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Filtration of *Bacillus subtilis* and *Bacillus cereus* spores in a pyroclastic topsoil, carbonate Apennines, southern Italy

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ABSTRACT

A comparative study on the filtration of *Bacillus subtilis* and *Bacillus cereus* spores in a pyroclastic topsoil was performed in laboratory using surfactant-free solutions and solutions with the surfactant sodium dodecyl sulphate (SDS) (anionic). The results of the column experiments demonstrate that the SDS does not significantly influence the retention of both *B. subtilis* and *B. cereus* spores. Since the SDS is adsorbed through hydrophobic interaction with the organic matter of soil media, these results suggest that hydrophobic interaction between spores and organic matter does not play a significant role on filtration processes within the studied topsoil. This statement is of utmost importance taking into consideration the hydrophobic nature of *Bacillus* spores and the very high organic matter content in the studied topsoil (20–34%). Conversely, the retention of the analyzed spores seems to be influenced by the pore size exclusion phenomenon.

1. Introduction

The genus *Bacillus* contains aerobic or facultatively aerobic, spore-forming and gram-positive bacteria, commonly associated with soil. Recent studies demonstrated their association with animal faeces [1,2] and showed that also spores of *Bacillus subtilis* are not transient passengers of the gastrointestinal tract but have adapted to carry out their entire life cycle within this environment [3].

Spores from a number of different *Bacillus* spp. are currently being used as probiotics and competitive exclusion agents [4,5], but some species (*B. anthracis* and *B. cereus*) are pathogenic to humans and/or animals [6].

Bacillus spores consist of several protective layers surrounding the nucleoid in the spore core [7–9]. This structural organization makes the spores extremely resistant to external physical and chemical insults and in part determines their exceptional longevity in the environment [9,10]. Due to their higher longevity, *Bacillus* spores can be used as effective tracers to analyze interaction between sources of fecal contamination and groundwater over wide distances [11–13]. Spores may also accelerate the transport of heavy metals in groundwater, as demonstrated investigating cadmium transport adsorbed on *B. subtilis* spores in column experiments [14]. Taking into consideration the hydrophobic nature of *Bacillus* spores, hydrophobic interaction may play a role in the adhesion of these spores to surfaces [15–17], therefore influencing filtration processes through soil media. However, *Bacillus* spore hydrophobicity varies among species and strains, with spores of *B. cereus* more hydrophobic than spores of *B. subtilis* [15,17].

Thus, we aimed in this study (a) to isolate and identify the main aerobic sporeformers belonging to the genus *Bacillus* from pasture areas in the Acqua dei Faggi experimental field site (southern Italy), and (b) analyze the role of hydrophobic interaction on filtration processes of *B. subtilis* and *B. cereus* spores through the pyroclastic topsoil. This second topic was analyzed through a comparative study on retention of *B. subtilis* and *B. cereus* spores within the pyroclastic topsoil using surfactant-free solutions and solutions with the surfactant sodium dodecyl sulphate (SDS) (anionic). The addition of surfactants to soil influences the interaction soil–microorganisms and the mobility of microorganisms in soil [18]. The SDS was chosen in such a study because it is known that it is adsorbed through hydrophobic interaction with the organic matter (OM) of different soil media [19]. Thus, taking into consideration the hydrophobic nature of *Bacillus* spores, the SDS allows for analyzing the role of hydrophobicity on spores retention.

2. Site description

The Acqua dei Faggi experimental site at Longano in the Molise region (southern Italy; Fig. 1) consists mainly of limestone (Cretaceous-Oligocene). The aquifer boundaries are fault zones that act as barriers to groundwater flow and compartmentalize

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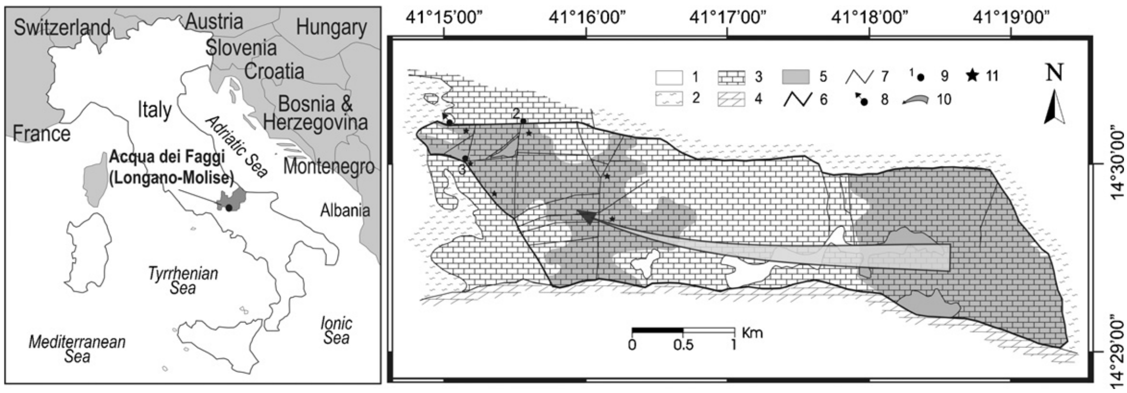

