (Hockberger, 2002). UV-A radiation by producing reactive oxygen species (ROS) leading to oxidative damage and causing single and double DNA strand breaks (Folkard et al., 2002). UV-B radiation (280-320 nm), although represents only 1-10 % of total solar energy, is involved in structural modifications of DNA, with lethal effects on different organisms (Hockberger, 2002). UV-C (200-280 nm) is higher than UV-A and UV-B energy. The oxygen and ozone in the atmosphere generally absorb these wavelengths. Their UV resistance is linked to the ability to induce genes for the production of metabolites with protective activity against the radiations (Gabani and Sing, 2013).

Ultraviolet radiation also causes direct damage to both DNA and proteins, in part due to oxidation (Daly, 2009; Chan et al., 2006). DNA damage due to UV-C radiation can include strand breaks (Setlow and Setlow, 1996; Setlow, 2001) and the formation of photoproducts, such as pyrimidine dimers, which form between adjacent pyrimidine bases on the same DNA strand (Cadet and Douki, 2010). These types of DNA damage interrupt the replication of DNA resulting in genome rearrangement as well as mistakes and/or the ceasing of DNA replication (Maloy, 1994).

The process of dimer formation in Bacteria and Archaea can be reversed by either photoreactivation or dark repair (Rastogi, 2006; Kowalski, 2009). Studies have shown a difference when comparing strains which have been exposed to UV-C and are then either incubated in the light (photoreactivation) or the dark (dark repair) for sufficient time to allow the cells time to recover, and DNA repair to occur. Those cells incubated without light can demonstrate up to an order of magnitude lower survival of cells compared to those incubated in the presence of light (Maloy, 1994).

Photoreactivation dimer repair firstly involves the formation of a complex between a photoreactivation enzyme (PRE), such as photolyase, and the dimer to be repaired (Maloy, 1994; Rastogi, 2006). Photolyase will cleave the dimer in the presence of cofactor folic acid, upon exposure to visible light the folic acid absorbs the light, the enzyme breaks the dimer light and the PRE is released. This process is directly related to the quantity of visible light received (Maloy, 1994; Rastogi, 2006).

Dark repair mechanisms (as the name suggest) do not require light and include; nucleotide excision repair (NER), post replication repair (PRR) /recombination repair and SOS repair. In *E. coli* NER involves the enzyme complex *Uvr*ABC endonuclease, proteins within this complex identify distortions in the double helix (*i.e.* thymine dimers), excises the damage and then a template DNA strand is used with the enzyme, DNA polymerase I, to fill the gap and repair the DNA (Yeung et al., 1997; Maloy, 1994; Cadet and Douki, 2010).

1.3.4 Planetary protection program

The exploration of the solar system has induced the spacefaring nations to commit to avoid biological contamination of other planetary systems and also to protect the Earth from potential harm caused by materials forward from space. Thus, the committee on space research (COSPAR) is an international group that design, for extraterrestrial missions, the measures for planetary protection (Kminek and Rummel, 2015).

The COSPAR divided the missions in five categories of contamination risks, and suggested planetary protection measures for each category.

The Planetary Protection Policy (currently approved version COSPAR, 2011; described by Kminek and Rummel, 2015) for special regions defines them the limits for: i) water activity; upper limit, temperature (-25°C; no upper limit defined); iii) Timescale within which limits can be identified: 500 years (Rettberg et al., 2016).

The Mars missions are considered as special issues for planetary protection indeed it is necessary protect the integrity of this planet from contamination because is knowledge as a region within which terrestrial organisms may be able to replicate, or a region which is interpreted to have a high potential for the existence of extant Martian life. The future missions with sample acquisition pose a number of specific protection issues about the possible contamination of Earth by Mars organisms on the other hand clearly it need to ensure that terrestrial microbes from acquisition missions do not contaminate samples that will be analyzed *in situ* or after return to Earth (Barengoltz, 2005).

Microorganisms, and especially bacteria producing endospores, may significantly contaminate space-qualified materials. During the Viking missions, to avoid the contamination of problematic microorganisms the spacecraft surfaces are treated with

chemical, physical, mechanical, and thermal processes (*e.g.*, high pressure, high temperature, UV, and gamma irradiation) (Puleo et al., 1978; Moisan et al., 2001; Kong et al., 2009; Heinlin et al., 2011, Stapelmann et al, 2013). The moderate levels of dry heat or chemical agents, such as hydrogen peroxide, that did not damage the spacecraft or the instruments, often not killed endospores (Venkateswaran et al.,2004 Benedikt et al., 2008; Heinlin et al., 2011). Therefore, these processes are not opportune because they have high cost, difficulty of application, induce changing properties of the materials with the deposition of residues on surfaces, and could increase the resistance of microbes to the disinfection process (Rutala and Weber, 2001). An innovative sterilization treatment is based on use of Plasma, to produce ionized gas particles, that reveled many advantages, such as cost-effective, fast, efficient, safe for spacecraft materials (Benedikt et al., 2008; Kong et al., 2009).

Different gas or a gas mixture could be used for the plasma sterilization to produce different biocidal agents, as chemically reactive species of nitrogen or oxygen, ions, and UV photons (Stapelmann et al., 2008; Ehlbeck et al., 2011; Heinlin et al., 2011). The combination of UV radiation and heat, among others, plays a major role in plasma sterilization efficacy (Heinlin et al., 2011). Although the biocidal mechanisms are not yet fully understood, probably a combination of different factors including reactive oxygen species, the factors involved in bactericidal and sporicidal effects are :i) UV radiation (including vacuum ultraviolet radiations UV ranged below 200 nm) that is capable of damaging nucleic acids (DNA, RNA) and proteins; (ii) the reactive nitrogen and oxygen species, such as OH^- radicals, which are linked to damages of membrane, proteins, RNA, and DNA; and (iii) the electrostatic force that induced microbial cells lysis due to the accumulation of charged particles coming from the plasma (Yasuda et al., 2010). To investigate the effectiveness of different plasma sources various studies were carried out on different bacterial species, *E. coli*, *B. subtilis*, *G. stearothermophilus*, as the most common biological contaminants (Benedikt et al., 2008; Klämpfl et al., 2012). To complicate matters, previous studies showed that numerous cultivable bacteria, isolated at the Jet Propulsion Laboratory (JPL), have unusual resistance to both physical and chemical antimicrobial agents (Link et al., 2004, Venkateswaran et al., 2016).

1.4 Bacillus *spores as resistant forms*

The ability to form spores was one of the identifying characters in the early classification of bacilli (Errington, 2003). Spores from bacilli are produced in response to a variety of environmental and nutritional stresses are known as the most resistant forms of life on Earth. The phenotypic diversity exhibited by the bacilli and the basal position of the phylum Firmicutes in the bacterial phylogenetic tree of life, suggests that the family of Bacillaceae is ancient and that bacilli may have ancestrally been entirely thermophilic (Fajardo et al., 2012). The major representative species of bacilli are *Bacillus antracis* and *B. subtilis*, the first was used by Koch to postulate the infection disease principles (Beierlein and Anderson, 2011) and the second was used to study the spore and sporulation process (Nakamura et al., 1999).

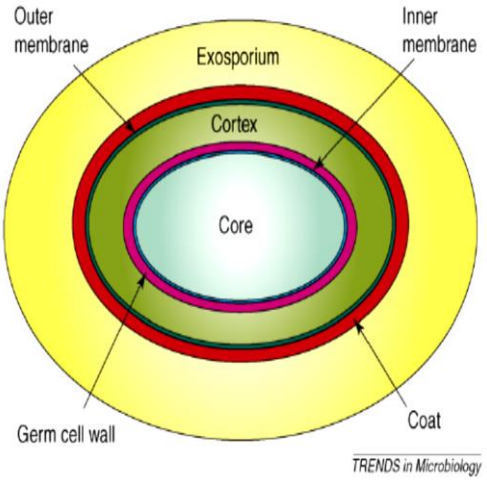
Spores can resist to different environmental stressors, such as physical and chemical insults including high and low temperature, salinity, ionizing and UV radiation and oxidizing agents. The resistance properties of spores are related to a multitude of mechanisms in order to protect macromolecules from damage during their dormancy period, before germination. These mechanisms include the structural properties of spores that are involved in their protection towards acute stresses, as well as against the damages accumulated during the time of dormancy.

Spores of *B. subtilis* are often used as model systems for studying the resistance mechanisms of bacilli, because of the relative simplicity of the organism and its amenability to the tools of traditional and molecular genetics (Piggot and Losick, 2002; Hilbert and Piggot, 2004). The formation of spores, triggered by nutrient limitation conditions (Nicholson et al., 2000), is induced by the program of gene expression and it is coordinated with the differentiation of other cells by intercellular signaling pathways. The process of spore formation involves three cell types known as the pre-divisional cell, the fore-spore, or “pre-spore”, and the mother cell. Under stress conditions, such as nutrient limitation, the cells carried out an asymmetric division in which a septum is formed near a pole, thereby creating dissimilar-sized progeny cells (Wang et al., 2006). This status originates the “fore-spore” and the mother cell. After morphogenesis stage, the fore-spore becomes the core of the mature spore, it is

dehydrated and the chromosome is complexed with proteins belonging to the family of small acid soluble proteins (SAPSs). To protect the DNA against many types of damages, the genomic structure is finally modified into a toroid-like structure (Setlow, 2006). The formation of spores is completed when the spore core is encased in protective outer layers of cortex and coat material. Finally, the spores are released by lysis of the mother cell.

The structure and chemical composition of spores of *Bacillus* species play their major roles in spore resistance to stress, that it is different from that of growing cells (Setlow 2006). The spores generally are composed by unique features and typical constituents (Fig.2).

a)



b)

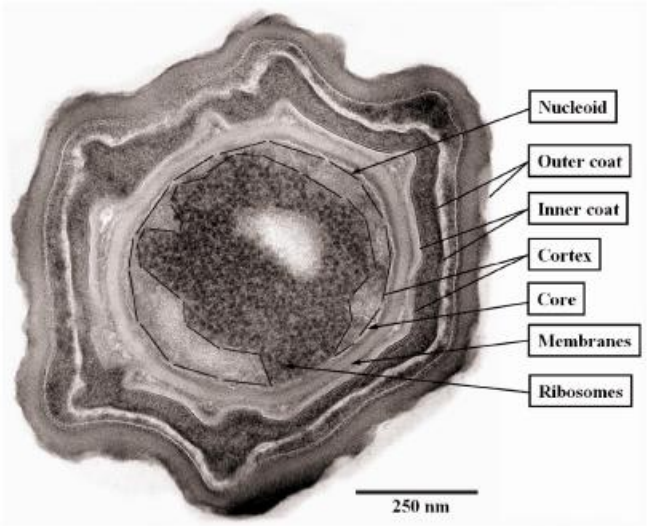


Fig. 2 a) Scheme of spore structure (from Setlow, 2006), b) Electron micrograph of *B. subtilis* 168 (wild-type) spore Frenkiel-Krispin et al., 2004).

The most external layer is the exosporium, composed of proteins and carbohydrate, this layer was found on spores of some species (*e.g. Bacillus anthracis*) but not in others (*e.g. Bacillus subtilis*). The function of the exosporium is unknown. The next layer contains more than 40 different proteins, almost all being spore-specific (Kim et al., 2007). The coat defends the spores against predatory and reactive chemicals (Setlow, 2006). Under the coat layer there is the outer spore membrane. This structure is essential in spore formation, when the spores are in a mature state the coat layer could lost its permeability properties. Experiment with spore coat-defective mutants showed the relative contributions of spore coat layers to UV and/or H_2O_2 resistance. In details, these observations showed that the coat layer is not involved in the UV-C (254 nm) resistance, whereas it plays a key role against the damages induced by UV-B/A (>290 nm) radiations, that represent the most relevant UV wavelengths in terrestrial environments.

It was found that pigmented spores show a higher resistance to UV radiation (>320 nm) than no-pigmented ones (Mitchell et al., 1986; Moeller et al., 2005). Spore pigments, such as the carotene and neoxanthin, may serve as UV-screening components and/or as antioxidant scavengers of radical oxygen species (ROS) (Edwards et al., 2006).

The spore core is the inner layer, exhibiting relatively low permeability to hydrophilic molecules more than 200 Dalton (Da) (Setlow, 2006). Usually the spores possess two different membranes: the outer membrane, which reacts as a functional membrane (Nicholson et al., 2000) and the inner membrane, that is also an intact membrane, significantly compressed in the dormant spore (Stewart et al., 1980). Since the spores are metabolically inactive, they have two ways to copy without deleterious effects the DNA in order to maintain their viability: either repair the DNA damages when spores germinate (return to vegetative life) before their inactivation results in cell death, and/or (ii) protect dormant spore DNA from damage (Moeller et al., 2012). Previous studies have been demonstrated that both strategies have a synergetic effect to ensure the spore resistance and survival (Setlow, 1995). However, the prevention of DNA

damages seems to play a main role in the spore resistance. Factors mainly involved in the protection of spore DNA could be found in the spore core: (i) pH of inner spore, (ii) water content and (iii) small molecules, typical of the dormant spore core. In *B. subtilis* spores the inner pH values ranged from 6.5 to 7.0, approximately one unit lower than the value in growing cells. However, no evidences on damages have been reported by the increasing or decreasing of inner pH values (Magill et al., 1996).

The spore coats seem to have some roles in spore resistance, as barrier that protects the spore structures against lytic enzymes (especially those degrading peptidoglycan) and various chemicals, *e.g.* hydrogen peroxide and alkylating agents. Moreover, spore coat layer plays a role in the resistance to bactericidal substances and in protecting spore DNA (Setlow, 2006). Despite the importance of this layer, there is no knowledge until now on the role of the single proteins constitutive the spore coat layer (Riesenman and Nicholson, 2000).

The low water content in the spore core represents an important factor that contributes to resistance of spores. It is much lower (28-50 % of wet weight) than both vegetative forms and the external layers of the spores (Setlow, 2006). This characteristic plays a major role in spore resistance to heat and ROS stresses. Furthermore, several studies reported that there is a correlation between the lower spore-core water content and the increasing of spore heat resistance among different *Bacillus* species (references in Setlow, 2006).

The spores core possesses very high levels of divalent ions, in particular Ca^{2+}, with the great majority of those cations (*e.g.* Na^{+}, K^{+}, Ca^{2+}, Mg^{2+} and Mn^{2+}) that can be varied, either by alterations in the metal ion content of the sporulation medium (Murrell, 1967; Slepecky and Foster, 1959). The increasing of mineralization in the spore core of *B. stearothermophilus* spores was associated with the increasing resistance to oxidizing agents (Marquis and Shin, 1994).

In the spore core high presence, about 10 % of the dry weight, of pyridine-2,6-dicarboxylic acid (DPA, dipicolinic acid) was detected (Douki et al., 2005). DPA chelated with divalent cations, predominantly Ca^{2+} (Setlow, 1994), is exclusive for spores but not for vegetative cells. DPA was also related with the spore heat resistance (Kimura and Sasakawa, 1975). *B. subtilis* spores lacking DPA, due to a specific

mutation in the *spo*VFA or *spo*VFB locus, showed decreasing in both heat and H_2O_2 resistance (Daniel and Errington, 1993), probably due to the increasing water content (Balassa et al., 1979). These spores didn't exhibit difference in UV resistance and are actually more UV resistant than their isogenic wild-type counterparts. Although DPA plays a valuable role in heat spore resistance, studies on spore of *B. cereus* lacking DPA showed that they were more heat resistant than those of wild type strain (Murrell and Scott, 1966).

The acid-soluble spore proteins (SASP), synthesized during sporulation, are a group of proteins able to saturate and chelate the sporal DNA (Nicholson et al., 1990). When the spores germinate, the SASP are degraded by protease, as reported for *B. subtilis* (Setlow, 2006)

The linkage of SASP proteins to DNA modifies the DNA-form, from B-like to the A-like helix conformation (Mohr et al., 1991). This transition form changes the DNA properties, including its photochemistry. Indeed, spores lack α/β-type SASP are much more sensitive to UV radiation and other chemical and physical stresses, as heat, oxidizing agents, and freeze-drying than those of the wild-type spores. No role of SASP has been demonstrated actually in the resistance against alkylating agents (Setlow, 2006). Until now 16 genes have been identified to encode for major or minor small, acid-soluble spore proteins in *B. subtilis* and, with the exception of *ssp*A (major α-type SASP) and *ssp*B (major β-type SASP), little work has been performed on the function of the other SASP proteins.

Spore-specific factors mentioned above play a role in at least one or more spore resistance stresses. They are involved differently, depending on the stress conditions and also on the bacterial diversity.

In table 5 the involvement of the various factors in spore resistance of *Bacillus* species to different treatments are summarized (Setlow; 2006; Nicholson et al., 2000; 2005).

Tab. 5 Role of various factors in the spore resistance of *Bacillus* spp. to different treatments. Factors: (+) involved; (-)not involved; (/) not demonstrated

Treatment	SASP	Core water	Core minerals	Spore coats	Sporulation conditions	Dipicolinic acid (DPA)
Desiccation	+	-	\	\	\	+
Dry heat	+	-	+	\	\	+
Wet heat	+	+	+	\	+	+
Ionizion radiation	-/+	-/+	\	\	\	\
UV radiation	+	-	\	\	-	+
Glutaraldehyde	-	-	\	+	\	\
Peroxides	+	+	\	+	\	\
Alkylating agents	+	\	\	-	\	\

The DNA of spores may accumulate different damages also with potentially lethal effects. Among the most common effects in spores exposed to physical and chemical factors able to block the critical pathways leading to DNA synthesis and RNA traduction there are: no-basis sites, helix-distorting lesions producing by the UV-induced spore photoproduct (SP), breaks of the backbone spore DNA (Friedberg et al., 1995). These damages could be repaired in germination process to allow the survival of spores to stress factors by the action of such mechanisms as nucleotide excision repair (NER), spore photoproduct lyase (SP lyase), base excision repair (BER), adaptive response, and oxidative stress response (Sancar, 1996).

When the replication machinery encountered noncoding lesions in the DNA constitute a block to further DNA synthesis. The DNA polymerase could dissociate and reinitiates replication on the other side of the lesion (Rupp and Howard-Flanders, 1968) this produce a daughter strand with gaps that could be “repaired” by process called recombinational repair (recombination-mediated DNA repair) (Walker, 1984). This type of mechanism was considered integral parts of an organism’s ability to survive to DNA stresses demonstrating that the actual damage is never physiologically removed but is diluted out by subsequent DNA replication.

Another system to repair DNA is called **photoreactivation**. This DNA repair mechanism reduces the deleterious effects inducing by UV irradiation (200-300 nm)

using the photolyase that enzymatically monomerized the cis-syn cyclo-butyl pyrimidine dimers (Friedberg, 1996).

The nucleotide excision repair (NER) system was able to repair noncoding lesions, *e.g.* UV-induced photoproducts (alteration of the nucleotide structure). This system in *B. subtilis* was reported as analogous to that in *E. coli*, which has been extremely well characterized (Friedberg, 1996). Whereas the photoreactivation process was not found in *B.subtilis* a Nucleotide excision repair genes was identified, mapped, cloned and sequenced (Kunst et al., 1997). However, the molecular details of NER was not been clarified. The knowledge until now demonstrated that a variety of bulky lesions are recognized and endo-nucleolytic cleavage of DNA is initiated both 3'and 5' to the lesion by the actions of UvrA, UvrB and UvrC proteins causing single strand nicks.

In *B. subtilis* a differently repair mechanism from NER was identified and named Base excision repair (BER). In this case, the bases are excised as free bases rather than nucleotides or oligonucleotides. In details, the first step was characterized by the hydrolysis of the N-glycosylic bond that links the inappropriate base or the lesion to the deoxyribose-phosphate backbone of DNA by a repair enzyme called DNA glycosylase. This last enzyme recognized the damaged base or incorrect base and cut the N-glycosylic bond remove the apurinic or apyrimidinic (AP) site in the DNA (Lindahl, 1982). The AP site was removed through the activities of one or more nucleases, after the site without bases was recognized by enzymes known as AP endonucleases and repaired as described for NER mechanism. It is interesting that the glycosylase could be considered as a base-specific excision repair protein (Radany et al., 1997).

Spores of *B. subtilis* and other *Bacillus* species were the much more resistant (up to 50-fold) to UV irradiation than their growing vegetative cells. Previous studies have been demonstrated as in vegetative cells of *Bacillus* species exposed to UV-C radiations the DNA possessed the *cis-syn* cyclo-butane-type pyrimidine dimers, the thymine-thymine (TT) dimers (Setlow, 2006). Whereas in spores the DNA damaging induced by UV irradiation was identified as 5,6-dihydro-5(α-thyminyl) thymine (THDT), known as the "spore photoproduct", SP (Moeller et al., 2012). Since the damage of spore DNA was different from vegetative spores they possess a specifically

dedicated system to the repair of spore photoproduct (SP): the SP lyase. This system was produced in the state of fore-spore and they were activated during the germination process (Fajardo-Cavazos and Nicholson, 2000). The SP-lyase is composed by two monomer one *spl*A of 79 amino and 9.2 kDa of unknown function, and *spl*B of 40-kDa with limited homology to the DNA photolyase/6-4 photolyase/blue-light photoreceptor proteins (Fajardo-Cavazos and Nicholson, 2000). SP lyase is activated during early germination to monomerize the SP dimer back to the two original thymine residues in an adenosyl-radical dependent ("Radical SAM") reaction (Nicholson et al., 1997; Rebeil and Nicholson, 2001).

SP-lyase was previously indicated as S-adenosylmethionine (SAM)-dependent that using radical mechanism to produce methionine and a 5'- adenosyl radical for catalytic reaction (Rebeil et al., 1998).

Damages producing by a variety of treatments, including UV, hydrogen peroxide, and cross-linking agents inducing the activation of regulation of SOS (a post-replication DNA repair system activates the DNA replication to bypass lesions or errors in the DNA) (Yasbin et al., 1993). This mechanism was characterized well in *E. coli* and in *B. subtilis* appears to be generally similar (Au et al., 2005). The signal that inducing activation of this system is the conversion of RecA into an activated form that involved the proteins LexA (Bohorquez et al ,2018). The DNA damage results in increased expression of many DNA repair genes, this expression is eventually shut down as DNA damage is repaired, RecA was inactivated, and functional LexA levels rise. Given what is known about this system in *E. coli* and *B. subtilis*, it is not surprising that treatments causing significant spore DNA damage result in induction of lexA- and recA-genes during spore outgrowth (Au et al., 2005).

Various authors reported that *B. subtilis* spores of a recA single mutation were no more sensitive to UV-C radiation, wet heat, hydrogen peroxide, formaldehyde and freeze drying than the wild-type spores (Setlow, 2006), whereas they were significantly more sensitive to dry heat and nitrous acid (Setlow, 2006) compared to their wild-type counterparts. Only few information is present about the recombination-mediated DNA repair of (germinating and outgrowing) spores after various treatments, including ionizing radiation and extreme desiccation.

Chemical agents such as methyl methane sulfonate could induce the alkylation damage. This condition induces the production of proteins that are able to accepting methyl groups from the DNA or remove the damaged bases of DNA (Lindahl et al., 1988).

The oxidative stress is fronted by two types of enzymes: (i) those able to remove the oxidizing agents such as superoxide dismutase and catalase, which prevent damage caused by reactive oxygen species and (ii) enzymes which actually repair deleterious lesions in cellular components such as alkyl-hydroperoxidase and glutathione reductase. Three catalase enzymes are known to be produced in *B. subtilis* 168 (Kunst et al., 1997). Since the spores are metabolically dormant, in *B. subtilis* did not found the major catalase KatA present in growing cells. However, the catalase KatX seems to be active during the germination process (Bagyan et al., 1998).

Genes *yko*U, *yko*V, codifying ligases enzymes, involved in the repair system nonhomologous-end joining (NHEJ) in *B. subtilis*, were related with the resistance to UV, ionizing radiation, and ultrahigh vacuum. Indeed, mutant spores for these genes were more sensitive to the stresses cited above than the wilde type strain (Moeller et al 2007; 2012). Furthermore, in *B.subtilis* genes *Dis*A, *Rad*A/*Sms*, *Rec*G, *Ruv*AB, *Rec*U, *Pol*Y1 or *Pol*Y2, codifying for RNA and DNA polymerases, are needed for spore survival after DNA damage to bypass the damage of DNA in replication after stresses exposition (Raguse et al., 2018).

Spores allow to bacilli to be widely distributed in natural environments, indeed the members of this family have been found in soil, sediment, air, fresh water, marine ecosystem, in activated sludge, in human and animal, and in various foods, as well as in unexpected environments such as those extremely hot as volcanic and hydrothermal vents and those extremely cold environments, as Antarctic and Artic soils. Microorganisms able to thrive in environments with parameters chemical and physical generally considered hostile to life are named extremophiles. Microorganisms, possessing optimal growth between temperature up to 45°C are called thermophiles, those with optimal growth temperature lower than 15°C (Morita, 1975) are named psychrophiles.

Spores have also been shown to be suitable dosimeters for probing terrestrial and extraterrestrial ionizing radiation in environmental and Astrobiology studies (Nicholson et al., 2000; Setlow 2006.). Ionizing radiation can damage cellular components through direct transfer of radiation energy into biomolecules (e.g., DNA, RNA, proteins) and indirectly by generating reactive oxygen species (ROS) from the radiolysis of intracellular H_2O (Cadet and Douki 2010). Considerable efforts have been invested in understanding the molecular mechanisms responsible for the almost unbelievable resistance of spores to environments which exist at (and beyond) the physical extremes which can support terrestrial life (Nicholson et al., 2000).

1.5 Spectroscopic techniques in biological studies

InfraRed (IR) spectroscopy, including Raman and Fourier transform, is an excellent method for biological analysis (Baker et al, 2014). Raman spectroscopy is optical technique that possess several properties very useful in astrobiology research. The technique is relatively fast and provide sensitive and specific biochemical information useful for characterizing both mineral phases and biological materials. In details, the analysis of inelastic light scattering from the vibrating molecules in the sample allows to: i) recognize and distinguish different substances, cells or bacteria in mixtures with a sensitivity that depends on their concentration (Managò et al., 2016; Rebrošová et al., 2017); ii) investigate biochemical changes at molecular level (De Angelis et al,. 2017; De Luca et al., 2014); iii) localize and map the biochemical content within a sample (Managò et al., 2018; Kosmeier et al., 2014). A typical Raman spectrum is used to provide structural information about biological system especially on the bonds regarding the main macromolecules as nucleic acids, proteins, carbohydrates and lipids, which are enabled to differentiate the cell types, physiological states, nutrient conditions, and phenotype changes (Managò et al., 2018). Raman spectroscopy presents some advantages in comparison with other biological techniques. First, time-consuming cultivation prior to measurements can be avoided since single-cell acquisitions are ensured by high numerical- aperture illumination and light-gathering optics. Second, sample preparation is limited to the isolation of cells from their native surroundings. Third, Raman spectroscopy is a completely label-free technique not requiring any labels or dyes. Indeed, the main feature of Raman spectroscopy is its intrinsic molecular specificity that allows to identify a specific molecule and/or to follow biological processes through the identification of the related biomarkers (De Luca et al., 2014a; Managò et al., 2018). The main limit of Raman spectroscopy concerns the weakness of acquired spectra and the fluorescence background when using lower wavelengths of laser (< 600 nm), especially in biological samples. Despite these drawbacks, the technique was successfully used in several works for investigating the resistance of spores to harsh conditions and stress. Dual-trap laser tweezers Raman spectroscopy (LTRS) and elastic light scattering (ELS), allowed the

investigation of dynamic processes during high-temperature treatment of individual spores of *Bacillus cereus*, *Bacillus megaterium* and *Bacillus subtilis* in water (Kong et al., 2011; Setlow 2006; Zhang et al., 2010; Stöckel et al., 2010).

The FTIR spectroscopy is a valuable technique to measures the wavelength and intensity of the absorption of IR radiation by a sample. Due to the introduction of mathematics of band-narrowing techniques (namely second-derivative analysis and Fourier self-deconvolution) and spectral subtraction the IR spectroscopy could be used in the conformational analysis of proteins (Yang et al., 2015).

The vibrational transitions, induced by the absorption of IR radiation, depend on the strength and polarity of the vibrating bonds, they are influenced by intramolecular and intermolecular effects.

The vibrational frequencies showed by proteins were Protein molecules exhibit many vibrational frequencies. Nine characteristic group frequencies arise from the polypeptide repeat unit, and they have been identified as follows: amide I (~1,650 cm^{-1}), amide II (~1,550 cm^{-1}), amide III (~1,300 cm^{-1}), amide IV (~735 cm^{-1}), amide V (~635 cm^{-1}), amide VI (~600 cm^{-1}) and amide VII (~200 cm^{-1}) (Yang et al., 2015). These amide vibrational bands can be described in terms of five in-plane (C=O stretching, C-N stretching, N-H stretching, OCN bending and CNH bending) and three out-of-plane (C-N torsion, C=O and N-H bending) displacement coordinates. The differential pattern in H-bonding and geometric orientations of amide bonds in α-helices, β-sheets, β-turn and random coil structures allow the different vibration frequencies to be associated with individual secondary structural folding. The amide I and amide II bands are the two major bands in the protein IR spectrum. The most sensitive spectral region to protein secondary structure compositions is amide I, which originates from the C=O stretching vibration of the amide group coupled with the in-phase bending of the N-H bond and stretching of the C-N bond (Bandekar 1992; Yang et al., 2015). These vibrations are found between 1700 and 1600 cm^{-1}, and they are directly related to the backbone conformation, each frequency corresponding to a particular structure. Amide II is more complex than amide I, and it is derived mainly from in-plane N-H bending (40–60% of the potential energy) and the C-N stretching vibration (18–40%). This band is conformationally sensitive, but it has been used very

little for protein structures. The other amide bands are currently of little use in protein structure analysis, because they are very complex and they depend on the details of the force field, the nature of side chains and hydrogen bonding.

FTIR is a useful tool for determining the secondary structure of proteins, and the amide I band has been widely used to quantify the secondary structural composition of proteins and polypeptides. However, analysis of the spectra in terms of protein secondary structure is not straightforward, which may present practical problems that should be fully realized by the practitioners. For example, amide I absorbance is usually a single broad band; therefore, contour, band-narrowing methods and curve fitting are required to resolve the overlapping bands. The purpose of this work is also to provide a practical guide to analyzing the secondary structure of proteins using FTIR techniques, including procedures for sample preparation, measurements, data analysis and all possible precautions that have to be taken to ensure reproducibility. Basic background information and information on recording and processing the FTIR spectra is also presented. Our protocol could help researchers perform basic experiments in this field in a manner that ensures that all steps are performed correctly.

1.6 Bacterial exopolysaccharides and spore resistance

Exopolysaccharides (EPSs) are high molecular weight carbohydrate polymers produced by microbial cells in response to stress condition to form a complex matrix called biofilm. EPS enhance the survival of bacteria influencing the physicochemical environment in proximity of the bacterial cell. Moreover, they assist the microbial communities to endure extremes of temperature, salinity, and nutrient availability (Poli et al., 2010). In response to stress the vegetative cells could be synthetize EPS and then sporulate producing spores immersed in sporulated matrix these spores may have greater resistance to environmental stresses such as heat (Elhariry et al., 201). EPSs display an important role in biofilm matrix in regard to the biochemical interactions between bacteria and surrounding cells (Dechoe et al., 1990). The hydrated biofilms offer a stable micro-environment in which extracellular enzymes can find storage and in the same time facilitating cellular uptake of small molecules (Poli et al 2010). EPSs are involved in the resistance to heavy metals and toxic substances (Gugliandolo et al.,

2012). Indeed, these exopolymers exhibit a polyanionic state in marine environment displaying a high binding affinity for cations as well as trace metals. Moreover, due to the contents of the acidic carboxyl groups attached the exopolysaccharides are ionizable at seawater pH (Poli et al 2010). Furthermore, EPS produced by some Antarctic bacterial isolates contain uronic acids and sulfate groups and may act as ligands for cations present as trace metals in the Southern Ocean environment, enhancing the primary production of microbial communities usually limited by poor availability of trace metals such as iron (Fe^{+3}).

The presence of EPS could be associated with the resistance to heat, desiccation or pH stresses of spores. New thermophilic and thermotolerant bacteria, isolated from the shallow hydrothermal vents of Eolian Islands (Italy), were able to produce novel EPSs potentially useful in various applicative fields (Gugliandolo et al 2012; Spanò et al 2013). More recently, a novel alkalophilic and thermophilic *Bacillus licheniformis* strain T14, isolated from a submarine vent off Panarea Island (Italy), has been reported as producer of a new exopolysaccharide (EPS-T14), by using relatively simple purification process (Spanò et al 2013). In details, EPS-T14, containing fructose, fucose and glucose as major monosaccharides, displayed structural characteristics (high carbohydrate content, 99% and high molecular weight, 1000 kDa), and interesting rheological properties (i.e. viscoelasticity) (Table 6).

Tab.6 Characteristics of EPS-T14 from *B. licheniformis* strain T14 (from Spanò et al., 2013)

	EPS-T14
Carbohydrate (%/v)	99
Protein (%/v)	1.2
Uronic acid (%/v)	0.5
Molecular weight	1000 kDa
Monosaccharide composition (ratio of relative proportion)	Fructose/fucose /glucose/galactosamine/mannose (1.0:0.75:0.28:tr:tr)
Saccharide repeating unit	Trisaccharide
Anomeric configuration	β-manno pyranoside
Specific viscosity	0.58η

Thermogravimetrical analysis	240 °C
Brine shrimp assay	Positive

2 Aim of the thesis

Extremophiles are ideal candidates to extend our knowledge on the limitations for terrestrial life, including sporicidal treatments, and on their ability to survive under conditions mimicking space environments, that is the major challenge in Astrobiology (Mesbah and Wiedel, 2008; reviewed in Horneck et al., 2010).