Fig. 1. Hydrogeological map (1: quaternary deposits; 2: low permeability rocks; 3: limestone; 4: dolostone; 5: pasture area; 6: aquifer boundary; 7: fault; 8: perennial spring; 9: seasonal spring; 10: main groundwater flow direction; 11: site of topsoil block collection) (after Naclerio et al. [26]).

the aquifer system. However, some fault zones allow significant groundwater flowthrough, and interdependence of hydraulic heads upgradient and downgradient of these faults has been observed [20]. The aquifer functions as a basin-in-series system, where seasonal springs occur along some fault zones [20]. At basin scale, the groundwater flows westwards towards the perennial spring (Fig. 1). Due to high fracture density and good interconnection of openings within limestone, diffuse infiltration and groundwater flow are observed in the unsaturated and in the saturated medium, respectively [21]. The limestone aquifer is laterally and vertically well connected in the subsurface and the fracture spacing is sufficiently dense to apply the continuum approach at the metric scale. The fractured limestone is hydraulically similar to granular porous media and Darcy's law can be applied to describe groundwater flow [22].

In pasture areas carbonate rocks (R) underlie a pyroclastic soil (*Vitric Andosols*) [23]), which is characterized by a profile A/R [24]. Horizon A is usually less than 15 cm thick. pH ranges from 5.1 to 5.4, while Total Kjeldahl Nitrogen from 0.5% to 0.6% [25]. The soil is characterized by high content in OM (20–35% [26]). Taking into account the high OM content and the fibrous structure it can be classified as organic soil (Pt) (Unified Soil Classification System [27]). Specific gravity values range from 2.06 g cm^{-3} to 2.36 g cm^{-3} and are compatible with values of alkali-potassic volcanic minerals typical of such soils of pyroclastic origin derived from eruptive centres of the Campania region (south of the Molise region). Grain size analyses show a global homogeneity testified by a narrow envelope of grain size curves with an uniformity coefficient (U) which ranges between 3 and 9 and by a prevailing sandy loam texture (as defined by the US Department of Agriculture [26,28]).

Recent studies suggested a significant microbial contamination of groundwater within the Acqua dei Faggi aquifer system, mainly due to cattle grazing (several hundred heads throughout the year [25,26,29]). Pasture areas cover 55% of the site, while a beech woodland covers 45% (Fig. 1).

3. Methodology

3.1. Preparation of soil samples, bacterial isolation, and growth conditions

Twenty soil samples were collected from the experimental field site. Soil aliquots were initially diluted 1:1 (wt:vol) in buffered peptone–water (Oxoid) and resuspended by vigorous vortexing until an evenly distributed suspension was obtained. Aerobic spore-forming isolates were then selected by heat treatment [30]. For heat treatment, the suspension was further diluted 1 : 10 in buffered peptone–water and incubated at 65 °C for 20 or 45 min. Subsequent plating of 0.1-ml aliquots of appropriate 10-fold serial dilutions in buffered peptone–water (up to 10^{-5}) was done aerobically on Difco nutrient agar or Luria-Bertani (LB) plates, both of which support germination. Although no quantification was attempted, a measurable number of colonies were routinely obtained on 10^{-2} to 10^{-3} dilution plates after 24–48 h of incubation at 37 °C. Colonies representing different morphologies were picked at random and purified by restreaking on agar plates of the same media. Production of spores by growth of the purified isolates on Difco sporulation medium (DSM) [30] plates was confirmed by phase-contrast microscopy before storage of the isolates at −80 °C in brain–heart-infusion broth (BHI, Difco) with 30% glycerol.

3.2. Taxonomic classification

Taxonomic classification of *Bacillus* isolates detected in soil samples was performed using the API 50 CHB system (bioMérieux) and by sequence analysis of one of the 16S rRNA genes amplified with two universal oligonucleotides: P1 (5-GCGGCGTGCCTAATACATGC) and P2 (5-CACCTTCCGATACGGCTACC), annealing to nucleotides 40–59 and 1532–1513, respectively, of *B. subtilis* rrnE.

The most representative non-pathogenic species and the most representative pathogenic species were then used to carry out the column experiments.

3.3. Column experiments

Spores filtration was investigated through column tests in topsoil blocks which were sampled at the Acqua dei Faggi experimental field site. According to described method [25,26], 12 intact soil blocks of *Vitric Andosols* were collected randomly from the test site in pasture areas, close to the sites where other soil samples were collected for previous studies (Fig. 1). To minimize disturbance of samples sod-covered blocks (181.36 cm^2 by 11 cm deep) were carved from undisturbed soil directly pushing permeameter cells used for column tests into the topsoil. All blocks were covered with plastic and transported to the laboratory where the experimental procedure started immediately at room temperature.

The column experiments were carried out in a standard permeameter. The water solution was applied on the top of blocks, while the outflow was collected at the bottom using sterile plastic tubes. A peristaltic pump was used to constantly push the water solution through the soil. 2000 ml of water solution were poured at a velocity of 2.5 mm h^{-1}, in soil samples S1 to S3 and C1 to C3, and at a velocity of 5.0 mm h^{-1}, in soil samples S4 to S6 and C4 to C6. Due to the clay content into the soil [26], a solution with 0.001 M $CaCl_2$ was used as rainwater to prevent dispersion of clays within the soil and the column plugging [31]. The interaction between *Bacillus* spores and soil blocks was investigated through two different strains for