Due to their ability to form metabolically dormant and resistant spores, *Bacillus* are ubiquitous in the biosphere and they are capable of persisting long term in extreme conditions. Therefore, bacilli could be successfully transferred through space to extraterrestrial environments (Benardini et al., 2003; Fajardo-Cavazos and Nicholson, 2006; Nicholson et al., 2009; Horneck et al., 2012; Khodadad et al., 2017).

On the other hand, bacterial spores have an enormous impact on many areas of human activity, since sterilization treatments are often compromised by the innate resistance of spores. Because of the germicidal power of surface radiation, such as UV-C, and oxidative chemicals, such as H_2O_2, these are commonly used to decontaminate low biomass and sterile sites, including spacecraft assembly facilities (Link et al., 2004; Kempf et al., 2005). Spores of *Bacillus subtilis* are considered the model for inactivation studies in laboratory work, due to their high degree of UV resistance, reproducible inactivation response, and their tractability in the laboratory (Nicholson et al., 2000; Setlow, 2016). *Bacillus pumilus* SAFR-032, *Bacillus horneckiae*, and *Bacillus nealsonii* were found as contaminants on spacecraft and in spacecraft assembly facilities (Smith et al., 2017), since their spores possess high levels of resistance to different contamination efforts, either chemical or physical treatments (Venkateswaran et al., 2003; Link et al., 2004; Vaishampayan et al., 2010).

The aim of the present research was to evaluate and compare the resistance of spores of bacilli isolated from extreme cold (Antarctic soils from Edmonson Point) and hot (shallow hydrothermal vents of Eolian Islands, Italy) environments to environmental stressors, sporicidal treatments and space simulating conditions. The final objectives are to:

i) evaluate the resistance of bacterial spores to environmental stressors and sporicidal treatments;

ii) evaluate the resistance of bacterial spores to space simulating environmental stresses, as those from planet surface of Europa, Enceladus and Mars;

iii) established some structural mechanisms involved in the spore resistance;

3 Material and methods

3.1 Sampling sites

The samples were collected from Edmonson Point, coordinates: Latitude 74°19′44.2″S- Longitude 165°07′59.7″E, deep 35 cm layer directly from the unfrozen part of the active layer at Edmonson Point in early January 2014 (see Fig. 1). Temperature was measured along depth, from surface to 35 cm.

Water and sediment samples were collected in June 2006 by SCUBA diving in the immediate vicinity of three vents, Campo 7 (latitude 38°37'59'' N–longitude 15°06'360''E) and Black Point (latitude 38°38'23''°N–longitude 15°06'28''E), located in front of Panarea Island near "Bottaro", "Dattilo", "Lisca Bianca" and "Lisca Nera" islets, and near at the site Vulcano– "La Roya" (14° 58' N –longitude 38° 25' W). Temperature and conductivity of thermal waters were recorded in situ by a digital thermometer (Hanna Instruments, Milan, Italy).

The physico-chemical characteristics of sampling sites are summarized in Table7.

Tab.7 Physical and chemical characteristics at the sampling sites

Strains	Location	Site	Deph(m)	Temperature (°C)	pH
APA	Shallow hydrothermal vent of Eolian Islands (Italy)	Campo 7	21.3	60	4.92
Gv	Shallow hydrothermal vent of Eolian Islands (Italy)	La Roya	3.0	55	7.6
P82	Shallow hydrothermal vent of Eolian Islands (Italy)	Campo7	8.0	60	4.92
SBP3	Shallow hydrothermal vent of Eolian Islands (Italy)	BlackPoint	23.0	130	3.3
T14	Shallow hydrothermal vent of Eolian Islands (Italy)	Bottaro	8.0	55	5.42
A30	Edmonson point (Antarctica)	EP1	0.35	-18	-
A34	Edmonson point (Antarctica)	EP1	0.35	-18	-
A45	Edmonson point (Antarctica)	EP1	0.35	-18	-
A43	Edmonson point (Antarctica)	EP1	0.35	-18	-
B58	Edmonson point (Antarctica)	EP1	0.35	-18	-
B51	Edmonson point (Antarctica)	EP1	0.35	-18	-

3.2 Phenotypic and genotypic characterization of isolates

The isolates reported in Table.7, were characterized for cell morphology, spore production and motility, Gram stained. Temperature and pH range for growth was determined following incubation of the strains for 3 days at 4, 25, 37, 40, 45, 50, 55 and 60 °C and pH 5.5, 6.0, 7.0, 8.0 and 9.0 in MB. Halotolerance was evaluated after incubation in Bacto Nutrient Broth (Difco) (NB) supplemented with 0, 2, 3, 5,7 or10% (w / v) NaCl. The growth ability was measured determining the turbidity at 600 nm with a spectrophotometer (Ultraspec 3000; Amersham Pharmacia Biotech, Freiburg, Germany) at 24 and 48h. Phenotypic characteristics were compared with those of reference strains:

3.3 Sporulation induction

The novel isolates were maintained in Tryptone Soy Agar with final concentration 1.5% of NaCl (TSA1). To induce the sporulation strains were cultivated in the agarized Schaeffer's medium (containing 0.1% KCl, 0.012% $MgCl_2$, 0.5mM $CaCl_2$, 0.01mM $MnCl_2$, 0.001mM $FeSO_4$, and 8 g/L NB) (Link et al., 2004; Nagler et al., 2014), and incubated at the optimal temperature for each strain, for at least 3 days. The spores were harvested by centrifugation (8000 rpm for 15 min) and then were washed 10 times and suspended in sterile distilled water to reach the >95% of spores, before storage at 4°C. To verify the sporulation process, the spores were observed using the phase-contrast microscope.

3.4 Purification of spores and assessment of vitality

Spores were purified and stored as described Moeller et al. (2007). To determine spore vitality, the suspension of spores will be titrated to serial dilution and plated on TSA1. For further experiments, suspensions of spores at final concentration of 1 x 10^7 spores per ml, were suspended in sterile distilled water.

3.5 Scanning electron microscopy analysis

The dimensions of spores were determined using scanning electron microscopy (SEM) (Zeiss, Sigma SEM). Spores from each strain suspension were posed to aluminum

stubs and dried at room temperature for 24h and then coated with a homogeneous layer (18 ± 0.2 nm) of Au–Pd alloy using a coating device (MED 020, Ba Tec AG, Tucson, AZ, USA) before SEM (Zeiss, EVO 40 EP) observations. To measure the size of spores was used the ImageJ software (Schneider et al 2012).

3.6 Resistance to:

3.6.1 Wet- heat

Resistance of spores to wet heat was measured as described by Melly et al. (2002), briefly, spore prepared in sterile distilled water (10^7 CFU/ml) were placed in Eppendorf tubes and exposed to 100°C. Aliquots (50µl) from spore those suspensions were taken at different time (0, 2.5, 5, 7.5, 10 min) and diluted 1:10 in sterile distilled water. To determine the spore survival, spore suspensions were plated on the appropriated medium and incubated overnight at optimal temperature for each strain.

3.6.2 Dry-heat

To measure spore resistance to dry-heat stress, air-dried spore samples were exposed to 130°C for different times until 60min, as described in Moeller et al. (2012). The spores were recovered from the steel disks after treatments, by a 10% aqueous polyvinyl alcohol (PVA) solution, and after air-drying, the spore–PVA layer was stripped off as described previously (Moeller et al., 2007). Spore survival was determined by plating onto TSA1 after serial dilutions, prepared in sterile distilled.

3.6.3 Acid and alkaline pH

To assay the resistance of spore to pH the suspension of spores 10^7 spores/ml were placed in sterile Eppendorf at two different pH conditions: acid value pH 3 and alkaline pH10. They were placed at room temperature for 30 min and stored for 8

days to verify how the pH influence the spore survival. Each sample was then diluted serially and plated onto TSA1

3.6.4 UV-C

An aliquot of each suspension of spores was exposed to UV-C(254nm) in aqueous and dry conditions according to Moeller et al. 2011. Each experiment was conducted in triplicate. Levels of spore survival were determined from appropriate dilutions in distilled water as colony-forming ability (CFU) after incubation overnight at each

optimal temperature on TSA1 plates.

Artificial UV-C radiation will be provided by a commercial low-pressure mercury arc lamp (Model NN 8/15, Heraeus, Berlin, Germany), to provide monochromatic 254-nm UV radiation. UV doses produced by the artificial UV and solar UV radiation sources were measured by using a model UVX radiometer (UV Products), UV doses will be reported in Joules per square meter (J/m^2).

An aliquot of each suspension of spores will be exposed to UV-C in aqueous and dry conditions according to Moeller et al. (2011). Each experiment will be conducted in triplicate.

To expose to UV-C the spores in aqueous condition a sterile Petri dishes (Nunc A/S, Roskilde, Denmark) were used as container of each suspensions (1 ml of 10^6spores/ml) and they were stirred continuously to ensure homogeneous exposure. The same experiment was conducted in air dry condition. The spore suspensions (100 µl of 10^7spores/ml) were posed on 7-mm-diameter steel disks airdried and exposed to UV-C. To recover the spores, the dry (mono-)layers were covered by a 10 % aqueous polyvinyl alcohol solution (PVA; obtained from DLR, Cologne, Germany) and air-dried under laboratory conditions for 1-2 days. After drying the spores-PVA layer was stripped off as described previously (Horneck and Bücker, 1983) and subsequently resuspended in 1 ml sterile distilled water,

resulting in > 95 % recovery of the spores (Horneck and Bücker, 1983; Horneck et al., 2001). This procedure (later called ‘PVA-stripping’) has no geno- or cytotoxic effects on the vitality of the spores (Horneck et al., 2001).

3.6.5 Low Pressure Ar-plasma (LPP)

To determine the resistance of air-dried spore monolayers to LPP, a double inductively coupled LPP reactor with argon as process gas. A roots pump (Edwards EH 500) with a rotary vane fore pump (Pfeiffer Duo 060 A) allows a low-pressure environment down to 5 Pa with flows up to 160 sccm (standard cubic centimeters per minute) of argon. Additionally, a turbo pump (Pfeiffer TPH 510) is used to evacuate the chamber below 0.1 Pa ensuring a high system purity. The spore samples were placed axially and radially in the center of the discharge with thin glass sample holders influencing the plasma as less as possible (flow rate: 100 sccm, pressure of 10 Pa, and power of 500Watt) as described in Halfmann et al. (2007) and Raguse et al. (2016).

3.6.6 Oxidizing agent H_2O_2

To measure spore resistance to H_2O_2, aliquots (50 µL) of spore suspensions (10^7 spores/mL) in sterile phosphate-buffered saline (PBS) were placed in Eppendorf tubes, plus 500 µL of 10% (v/v) H_2O_2 solution (Merck KGaA, Darmstadt, Germany). After incubation at room temperature (20°C- 25°C), 100 µL of each sample was removed at various times (0, 2, 5, 10, 30min) and immediately diluted 1:10 with a solution of bovine catalase (100 µg/mL in PBS; Sigma-Aldrich, St Louis, MO) for the purpose of neutralizing the H_2O_2 (Riesenman and Nicholson, 2000; Moeller et al., 2012).

3.7 Simulating space conditions

3.7.1 Galactic cosmic rays: *Heavy ions, Starlife project*

To irradiate the spores with heavy ions (Ar, Fe and He) were prepared the

suspensions of 10^8spores/ml. The exposition was performed at the Heavy Ion Medical Accelerator (HIMAC) at the National Institute for Radiological Sciences (NIRS) in Chiba, Japan. A large and uniform irradiation field is provided for biological and biomedical research. Efficiency functions in dependency on ion species and linear energy transfer (LET) in the LET range from 2-200 keV/µm (Table 8 and Figure3) were recorded for studying the inactivation. Detailed information concerning the irradiation geometry of the HIMAC facility, beam monitoring, dosimetry and dose calculations were described in detail in Okayasu et al. (2006).

Maximum intensity was used between 3-5 Gy/min. The experiments were repeated at least three times to reduce statistical uncertainties.

Tab.8 Ions, energies and linear energy transfer (LET) for the spore inactivation

Ion	Energy(MeV/n)	LET(keV/µm)
Argon	500	90
Helium	150	2.2
Iron	500	200

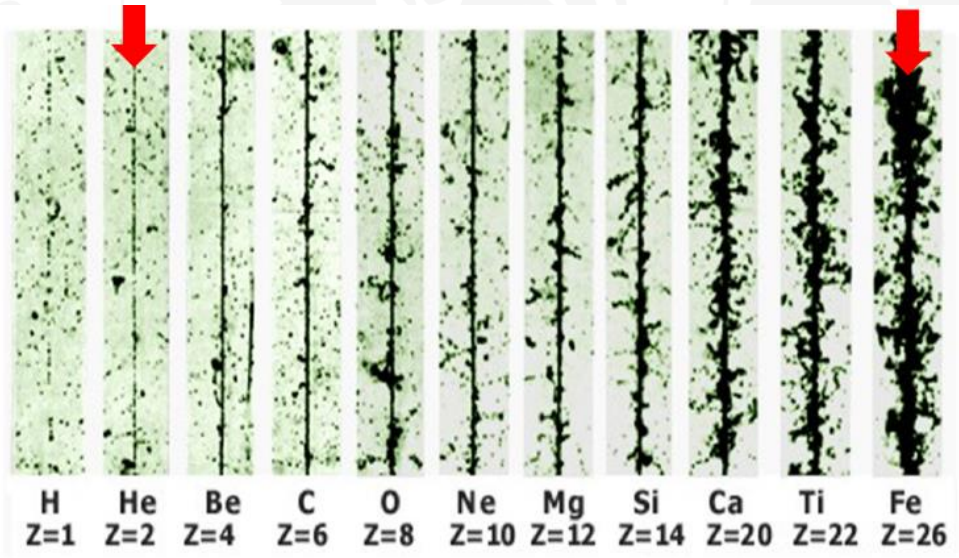


Fig 3 radiation particle track thought the inorganic matter (Hassler et al., 2014)

3.7.2 Exposure to ionizing radiation

For studying the effects of ionizing radiation on spore survival, X-rays (150 keV/20mA) were generated by an X-ray tube (Gulmay Medical RS225; X-Strahl, Surrey, United Kingdom) (Moeller et al., 2007, 2014). Radiation dosimetry and dose

calculations were used as described in detail by Micke et al. (1994). Air-dried spore monolayers were exposed to different doses of X-rays as described previously (Moeller et al., 2014).

3.7.3 Resistance to high vacuum exposure

Air-dried spore-monolayers were exposed to high vacuum produced by ion getter pumping system (400 l/s; Varian s.p.a. Via Varian, Leini, Torino, Italy) reaching a final pressure of 10^{-7} Pa (according to Horneck, 1993; Munakata et al., 1997). The 50 µl of spore suspensions were posed and airdried in a steal discs overnight than they were exposed for 7 days to vaccum chamber. To recover the spores the metal discs were resuspended in sterile water and vortexed at least for 3 min.

3.8 Numerical and statistical analyses to evaluate the surviving rates

The surviving fraction of spores from each strain will be determine from the ratio N/N_0, where N is the number of CFU of the irradiated sample and N_0 that of the non-irradiated controls. Furthermore, spore inactivation curve will be obtained as

described by Moeller et al. (2011). The results with the treated spores were compared statistically by using Student's t test (Moeller et al., 2009). Values were analyzed in multigroup pairwise combinations (i.e., either comparison with regard to the spores resistance of *B. pumilus* SAFR-032 or comparison with regard to the resistance to respective type strain, and differences with p-values of >0.05 were considered statistically significant (Moeller et al., 2014). No multiple testing corrections was applied

3.9 Spores Spectroscopy analysis

Spore suspension of treated and untreated spores were analyzed by Raman spectrometer using 4.5 mW (50% of laser power at source) of 514 nm radiation from an argon-ion laser used for acquiring the spectra. An accumulation time of 80 s for each acquisition was recorded per a single measurement on each sample area. The processing of Raman spectral data was performed with a Matlab script. Basically,

the spectra were background corrected by subtracting a third-order polynomial fit, normalized with respect to the total area under the curve and scaled (Caccamo et al., 2018; Managò et al. 2016; Teh et al. 2008).

Air-dried spores of SBP3 were analyzed by the Attenuated Total Reflectance-FTIR (ATR-FTIR) in comparison to *B. pumilus* SAFR032 and *B. subtilis* 168. The spectra were collected using a Bruker Vertex- FTIR system equipped with a Golden Gate diamond attenuated total reflectance crystal and a liquid nitrogen cooled mercury-cadmium-telluride detector. The FTIR spectra recorded in the wavenumber range of 400−4000 cm^{-1} with a resolution of 4 cm^{-1} for 50 scans were baseline corrected using OPUS software, which is integrated in the system. Forty spectra (10 biological replicates each for four different sample preparations) were collected from untreated control samples and heated samples using each technique.

Commercially available software (Matlab and OriginLab) was used for all data processing. Spectral data were processed in the 2000 to 400 cm^{-1} range that covers the fingerprint region of most biological materials (Nawaz et al. 2010). The subtraction of FTIR signal contributions of the water peak (in the range of region 3700−3000 and/or 1700−1600 cm^{-1}) from spectra of bacterial cell suspensions was performed as previously described (Hlaing et al., 2017). Briefly, an appropriate subtraction factor of the bulk water spectrum was used to enhance the signal of the adsorbed bacterial-related peaks over the water peak and to facilitate the quantitative peak evaluation (Kong and Yu 2007). The total intensity normalization method was applied to the background-subtracted spectra to account for variations in intensity. Second derivatives of the spectra were calculated using nine smoothing points for the Savitsky-Golay process (Bocklitz et al., 2011; Moritz et al., 2010; Savitzky and Golay 1964).

The *Bacillus* spores of SBP3 strain were analyzed by using Raman spectrometer in comparison with and *B.subtilis* 168. All the spectra were obtained by collecting 48 scans with a resolution of 4 cm^{-1} in the 4000 to 400 cm^{-1} wavenumber range. The following data pre-processing procedures were applied: i) a baseline correction, in order to diminish the dissimilarities between spectra due to baseline shift; ii) a smoothing treatment, to reduce the instrumental noise; iii) a first derivative

treatment, to correct baseline shift together, and a second derivative treatment, to better discriminate features associated with spectra; iv) a spectral normalization, to correct the path-length variation and to reduce the differences among single measurements.

3.10 Thermal analysis of EPS with Attenuated Total Reflectance Fourier Transform Infra-Red (ATR-FTIR) spectroscopy

To investigate the thermal properties of exopolysaccharides synthetized by vegetative cells, that could cover the spores, ATR-FTIR Vertex 70V spectrometer (Bruker Optics) using Platinum diamond ATR was employed to collect spectra on EPS-T14, fructose, fucose and glucose, in the temperatures range from 20°C to 80 °C. All the spectra were obtained by collecting 48 scans with a resolution of 4 cm^{-1} in the 4000 to 400 cm^{-1} wavenumber range. The following data pre-processing procedures were applied: i) a baseline correction, in order to diminish the dissimilarities between spectra due to baseline shift; ii) a smoothing treatment, to reduce the instrumental noise; iii) a first derivative treatment, to correct baseline shift together, and a second derivative treatment, to better discriminate features associated with spectra; iv) a spectral normalization, to correct the path-length variation and to reduce the differences among single measurements (Gautam et al., 2015).

The intramolecular OH-stretching band frequency shift evaluation and the wavelet cross-correlation analysis were performed by MATLAB 2016a software (Mathworks, Natick, USA). The spectral distance calculation was performed by Origin 9 software package (OriginLab Co., Northampton, USA). In order to characterize the interaction mechanisms among the three major components of EPS1-T14, the difference between the experimental spectrum and the ideal spectrum of EPS-T14, obtained as a weighed sum of the three component spectra (see eqn.1) was carried out, as shown in eqn.:

$$I^{ideal}(\omega) = \alpha I^{fucose}(\omega) + \beta I^{fructose}(\omega) + \gamma I^{glucose}(\omega)$$

where α, β and γ represent the weight factors of the three different components, i.e. fructose, fucose and glucose, respectively.

$$\Delta I(\omega) = I^{experimental}(\omega) - I^{ideal}(\omega)$$

A first and the second-order derivative spectra treatments were employed to calculate the band center frequency. The hypsochromic shift of the band maximum to higher frequencies was used to highlight the change of H-bond crosslinks among the structural components of EPS-T14 at increasing temperature. The thermal properties of EPS-T14 were evaluated by analyzing the OH stretching contribution shift.

To investigate the temperature-induced structural rearrangements of EPS-T14 H-bond network, the Spectral Distance (SD) of the intramolecular OH stretching band contribution was calculated in the temperature interval (from 20 °C to 80 °C), as it follows:

$$SD = \left\{ \sum_{\nu} \left[I(\omega, T) - I\left(\omega, T = 20\,^{\circ}C\right) \right]^2 \Delta\omega \right\}^{1/2}$$

where I(ω) is the absorbance at frequency ω and Δω is the instrumental frequency resolution. In order to extract further quantitative information on the whole EPS-T14 thermal stability, for the SD versus temperature data fit, the following expression has been applied:

$$SD(T) = A\left(1 - \frac{1}{1 + e^{-B(T - T_0)}}\right) + (C - DT)$$

Here "A" represents the sigmoid amplitude, whose inverse A^{-1} is connected to the thermal restraint. B is the sigmoid steepness; T_0 represents the temperature value of the sigmoid inflection point and, finally, C-DT considers the low temperature vibrational contribution. In order to quantitatively evaluate the correlation degree among the registered spectra at different scales, a Wavelet Cross-Correlation (XWT)

analysis between the different couples of spectra was performed (Valeda et al., 2012).

4 Results

4.1 Phenotypical and genotypical characteristics of the isolates

The eleven isolates from shallow hydrothermal vents of Eolian Islands and from Antarctic soils were investigated (Tab. 9).

Tab.9 List of isolated and reference strains used in this study

	Strains	Optimal Growth Temperature (C°)	Optimal Growth NaCl(%)	Optimal growth pH	Species	Origin
Isolates	APA	45	3	7	*B. oceanisediminis*	Shallow hydrothermal vent of Eolian Islands (Italy)
	Gv	60	2	2	*Geobacillus vulcanii* DSM 13174	Shallow hydrothermal vent of Eolian Islands (Italy)
	P82	50	2	5	*Bacillus* sp.	Shallow hydrothermal vent of Eolian Islands (Italy)
	SBP3	50	2	8	*B.horneckiae*	Shallow hydrothermal vent of Eolian Islands (Italy)
	T14	50	5	8	*Bacillus licheniformis*	Shallow hydrothermal vent of Eolian Islands (Italy)
	A30	15	3	7	*Bacillus* simplex	Edmonson point (Antarctica)
	A34	15	2	7	*Bacillus* simplex	Edmonson point (Antarctica)
	A45	15	2	7	*Bacillus* simplex	Edmonson point (Antarctica)
	A43	15	0	7	*Bacillus* simplex	Edmonson point (Antarctica)
	B58	15	2	7	*Bacillus* simplex	Edmonson point (Antarctica)
	B51	15	2	7	*Bacillus* simplex	Edmonson point (Antarctica)
Reference strains	Gs	60	0	7	*Geobacillus stearothermophilus* DSM22	
	Bho	30	0	7	*Bacillus hornekiae* DSM 23495	
	Bsu	30	0	7	*Bacillus subtilis* 168	
	Bne	30	2	7	*Bacillus nealsonii* DSM 15077	
	Bpu	37	0	7	*Bacillus pumilus* SAFR032	

All the five isolates from hydrothermal vents origin (APA, Gv, P82, SBP3 and T14) were thermophilic (optimum temperature ≥45°C), whereas the six isolates from Antarctic soils (A30, A34, A43, A45, B51 and B58) were psychrotolerant (optimum temperature ≤15°C). The isolates were halotolerant, T14 was halo-alkalophile (NaCl 5% and pH 8); SBP3 was thermo-alkalophile (50°C and pH 8); Gv was thermo-acidophile (60°C and pH 2). Except for *Geobacillus vulcani* (Caccamo et al., 2000), the partial 16S rRNA gene sequence analysis revealed that all the isolates belonged to the genus *Bacillus*. Strain APA was phylogenetically similar (99%) to *B.oceanisedimis* DSM2771, SBP3 was related (99% of similarity) to the astrobiological relevant species *B.horneckiae* DSM23495 and T14 exhibited similarity (98%) to *B.licheniformis* DSM13. Each of the six Antarctic strains shared high sequence similarity levels (from 97 to 99%) with *B.simplex* DSM1321.

4.2 Production and morphological studies of the spores by scanning electron microscopy

The spores from all strains were purified as described by Nagler et al. (2014) and checked with phase contrast microscopy. Morphology and dimensions of spores were observed by scanning electron microscopy (SEM) in comparison with those of *B. subtilis* 168 and *G. stearothermophilus* DSM 22 (Fig.4).

The spores from *B.oceanosediminis* APA (Fig.4c), *B.horneckiae* SBP3 (Fig.4d), *G. stearothermophilus* (Fig.4f) shown immersed into an external matrix to the spores.

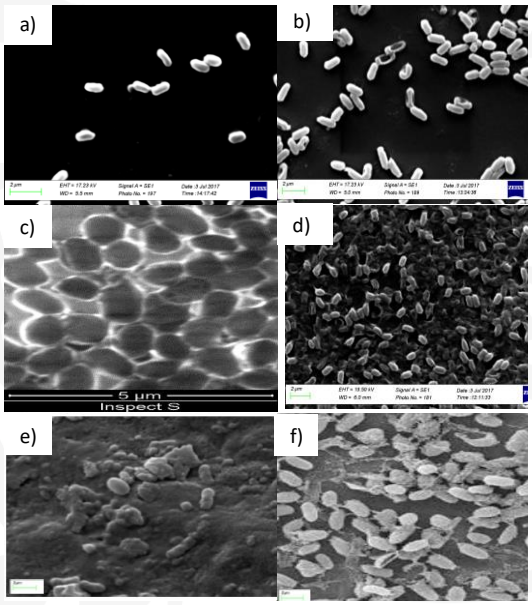


Fig.4 Scanning electron-micrographs (bar 5µm) of spores from *B. licheniformis* T14 (a), *B.subtilis* 168 (b), *B.oceanisediminis* APA (c), *B.horneckiae* SBP3 (d), *G. vulcani* DSM 13174 (e) and *G. stearothermophilus* DSM 22 (f).

Tab.10 Spore length and volume from different *Bacillus* species cultured under different conditions

	Length (μ m)	Spore volume (μm^3)
T14	0.944 ±0.05	0.578±0.026
Bsu	1.2±0.23	0.747±0.069
APA	0.921±0.054	0.8±0.025
SBP3	0.820±0.08	0.589±0.012
G.V.	1.4±0.012	1.19±0.034
Gs	1.32±0.029	1.11±0.02

Results revealed that the average size (Tab.10) depends on the genus level of strain. Indeed, the two *Geobacillus* showed larger spores than those of *Bacillus*. Spores from SBP3 and T14 were the smallest spores.

4.3 Resistance to environmental and sporicidal treatments

4.3.1 Resistance to wet

As showed in Fig.5, spores from the thermophilic Eolian strain *G. vulcani* DSM 13175 (Gv) were the most resistant to wet-heat, followed by those from SBP3, T14, and P82, and the psychrotolerant strain A43. All of these were more resistant than spores from *B. subtilis* 168 (Bsu).

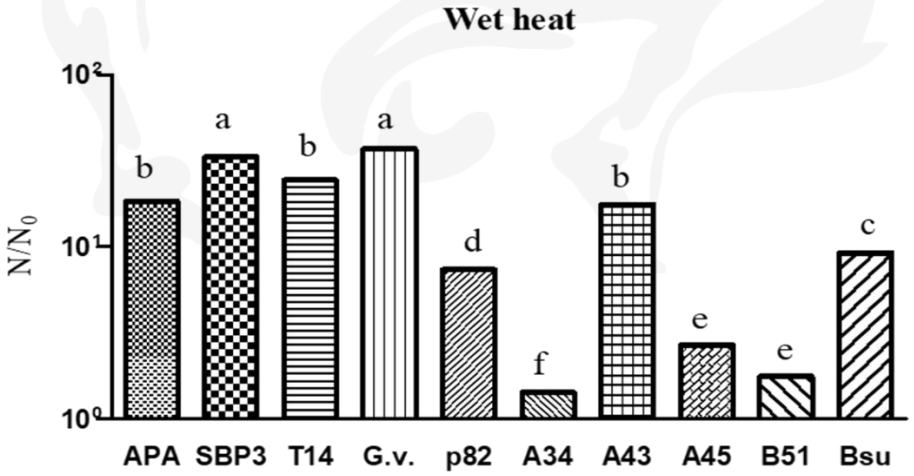


Fig.5 Comparison of the spore resistance to wet-heat. The lowercase letters above the bars denote groups significantly different by ANOVA ($P < 0.05$).

In order to study the impact on spore viability of different environmental, artificial and spaceflight-relevant conditions, spores, either in suspension or as an air-dried monolayer, of different *Bacillus* strains were tested. The resistance to environmental stressors and simulating space environments (wet-heat, dry-heat, X-rays, desiccation) of spores, was expressed as LD_{90} values or CFU in %.

The spores from hydrothermal vent are expected to possess the highest resistance to wet-heat indeed SBP3 exhibit the highest level of resistance to wet-heat similar to Bpu. The level of resistance of SBP3 was higher than its closest strain Bho that results the most sensitive (Fig.6).

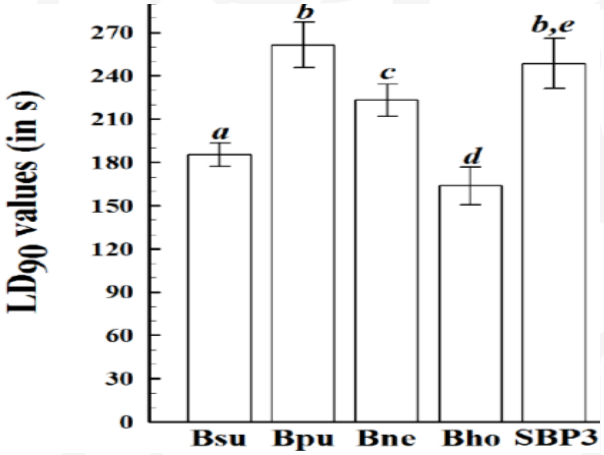


Fig.6 Comparison of the spore resistance to wet-heat. The lowercase letters above the bars denote groups significantly different by ANOVA ($P < 0.05$).

4.3.2 Resistance to dry heat stress

The spores from eolian strain Gv showed the highest resistance to dry-heat similar to those of the reference strain Gs. The other eolian strains SBP3 and T14 possessed similar resistance degree and they were more resistant than biodosimetry strain Bsu (Fig.7).

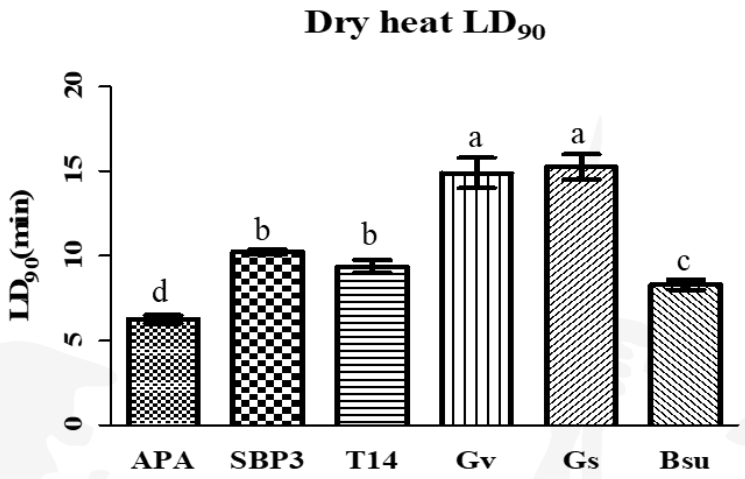


Fig.7 Comparison of the spore resistance to dry-heat. The lowercase letters above the bars denote groups significantly different by ANOVA ($P < 0.05$).

Spores from SBP3 were more resistant to dry-heat than those of its closest related strain Bho, whereas they were similar to those of Bne. Bpu showed the highest resistance degree (Fig.8).

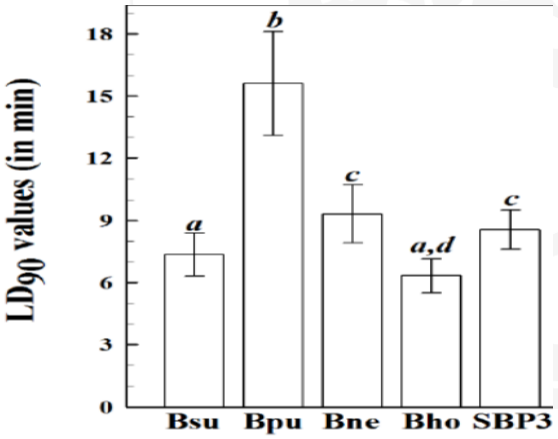


Fig.8 Comparison of the spore resistance to dry-heat. The lowercase letters above the bars denote groups significantly different by ANOVA ($P < 0.05$)

4.3.3 Resistance to low and high pH values

The spores from extremophilic strains were exposed to pH3 and pH10 values (Fig.9) to simulate acidic and alkalophilic environments similar to the planet surfaces of Enceladus and Mars, respectively.

All spores were more resistant to pH 3 than those of the biodosimetry strains Bsu, except for Gv that responded in a similar way. The spores A43 showed the higher

resistance to pH10 values after 30 min (Fig.9). It is notable that all the spores from both extreme environments were able to resist better than those of Bsu.

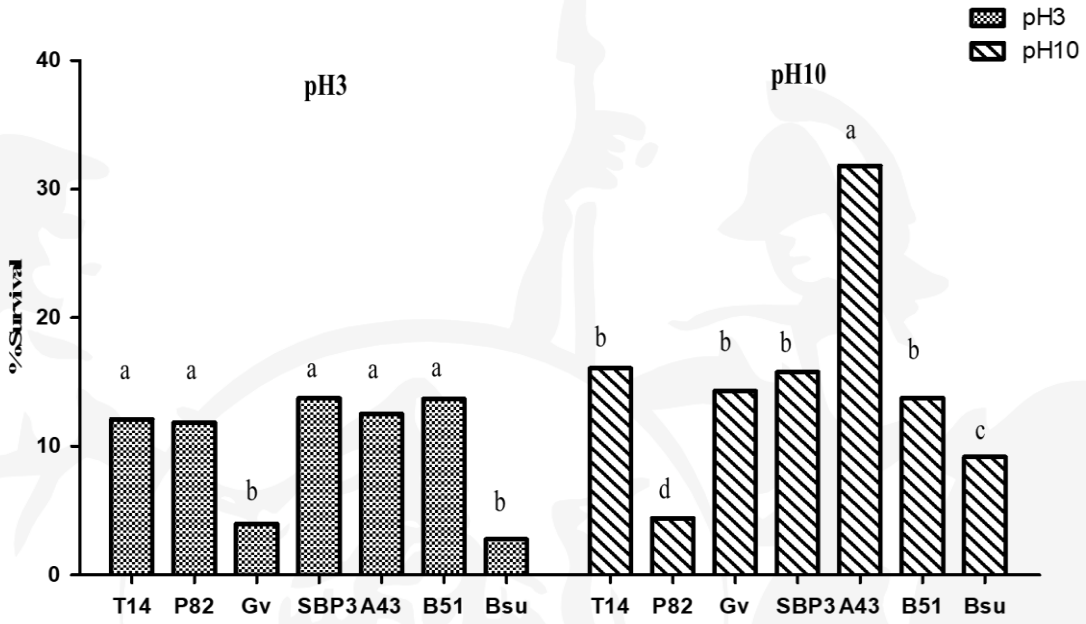


Fig.9 Resistance to pH3 and pH10 of spores from thermophilic and psychrotolerant strains. The lowercase letters above the bars denote groups significantly different by ANOVA ($P < 0.05$).

4.3.4 Resistance to UV-C

The survival of each strain was showed in survival curves obtained through the best-fit curves (Fig 10). They were used to calculate LD_{90} values (as fluence dose) (Fig.10). Spores from SBP3 were the most resistant to UV-C (197 J/m^2), followed by those of the biodosimetry strain Bsu (192 J/m^2), the Eolian strain T14 (LD_{90}=187 J/m^2) and the Antarctic strain A34 (185 J/m^2) (Fig.11). However, the statistical difference of the resistance degree among these strains was not significant ($P > 0.05$).

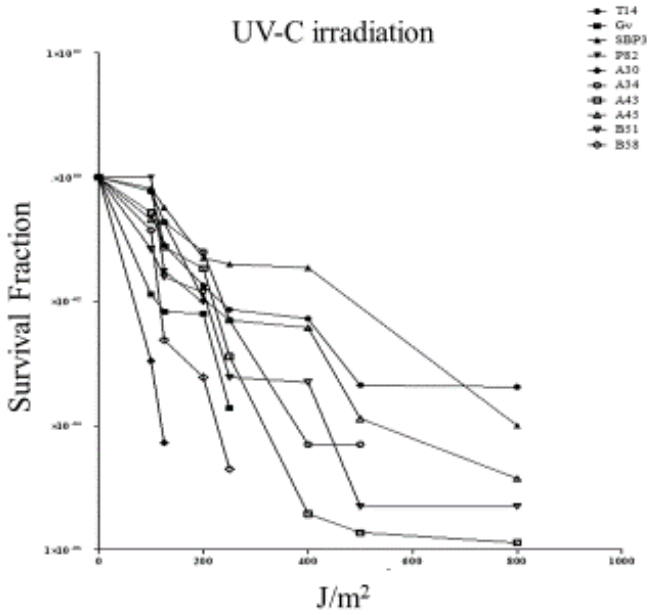


Fig.10 Survival curves of air-dried spores of *Bacillus* sp. strains in response to 254-nm UV-C. The surviving fraction was determined from the quotient N/N_0, with N being the number of CFUs of the treated sample and N_0 the CFUs of the non- treated controls. By plotting the logarithm of N/N_0 as a function of each treatment survival curves were obtained.

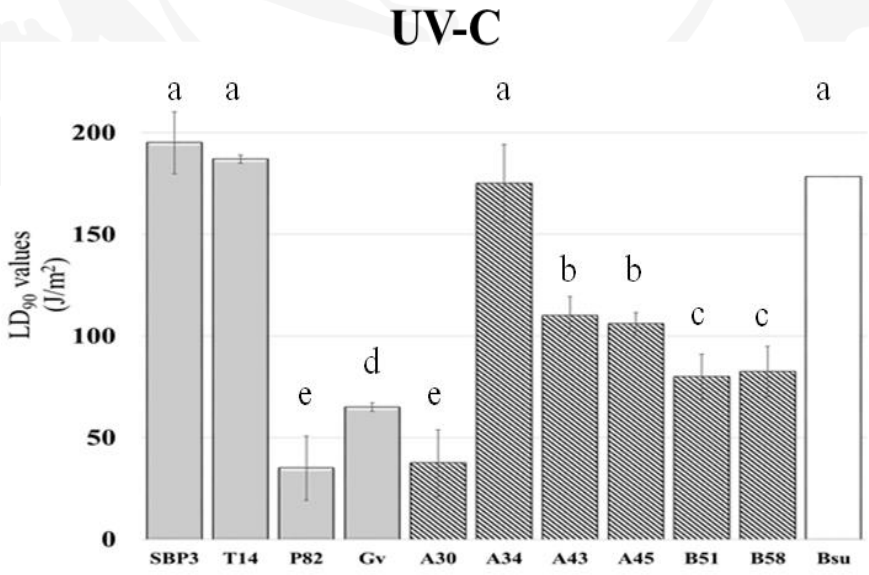


Fig.11 Spore resistance to UV-C of spores from thermophilic and psychrotolerant strains expressed as LD_{90}. The lowercase letters above the bars denote groups significantly different by ANOVA ($P < 0.05$).

The samples were exposed in two different condition in dry and aqueous suspensions. In both conditions the spores from Bpu resulted the most resistant followed by Bne, Bsu and SBP3. Therefore, the spores from Bpu, Bne and Bho respond in similar way in both conditions whereas those from Bsu and SBP3 showed a higher degree of resistance when were exposed in dry condition (Fig.12).

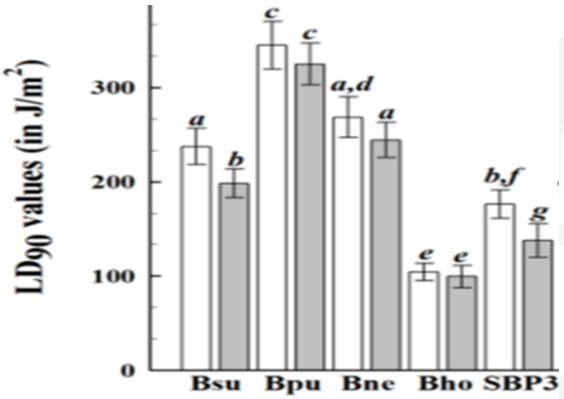


Fig.12 Comparison of the spore resistance to UV-C in dry (white bar) and aqueous (grey bar) conditions. The lowercase letters above the bars denote groups significantly different by ANOVA (P <0.05).

4.3.5 Resistance to low pressure plasma (LPP)

The LPP treatment showed a high efficiency to kill the spore. SBP3 showed similar resistance to Bne and they were the second only to Bpu. Therefore, Bsu and Bho exhibited the lowest resistance degree (Fig.13).

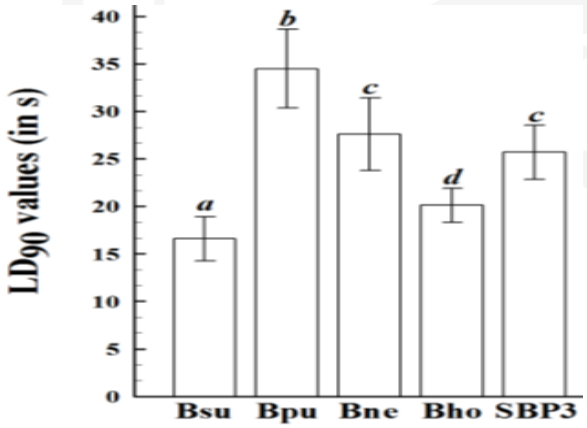


Fig.13 Comparison of the spore resistance to LPP. The lowercase letters above the bars denote groups significantly different by ANOVA (P <0.05).

4.3.6 Resistance to hydrogen peroxide (H_2O_2)

The comparison of resistance to H_2O_2 revealed as Bpu was the most resistant, however Bne, Bho and SBP3 showed similar response and higher resistance level than those of Bsu (Fig.14).

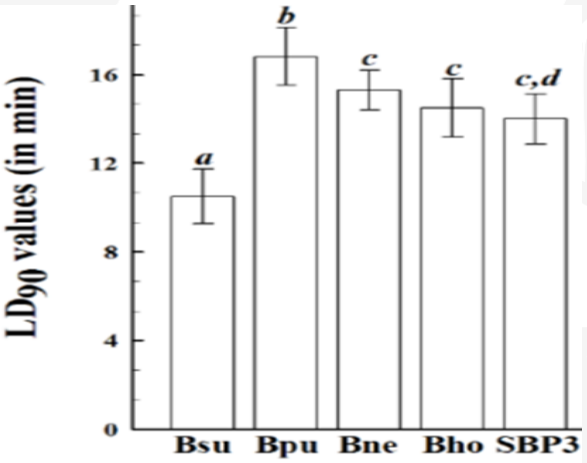


Fig.14 Comparison of the spore resistance to H_2O_2The lowercase letters above the bars denote groups significantly different by ANOVA ($P < 0.05$).

4.4 Resistance to simulating space conditions

4.4.1 Heavy ions: Starlife project

In the frame of the Starlife radiation experiments, a set of different space simulating radiations has been studied to increase our knowledge on their biological effects. Five strains were investigated for their resistance to irradiation of He, Fe and Ar. Fig. 15 showed the survive fractions under exposure to He, Fe and Ar at 250 Gy and 1000 Gy doses.

Spore resistance to exposure to He and Ar of the thermophilic strains T14 and SBP3 was highest, but showed different response to Fe (Fig.15), with T14 the highest resistant. On the contrary, the spores from the psychrophilic strains A34 and A43

showed different resistance to He and Ar, while showed similar resistance degrees to Fe.

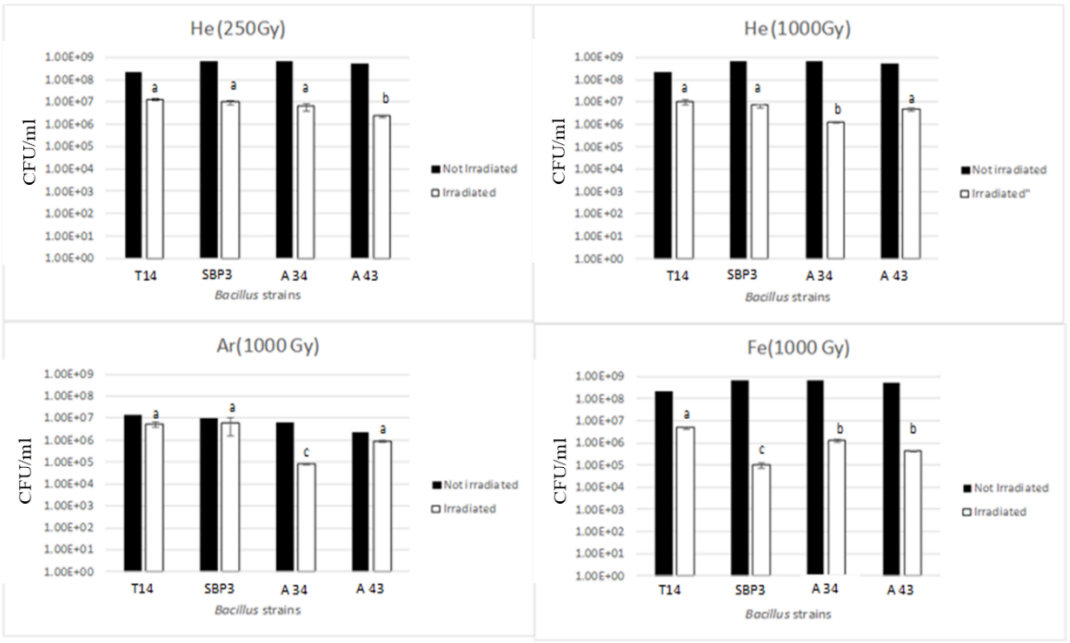


Fig.15 Spores resistance to heavy ion particles (He 250 Gy, He 1000 Gy, Ar 1000 Gy, Fe 1000 Gy) of the different tested strains. The lowercase letters above the bars denote groups significantly different by ANOVA ($P < 0.05$).

The spore resistance (expressed as LD_{90}) to He irradiation of strain Gv was similar to that of T14 and SBP3 strains (Fig.16), while Gv showed different response to Fe irradiation, with Gv the lowest resistant.

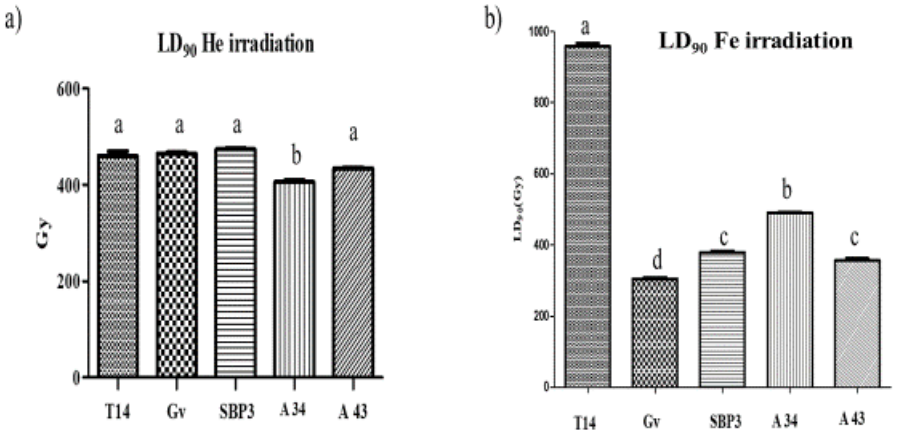


Fig.16 Spore resistance, expressed as $LD_{90,}$ to: a) He and b) Fe The lowercase letters above the bars denote groups significantly different by ANOVA (P <0.05).

4.4.2 Exposure to ionizing radiation

The spores were exposed to X-rays radiation and, as showed in Fig.17, Bpu exhibited a highest level of resistance. Spores from SBP3 were more resistant than those of Bho whereas are less resistant than those of Bsu and Bne.

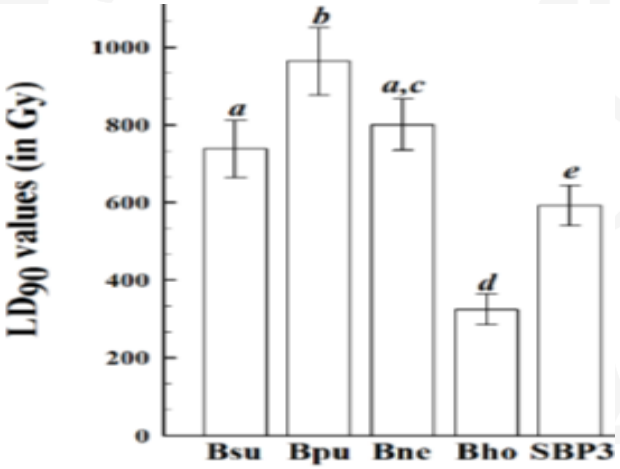


Fig.17 Comparison of the spore resistance to X-rays. The lowercase letters above the bars denote groups significantly different by ANOVA (P <0.05).

4.4.3 Desiccation by Ultra-vacuum pressure

The desiccation induced by the high vacuum poorly affect the spores of Bpu, Bne and Bsu that showed higher viability instead SBP3were influenced by this treatment although its spores showed higher resistance degree than Bho (Fig.18).

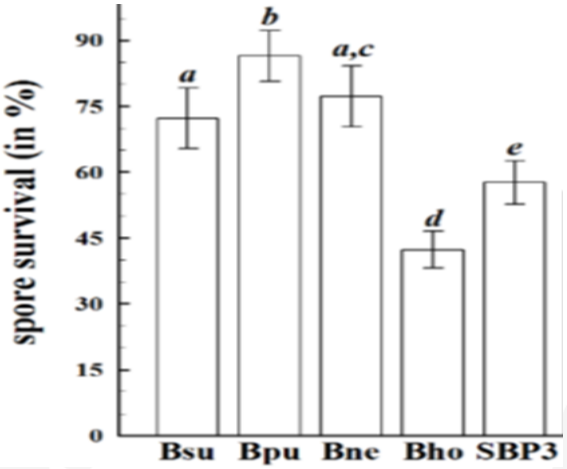


Fig.18 Comparison of the spore resistance to ultra-high vacuum. The lowercase letters above the bars denote groups significantly different by ANOVA (P <0.05).

4.5 Spores spectroscopy analyses

4.5.1 Resistance of spores of a thermophilic strain of shallow-vent origin evaluated by spectroscopic technique.

The results from heat treatment of spores showed (Fig.19) that increasing treatment times generally increased spore inactivation. SBP3 exhibited higher thermal resistance to 95°C in wet heat condition. The spores from SBP3 are similar to those of Bsu until 10 min, at the maximum time of exposition (30 min) the difference of survival spores between SBP3 and Bsu were higher than 1 log scale between SBP3 and Bsu.

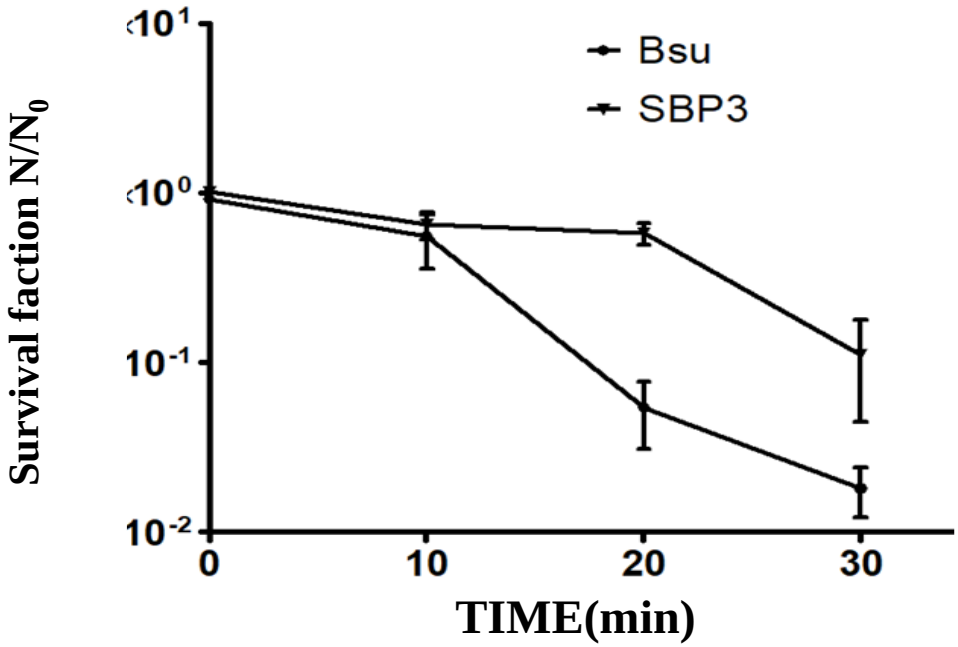


Fig.19 The surviving fraction was determined from the quotient N/N_0, with N being the number of CFUs of the treated to heat 95°C to wet heat condition and N_0 the CFUs of the non- treated controls. By plotting the logarithm of N/N_0 as a function of survival curves were obtained.

The investigation by SEM showed as the environmental strain of SBP3 possess a sticky layer (Fig 20a) commonly attributed to exosporium, similar to its related strain *B.horneckiae* DSM 23495^T; (Vayshampayan et al., 2010). The Bsu didn't exhibit exosporium (in Fig.20b), this structure, together the coat, is known to be composed of several layers containing spore-specific proteins (Lai et al., 2003).

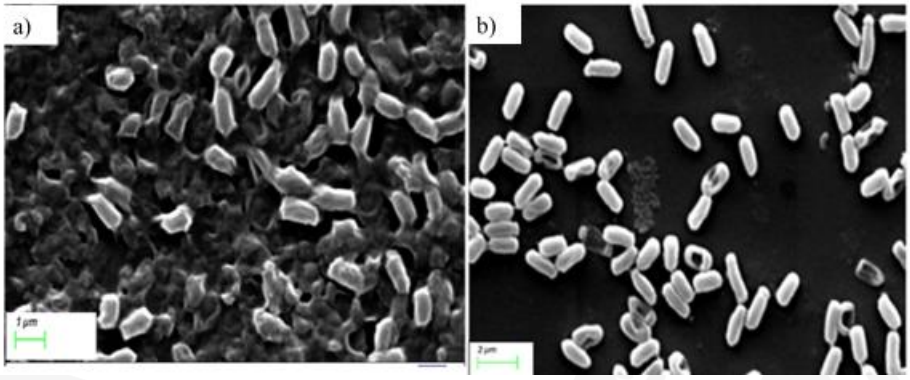


Fig.20 Micrograph from scanning electron microscopy of spores from a) *B. horneckiae* SBP3 and b) *B. subtilis* 168

4.5.2 Vibrational spectroscopy analyses

The spores from the two strains after wet-heat treatment, were harvested and analyzed using Raman spectroscopy. The Raman spectra from spores of *B. horneckiae* SBP3 and *B. subtilis* 168 were obtained before and follow the thermal challenge (shown in Fig.21a and Fig.21b) These spectra showed the characteristic Raman bands associated with the spores components, such as carbohydrates, lipids, proteins, and nucleic acids (Table 11) as reported by de Gelder et al 2007. To elucidate the difference between untreated and treated spectra, the difference spectrum was obtained by subtracting the untreated from treated spectra (Fig.21b Fig.21d).

The Raman spectra after different heat treatment displayed some obvious differences in SBP3 spores (Fig.21a). In particular, the spectra of SBP3 after heat treatment showed a little shift at 667 cm^{-1} related to Ca-DPA, 715 cm^{-1} (ring breathing mode of adenine), 899 cm^{-1} bands associated with the DNA structures, 1192 cm^{-1}, and 1654 cm^{-1} from amide I bands intensities both associated with C-N stretching vibrations, related to proteins changes.

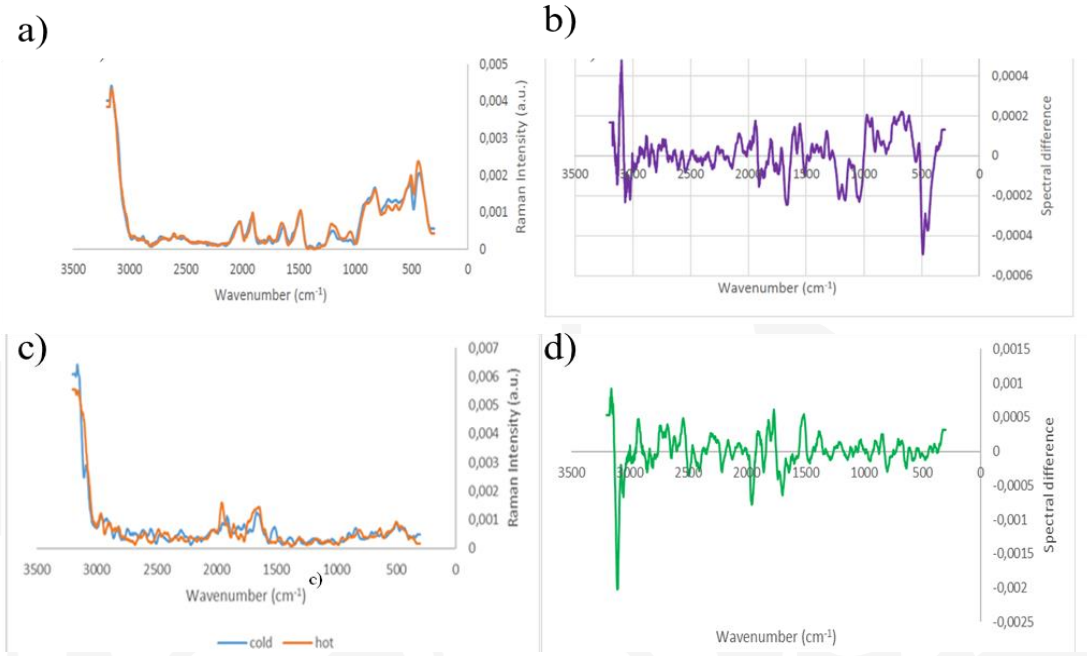


Fig.21 Spectra from spores treated and untreated of a) *B. horneckiae* SBP3 and c) *B.subtilis* 168 and their relative difference spectrum (b, d).

Spectra from spores of Bsu in Fig.21c showed several shifted peaks at higher wavenumber in the heat treatment (in red) compared to the control sample, without any treatment (in blue). The peaks variations were noticeable at 667 cm^{-1} (C-S stretching mode of cytosine), 715 cm^{-1} (ring breathing mode of adenine) 835 cm^{-1} (ring breathing mode of tyrosine and stretching mode of proline), the variations in the range 832-835 cm^{-1} (the DNA B-form) can be taken as markers for the conformational changing in the DNA caused by thermal treatment. Moreover, the shifts were highlighted at 1065 cm^{-1} related to C-N stretching vibrations of protein tertiary structures, 1186 cm^{-1} C-C associated to skeletal vibrations 1178-1182 cm^{-1} from C-H bending and 1660 associated to amide I.

Tab.11 Raman peak assignments reported by De Gelder et al. (2007)

Raman shift (cm^{-1})[a]	Band assignments
527	ν(S–S) stretching (cysteine in spore coat)[b]
622	Phenylalanine
638	ν(C–S) stretching (cysteine in spore coat)[b]
643	Tyrosine
661	CaDPA
723	Adenine, coenzyme A, acetylcoenzyme A
783	Guanine, uracil, citric acid
822–827	CaDPA and tyrosine
853	Tyrosine
1,004	Phenylalanine
1,018	CaDPA
1,031	$\nu(PO_2^-)$ symmetrical stretching
1,250–1,300	Amide III, $\delta(CH_2)$ deformation
1,397	CaDPA
1,448	CaDPA, $\delta(C–H_2)$ deformation (e.g., from fats)
1,576	CaDPA, guanine, adenine
1,616	Tyrosine
1,650–1,680	Amide I

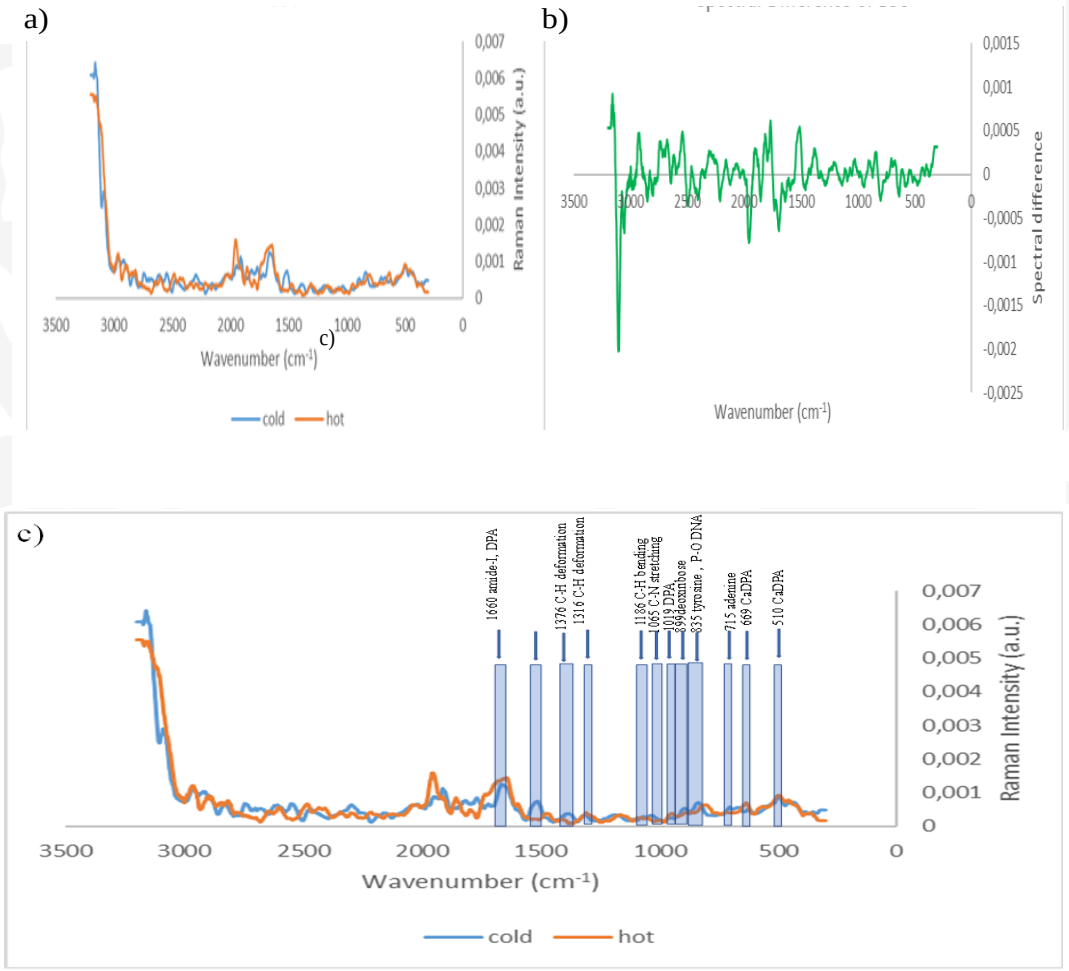


Fig.22 a) Mean Raman spectra of the *B. subtilis* 168 spores untreated(blue) and treated (red) b) Difference spectrum obtained by subtracting the spectra of untreated and treated c) packs assignment with major variation

To elucidate the structural properties of spores from SBP3, FTIR technique was used. The spectra obtained from the different spores are shown in Fig. 23. The FTIR spectra showed the dominant peaks associated with carbohydrates, lipids, proteins, and nucleic acids as reported by Martínez et al. (2016) (Table 12). The three different strains showed differences in the region of the wavenumber 1800−1400 cm^{-1} attributed to amide I and amide II bands (arises from C-N stretching and CHN bending vibrations). The spectra also showed differences at the wavenumber of 1200 cm^{-1} (asymmetric vibrational mode of the phosphate backbone of DNA, PO^{2-}) and in the range of 1200−1000 cm^{-1} which are associated with C–O, C–C stretching, and C–O–H deformation vibrational modes of carbohydrate. The spectra of SBP3 showed all the characteristic regions associated with microbial spectra, including the amide, fatty acid, and polysaccharide peaks (Riddle et al., 1956; Naumann et al., 1982, 1991; Naumann, 1984). The FTIR displays the follow differences in structures of spores: i) the regions attributed to the diaminopimelic acid (DPA) of the quartet of peaks between 650 and 775 cm^{-1} attributed to Ca-DPA (Johnson et al., 2009, 2010), ii) the spectra of SBP3 and Bsu display differences in 1700-1750 cm^{-1} assigned to lipids that could suggest as the composition of membrane are very dissimilar and iii) the band of 1047 cm^{-1} in the spectrum of SBP3 highlights the polysaccharide presence, probably related to the exosporium layer. The spectrum of SBP3 showed similar regions to the spectrum of *B. pumilus* SAFR032, that is well known for its multi-resistant ability to different stressors.

Tab.12 bands assignments of FTIR reported by Martínez et al. (2016).

Nominal frecuency of bands (cm^{-1})	Assigment[a]
3600	O-H stretching of water
3400	H-bonded OH groups of alcohols, phenols and organic acids, as well as H-bonded N-H groups
2971	Methyl (-CH_3) asymmetric stretching of lipids
2917	Methylene (-CH_2) asymmetric stretching of lipids
2850	Methylene (-CH_2) symmetric stretching of lipids
1700–1750	C = O stretching vibrations of carboxylic groups involved in an ester linkage
1660–1628	C = O vibrations of primary amides at sludge
1548	C = O vibrations of primary amides
1450–1410	CH_2 scissor deformation vibrations
1230	Phospholipids (PO2) asymmetric stretching, protein amide III band (C-H and N-H)
1070–1048	–C–O–C of carbohydrates, Si–O–C groups
888–738	Scissoring deformation of CH_2

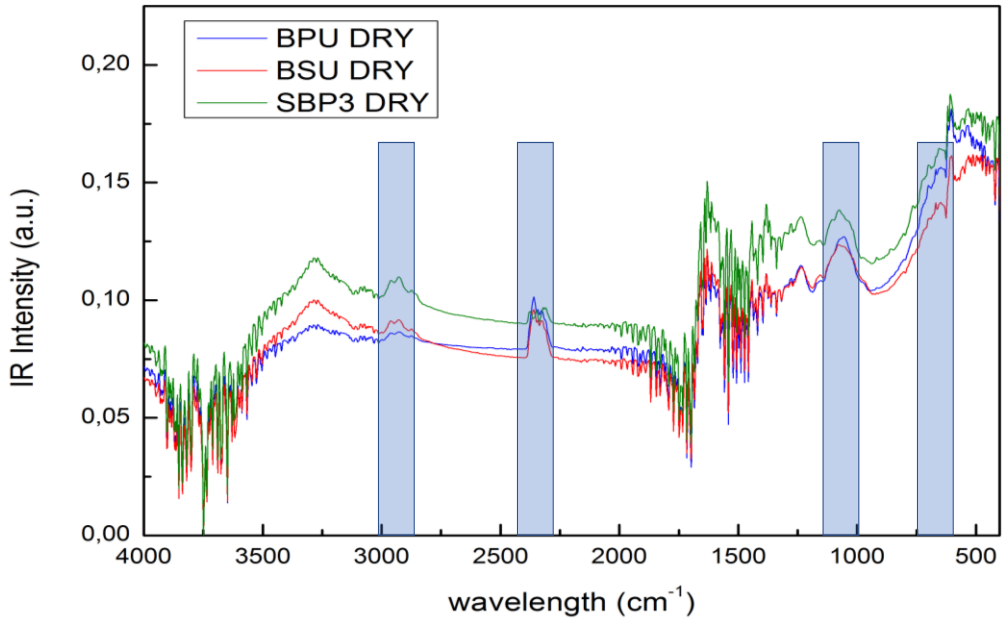


Fig. 23 FTIR spectra of spores from *B. horneckiae* SBP3, *B.subtilis* 168 and *B. pumilus* SAFR032

4.6 Thermal analysis of EPS-T14 by Attenuated Total Reflectance Fourier Transform Infra-Red (ATR-FTIR) spectroscopy

The thermal restrains of the whole biopolymer EPS-T14 and of its main monosaccharides components (*i.e.* fructose, fucose and glucose) was investigated using a biophysical approach able to provide a measure of the system sensitivity to temperature changes. The Attenuated Total Reflectance Fourier Transform Infra-Red (ATR-FTIR) spectroscopy was applied over a temperature range from 20 °C to 80 °C, and each spectrum was analyzed by using innovative mathematical tools: i) non-ideal spectral deviation; ii) OH-stretching band frequency center shift; iii) spectral distance; and iv) wavelet cross-correlation analysis.

Many EPSs from thermophilic bacteria have been reported to be thermostable, as determined only by using the TGA technique, with the highest thermostability registered for the exopolymer from *Geobacillus tepidamans* V246 (280 °C) (Magazù et al.,1998), followed by those from *G. thermodenitrificans* B3-72 (240 °C) and *Bacillus licheniformis* T14 (240 °C) (Spanò et al 2013).

Since thermostability represents a prerequisite in applicative areas that require high temperature processes, in this study the structural changes of EPS-T14 at increasing temperatures have been monitored by using the ATR-FTIR spectroscopic technique, coupled with innovative technique (non-ideal spectral deviation, hypsochromic frequency center shift, spectral distance and wavelet cross correlation analysis).

The spectra obtained by means of ATR-FTIR technique on EPS-T14 and on its main components (*i.e.* fructose, fucose and glucose) (Fig.24) showed the following main features: i) an intense band in the region between 950 cm^{-1} and 1200 cm^{-1}, corresponding to the –C–O, C–CH stretching and to the CO and C–O–C bending of the glycoside ring (Migliardo et al., 2014); ii) at the from 1300 cm^{-1} to 1450 cm^{-1} wavenumber range, bands related to the C–CH and O–CH stretching and –CO stretching are observed (Veleda et al., 2012) a broad and intense band in the region between 2500 cm^{-1} and 3600 cm^{-1} which is composed by several bands ascribed to the –OH stretching of the polymer glycoside ring, and to the –OH stretching of free water and of water involved in hydrogen bonds (Coates,1996).

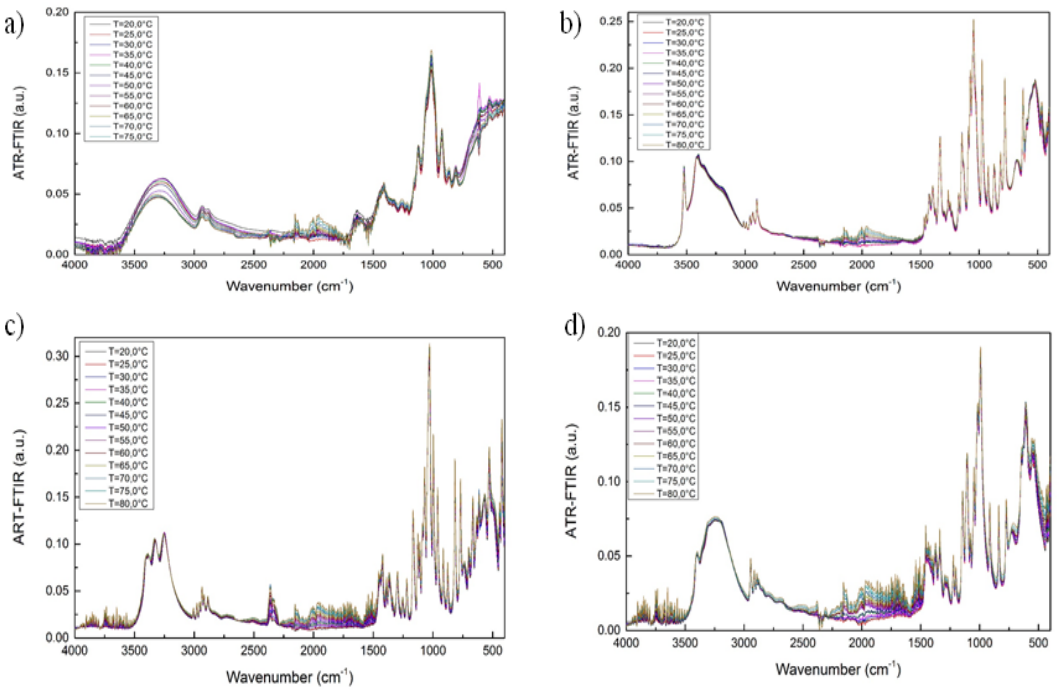


Fig. 24. ATR-FTIR spectra of whole EPS-T14 (a) and its main monosaccharidic

components: fructose (b), fucose (c) and glucose (d) in the $400<\Delta\omega<4000$ cm^{-1}spectral range for positive thermal scans from 20 to 80 °C. The EPS-T14 ideal spectrum, reconstructed starting from the spectra of its main constituents, is shown in Fig. 25, while the spectral deviation, *i.e.* the difference between the experimental spectrum and the ideal spectrum is reported in Fig. 26. As it can be seen from the figure, marked deviations from the ideal behavior are registered in the whole investigated spectral range. Such a circumstance suggests that remarkable interactions take place among the constituents. In particular, although in the range from 1800cm-1 to 2800cm-1 spectral range the evaluated ideal spectrum is almost coincident to the experimental one, remarkable differences occur in the OH-stretching region, which is connected with the system H-bond network.The non-ideal spectral deviation revealed that the real spectrum of EPS-T14 is not the simple sum of its components, but it seems to acquire new properties and different responses at increasing temperature. This different thermal behavior could be ascribed to the interaction of its three main monosaccharidic components (i.e. fructose, fucose and glucose), that contribute differently to create the complex unique matrix of EPS-T14

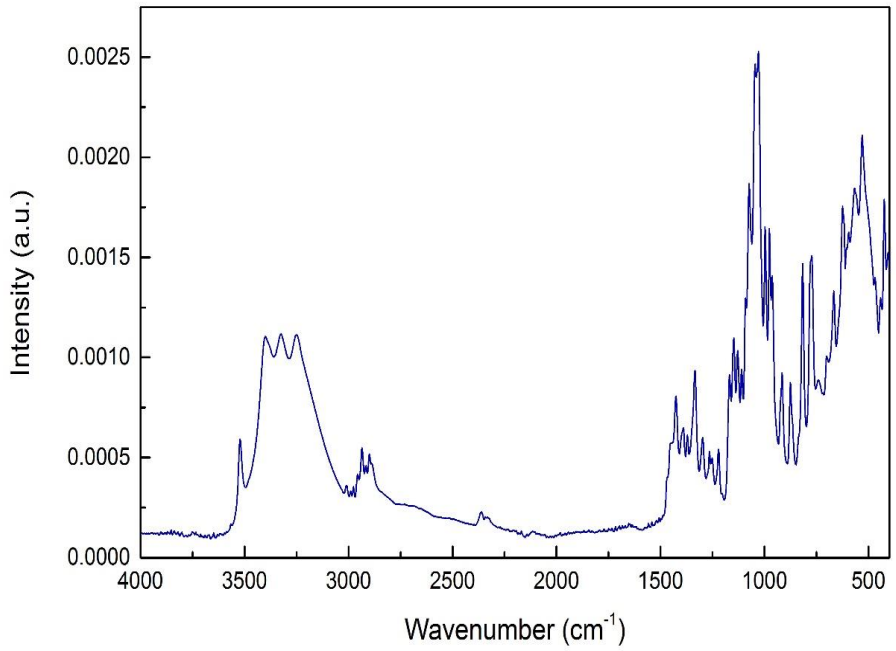


Fig. 25 Ideal spectrum of EPS-T14 reconstructed from the spectra of its main constituents, obtained using ATR-FTIR in the $400<\Delta\omega<4000$ cm^{-1}spectral range at 25 °C.

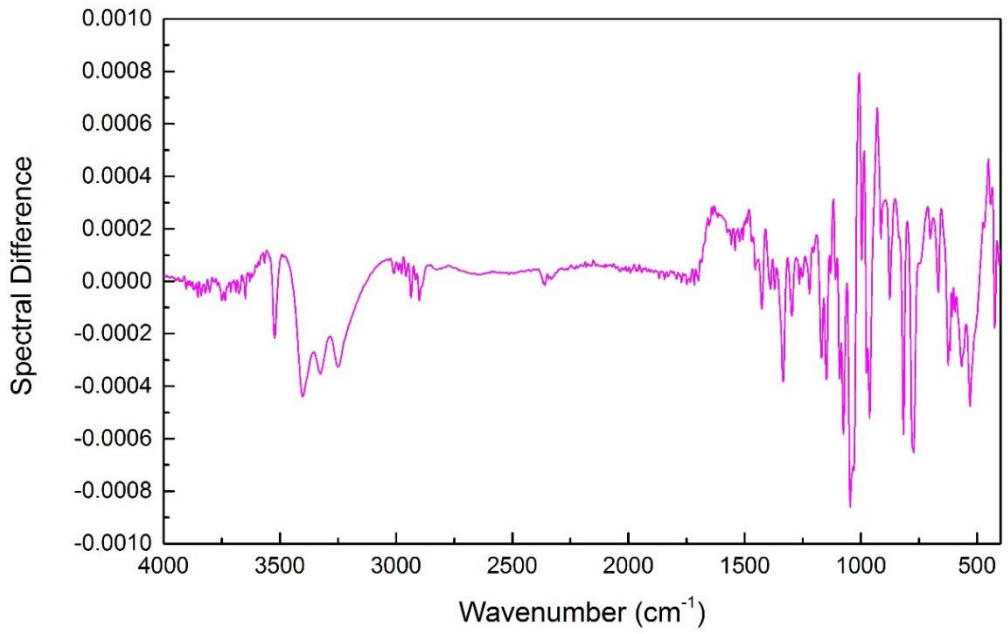


Fig. 26 Difference spectrum obtained by substracting ideal spectrum to experimental spectrum of EPS-T14

3D-FTIR spectra of EPS-T14 in the $3000 < \Delta\omega < 3600$ cm^{-1} range as a function of temperature are reported in Fig. 27a. The EPS-T14 OH stretching band frequency shift in the temperature range from 20 °C to 80 °C changes from 3271 cm^{-1} to 3313 cm^{-1}. Furthermore, this registered hypsochromic frequency shift changes linearly (5) with the increasing of the temperature, as shown in Fig. 27b. In order to fit the data, the following equation was employed:

$$\omega(T) = A + BT \qquad (5)$$

The thermal restraint determined by hypsochromic frequency center shift was equal to 1.49.

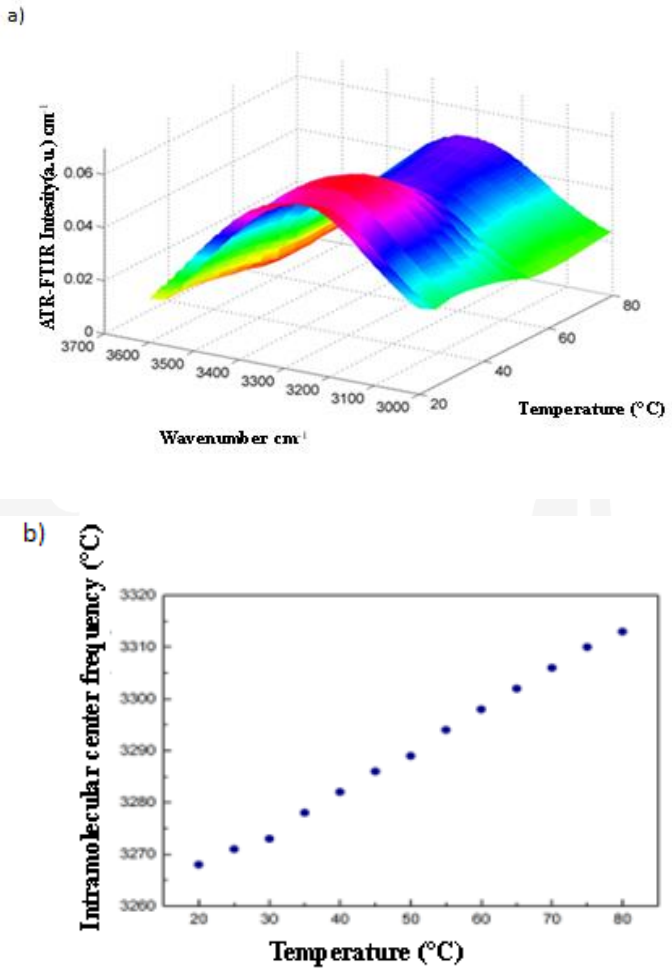


Fig. 27 Hypsochromic frequency shift: a) 3D FTIR spectra of EPS-T14 in the $3000 < \Delta\omega < 3600$ cm^{-1} range as a function of temperature and intensity, and b) the center frequency of OH stretching band of EPS-T14 as a function of temperature. Black dots: experimental data.

The spectral analyses (hypsochromic frequency center shift and spectral distance) convey coherent information on the thermal evolution of EPS-T14, since both showed that the whole biopolymer was stable at the maximum value of the tested temperature (80 °C).

Spectral distance analysis of the spectra of EPS-T14 at increasing temperatures showed a sigmoid trend, with an inflection point at T=50 °C (Fig.28).

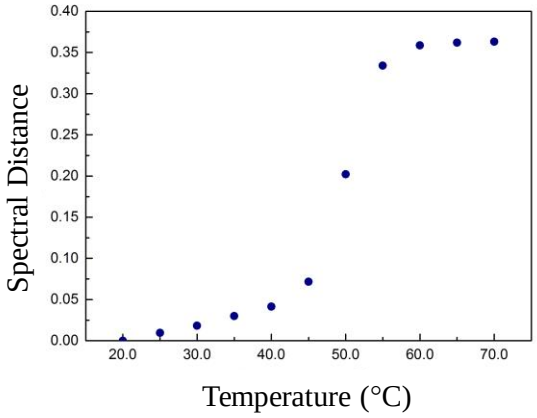


Fig.28 Spectral Distance of the intramolecular OH stretching bands of the EPS1-T14 at the increasing temperature.

The SD versus temperature behavior followed a logistic function trend allowing to extract quantitative information on the relaxation amplitude, as well as on its temperature localization. The analysis by wavelet tools revealed different pathways of EPS1-T14 and its components (Fig.29), where the shaded region indicates the Cone Of Influence (COI). This advanced investigation indicated that the spectrum of fucose is more closely correlated to that of the whole EPS-T14, whereas glucose is the less correlated. This finding suggests that fucose plays a key role in the EPS-T14 structure.

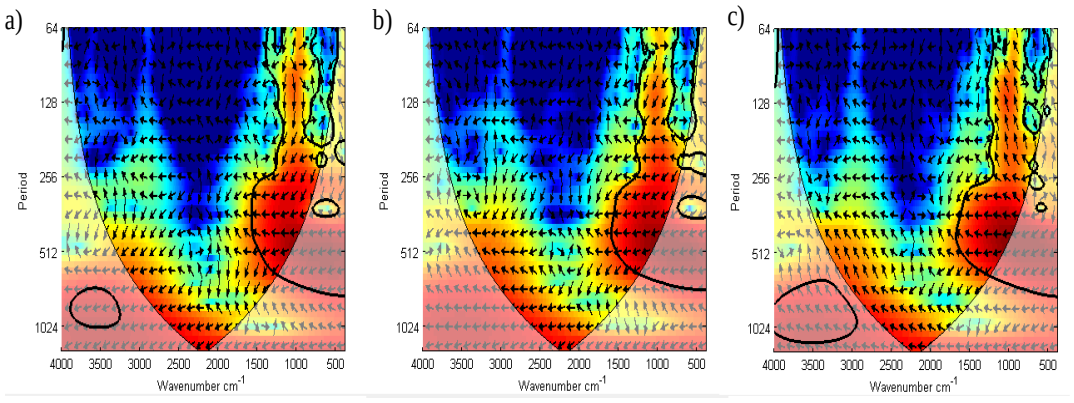


Fig. 29 Wavelet Cross Correlation between the whole EPS1-T14 and fructose a), fucose b) and glucose c) at 25 °C.

5 Discussions

The increasing knowledge of microbial physiology in extreme environments has led numerous scientists to consider the possibility of finding life in various planetary bodies within the Solar System as Europa, Enceladus (Jupiter and Saturn satellites), and Mars (Nicholson et al., 2000, 2009; Horneck et al., 2010; reviewed in Fajardo-Cavazos et al., 2016). However, robotic probes, including their components and assembly facilities, which used to detect biosignatures in extraterrestrial environments, are in need of new cleaning and sterilization procedures to avoid contamination with terrestrial organisms and subsequent false-positive detection (reviewed in Link et al., 2004; Crawford, 2005; Kempf et al., 2005; Nicholson et al., 2009; Khodadad et al., 2017). On the other hand, the identification of biosignatures and set up of analysis technique, including IR spectroscopy are developed to search the trace of life in the Universe.

Since cold (Antarctica soils) and hot (Eolian shallow hydrothermal vents) environments have parallel conditions to those of extraterrestrial environments, microorganisms inhabiting extreme conditions could help to gain new knowledge for understanding the environmental constraints for life with broader implications to the field of Astrobiology.

Our results indicate that strains *Bacillus horneckiae* SBP3, *B.licheniformis* T14, also *Geobacillus vulcani* (GV), isolated from the Eolian hydrothermal vents, were more thermotolerant than their closest phylogenetic relatives. As inhabitants of hot acidic and salty hydrothermal vents the strains possess a great physiological versatility that allow them to adapt to the severe vent conditions (Caccamo et al., 2000; Gugliandolo et al., 2012). Despite belonging to the same species, *Bacillus horneckiae* SBP3, isolated from a vent with the highest temperature registered (130°C), and *Bacillus horneckiae* DSM 23175^{T} responded differently to heat. This is reflected in the measured fatty acid content of the two strains (Tab 13). Membrane lipids of the strain SBP3 may be designated as thermophilic lipids, by displaying the highest iso-C15 (63%) and the low anteiso-C15 (9%) fatty acids content (Tab 13).

Tab.13 Comparison of the fatty acid methyl esters membrane composition of SBP3 from a hydrothermal vent off Panarea with its closest phylogenetic relative strain *Bacillus horneckiae* (Bho) and the bacilli space spacecraft associated: *B. pumilus* SAFR032 (Bpu) and *B. nealsonii* (Bne).

	SBP3	Bho	Bpu	Bne
Fatty acid				
straight-chain saturated				
14:0			3	12.3
16:0	0.8	0.5	6.2	5.2
Terminally branched saturated				
12:0 iso			1.4	
13:0 iso			7.6	2.9
14:0 iso	3.4	4.0	5.0	6.6
15:0 iso	63.1	54.3	20.9	26.4
16:0 iso	3.5	2.5	10.2	2.1
17:0 iso	2.0	1.3	6.9	
13:0 anteiso		19.9	1.9	
15:0 anteiso	8.8	2.3	4.5	32.2
17:0 anteiso	1.8		2.2.	3.0
16:1ω7c alcohol	7.8	6.8	4.6	1.8
17:1 ω10c alcohol	3.7	2.3	2.2	3.0

Thermophiles, able to grow between 45°C and 75°C, possess dominant iso-C15 fatty acid content (30-50%), whereas the anteiso-C15 fatty acid are prevalent in psychrophiles (Koga, 2012). In contrast to strain SBP3, membrane lipids of *B. pumilus* and *B. nealsonii* displayed significant lesser content of iso-C15 fatty acids (Tab. 13). The fatty acid content of the SBP3 strain is, therefore, highly concordant with the high temperature (130°C) vent environment from which it was isolated, suggesting potential membrane adaptations to the extreme temperature.

To expand our knowledge on the limitation of terrestrial life, including sporicidal treatments and on the ability to survive under conditions mimicking space

environments in this work spores from strains isolated from the shallow hydrothermal vent and Antarctica soils, were evaluated for the resistance to: i) environmental stresses (as heat and acid or alkalophilic conditions) that could also simulate conditions in the celestial bodies Europa, Enceladus and Mars and ii) simulating space conditions that are not reached on the Earth surface (as UV-C, X-rays radiation, heavy ions particles, and iii) traditional and novel procedures to decontaminate spacecraft environment (*e.g.* hydrogen peroxide).

The spores from hydrothermal strains of SBP3 and T14 were frequently the most resistant to all tested stresses compared to environmental strains (Tab14).

Table 14 Summarize of the resistance pathway of the spores tested. In green the most frequently resistant spores from environmental isolates and in yellow the most resistant reference strain.

	Strains	Treatment											
		Enviromental stressors				Sporicidial treatments and simulating space enviroments stressors							
									Irradiation				
		Wet-heat	Dry-heat	Acid pH	Alkalinic pH	UV-C	H_2O_2	LPP	Ar	Fe	He	X-rays	Dessication
Isolates	APA	+++	+	++	++	+	+	+	nt	nt	nt	+	+
	Gv	++++	+++	+	+++	+	nt	nt	+	nt	nt	nt	nt
	P82	+++	nt	+++	+	+	nt	nt	nt	nt	nt	nt	nt
	SBP3	++++	++	+++	+++	+++	+++	+++	+++	+	+++	+++	++
	T14	+++	++	+++	+++	+++	nt	nt	+++	+++	+++	nt	nt
	A30	nt	nt	nt	nt	+	nt	nt	nt	nt	nt	nt	nt
	A34	+	nt	nt	nt	+++	nt	nt	+	++	++	nt	nt
	A45	+	nt	nt	nt	++	nt	nt	nt	nt	nt	nt	nt
	A43	+++	nt	+++	++++	++	nt	nt	++	++	+++	nt	nt
	B58	+	nt	nt	nt	+	nt	nt	nt	nt	nt	nt	nt
	B51	+	nt	+++	+++	+	nt	nt	nt	nt	nt	nt	nt
Reference strains	Gs	++++	+++	nt		++	nt	nt	nt	nt	nt	nt	nt
	Bho	++	+	nt		++	+++	++	nt	nt	nt	+	+
	Bsu	++	+	+	++	+++	++	++	nt	nt	nt	+++	+++
	Bne	+++	+++	nt		++++	+++	++++	nt	nt	nt	+++	+++
	Bpu	++++	+++	nt		++++	++++	++++	nt	nt	nt	++++	++++

nt = not tested
+ = less resistant
++ = resistant
+++ = high resistant
++++ = higher resistant

When compared to the spores from biodosimetry strain *B. subtilis* 168, spores from SBP3, T14, Gv and P82, isolated from shallow hydrothermal vents, and those from the Antarctic strain A43 were more resistant to both dry- and wet-heat stresses.

The heat resistance is considered the hallmark property of bacterial spores, which represent the main issue for microbial contamination in different human activities and also, in the planetary protection field. In the aerospace industry microorganisms could have uniquely disastrous effects, since spacecraft could contaminate other cosmic bodies with terrestrial microbes, if the space-bound vehicles are not sufficiently decontaminated before launch (Carlson et al., 2018). Spores of *Geobacillus* spp are more resistant to high temperature than those of *Bacillus* spp, nevertheless, spores of *B. atrophaeus* ATCC 9372, indicated as the official bioindicator in dry-heat sterilization processes (US Phamacopeia, 2007), were reported to possess similar dry thermal resistance of *G.stearothermophilus* (Wood et al. 2009), used to validate steam sterilization procedures.

The adaptation to high temperatures of spore's structures, such as the lipid composition of membranes, the high stability of proteins and of DNA, could allow the thermophilic strains to resist to extreme environmental conditions registered at shallow hydrothermal vents.

Across all treatments, strain SBP3 shared comparable spore resistance with its closely related type strains as well as with space-relevant *Bacillus* species. Spores of the SBP3 strain demonstrated their ability to survive to various stress conditions at a higher degree, in many cases, than those of the current space-biological model system *B. subtilis*168. As reported by Horneck (2012) spores from *B.subtilis* 168 was able to resist to real space environment for more than 300 days..

Differently from wet-heat, the **dry-heat** stress could kill the spores through oxidation processes (Russel, 2001) and DNA disruption (Setlow, 2006). Spores from thermophilic strains SBP3, T14 and Gv able to resist to dry heat were also resistant to desiccation, confirming data previously reported by several authors (Di Donato et al., 2018; Mastascusa et al., 2014; Nicholson et al., 2000). As revealed for *B. subtilis*168 spores, the mechanisms involved in dry-heat and desiccation treatments were attributed to the α/β type small acid soluble proteins (SASP) (Setlow, 2006). Interestingly, the spore resistance to heat seems to be related also to the dimension of spores, since those from *Geobacillus* showed higher volume than those from *Bacillus*,

probably due to the different water contents in the spore-core. However, these findings need to be deeper investigated in the next future.

The high resistance to **wet heat** of spores from SBP3, could be linked to their relatively low core water content and to the intrinsic thermostability of their macromolecules developed to inhabit the high temperature vents. It has been reported that thermophiles invariably have more wet heat resistant spores than those of mesophiles (Nicholson et al., 2000). Contrary to what may be expected at elevated temperatures in dry conditions, spore inactivation by wet heat treatment does not occur through DNA damage (e.g., depurination) (Coleman and Setlow, 2009). Rather, evidence suggests that wet heat treatment targets spore proteins through denaturation processes (Belliveau et al., 1992; reviewed in Setlow, 2016). As previously reported, spores of *B. subtilis* are well protected against DNA damage caused by wet heat treatment due to the saturation of the spore DNA with small acid soluble proteins (SASPs) and the high levels of divalent mineral ions in spores (mainly Ca^{2+} and Mg^{2+} and to a lesser extent Mn^{2+}) (Nicholson et al., 2000). In general, the higher the levels of mineral ions associated with the spore core, the greater the wet heat resistance of the spores (Melly et al., 2002; Setlow, 2016). Continued investigation into these mechanisms could offer greater understanding of spore resistance to thermal extremes.

Vibrational spectra of spores in this region are dominated by the amide I and II normal assigned to proteins. The greater variation for Bsu are in the region of amide I and amide III at 1376 cm^{-1} that indicated the deep denaturation of proteins in both conformation helix and beta sheet.

The shifted peaks associated with protein were noticed as the major contributors to the Raman spectral differentiation among heated spores and control untreated samples. The denaturation of the proteins linearly decreased as protein structural band at 1645 cm^{-1}. The ATR- FTIR spectra showed some differences about the species of SBP3 and Bsu that suggesting a deeply different composition of spore structures. SBP3 showed some similar peaks with *B. pumilus* SAFR032 among the most resistant *Bacillus* spores find until now. Therefore, the exosporium that appears evident in SBP3 SEM micrography (Fig.20) contribute to resist of a multiple stress condition but it is not

enough to defender the spores by the damage induced by high temperature. As reported in previous studies in *B. subtilis* spores low core water content, high levels of dipicolinic acid

(DPA) in chelation with divalent cations (Ca^{2+}-DPA), abundant small acid-soluble spore proteins (SASP), and the intrinsic stability of spore proteins overall were involved in spores' resistance to different factors including wet heat (Setlow 2006).
SBP3 spore resistance to **LPP, H_2O_2,** was comparable with or exceeding that of *B. subtilis* 168 under all simulated extraterrestrial stressors. Because of similar spore resistance patterns, we may suppose that similar mechanisms of resistance could be involved. *B. subtilis* spore resistance to chemical, heat, and LPP exposure is known to be due to the structure of the coat layers, the impermeability of the spore core, and the low water content of the spore (Setlow, 2016) rather than by the presence of enzymes involved in the reactive oxygen species detoxification, such as catalases and superoxide dismutase (Moeller et al., 2014).
For spores of *B. pumilus* SAFR032, two additional catalases have been isolated and characterized that confer an increased H_2O_2 resistance (Gioia et al., 2007; Checinska et al., 2012; Tirumalai et al., 2013). It should be kept in mind that multiple mechanisms as suggested by Setlow (2006) may be involved in spore resistance to specific agents, since the different responses to **UV** and **ionizing radiation** exposure could be due to (i) the content of α/β-type SASPs, core water content, level of mineralization, or number of chromosomes and to (ii) the efficiency of the DNA repair processes during spore outgrowth (Setlow, 2016).
In the frame of the international project "Starlife", spores from the strains T14, SBP3, Gv, A34 and A43 were exposed to heavy ions particles (He, Ar and Fe). Although the heavy ions particles only contribute to roughly 1% of the flux of cosmic radiation, they are considered as a potential major concern to living beings in space, especially for long-term missions beyond the protection of Earth's magnetosphere (Durante and Cucinotta, 2011). In this experiment the doses of radiation of heavy ions particles using to irradiate the spores were equivalent at least to 10 years on the surface of Mars and 5 years in space environments (Hassler et al.,2014). All spores were resistant to heavy ions particles to maximum dose. Interestingly, all spores were resistant to Fe ions

exposition suggesting the possibility of interplanetary movement of these spores. Based on the observed resistance of spores from environmental strains to Fe and He ions, our results partially confirm that the sporicidal action increased in the order X-rays > Helium ions > Carbon ions > Silicium ions > Iron ions as previously assessed (Hassler et al.,2014). Therefore, the spore inactivation is closely related to the linear energy transmission (LET) of the applied heavy ion species (Hassler et al., 2014).

Spores from *B. licheniformis* T14 were more heat resistant than those from its closest phylogenetically related strain *B. licheniformis* DSM13 (data not shown), suggesting that the genetic make-up at species level is not sufficient to determine the degree of heat resistance. StrainT14 has been reported able to produce an exopolysaccharide (EPS-T14) (Spano et al.,2013). This exopolymer is produced by vegetative cells in response to stress condition to form a complex matrix called biofilm.

This polymer, synthetized during the sporulation process (Marvasi et al.2010), could confer resistance to heat stress and protection against heat damage and desiccation of spores (Elhariry, 2008). The high thermostability of EPS-T14 has been recently demonstrated by ATR-FTIR analysis (Caccamo et al 2018). In details, the high resistance of the biopolymer at the increasing temperature could be explained by the increasing H-bounded connectivity. Since the frequency center linearly shifted with the increasing temperature, we may suppose that all the rheological properties (i.e. viscosity, density, etc) of EPS-T14 could be maintained at 80 °C. Based on results obtained by TGA analysis, the decomposition temperature of EPS1-T14 (240 °C) appeared higher than that of fucoidan (210 °C) (Saravana et al 2016). However, to our knowledge, no data on the thermal behavior of fucoidan have been until now reported for further comparison. Nowadays, chitosan is the most characterized polysaccharide in the field of drug and food deliveries, however its uses are still limited, due to its relatively lower thermal stability (below 40 °C), as spectroscopically determined (Szymańsk et al 2015). The wavelet analysis revealed that the fucose spectrum was correlated to that of the whole EPS-T14, suggesting that fucose may be involved in both biological and structural properties of EPS-T14. For its physicochemical properties and thermo-stability EPS-T14 could be also considered as useful biopolymer for nanoparticle composites.

Strains SBP3 and T14 isolated from the hydrothermal vents could represent novel bacterial models for the investigation of spore responses to space environmental stressors and sterilization treatments (Friedline et al., 2015; Mandic-Mulec et al., 2016). Because of its resistance to wet heat, radiative stressors, and oxidative stressors suggested from this study, SBP3 could be a superior bacterial model organism for future investigations into the potential for contamination of an ocean world, which could likely harbor these environmental stressors. Such environments, in particular, may play increasing roles in future space exploration and the search for life on ocean worlds (Rummel and Race, 2016; Lunine, 2017).

Conclusions

The spores from the bacilli isolated from Edmonson point and Eolian shallow hydrothermal vents could represent new model to study the different response to stress conditions also beyond the Earth.

The strains adapted to cold environments produce spores more sensitive to heat, even if the strain A43 possesses similar pathway resistance to thermophilic strains suggesting that the mechanisms involved in the heat spore resistance could be independent from the sporulation conditions (also find in environment), as suggested previously (Setlow, 2006).

Spore formers and thermophiles are among the select bacterial groups able to survive adverse environmental extremes, presenting implications for planetary protection initiatives (Gosh et al., 2010). The polyextremophilic strains T14 and SBP3 were able to produce spores with astounding resistance to different stresses including radiations as UV-C, X-rays and Heavy ions particles. Spores from SBP3 exhibited more resistance than those from its closest strain *B. horneckiae* DSM 23475^T and showed similar resistance to the relevant astrobiology species *B. nealsonii* and *B. subtilis*168, also in decontamination treatments (Low Pressure Plasma, and H_2O_2).

In order to understanding the mechanisms involved in the multi-resistance of the spores from SBP3, the spore structural composition was deeply investigated by FTIR and Raman analyses in comparison to *B. subtilis* 168. Results indicated that the resistance to heat stress of spores from SBP3 could be related to the protein stability.

In this work, we suggest that exopolysaccharides, produced by the vegetative cells, could also protect the spores immersed in the biofilm matrix. As reported for the EPS-T14, its thermostability could provide an efficient barrier against the desiccation and heat stress.

This study encourages future research, especially regarding the mechanisms involved in spore resistance to extreme conditions which is currently largely confined to *B. subtilis* spore mechanisms but almost unknown for spores of other *Bacillus* species, including SBP3 and T14 especially in perspectives of novel exploration in ocean world as reported in a recent article by Hendrix et al. (2018).

Due to the extreme resistance of the dormant spores could represent the standard for the search of life in habitability celestial bodies such as Mars Europa and Enceladus and also a novel biodosimetry strains for biosecurity in Planetary Protection Program to avoid the space contamination.

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