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# Accelerated aging caused diversity and specificity loss in the bacterial communities of *Brassica napus* seedlings

Nina Bziuk[1,2,*], Simon Görtz[3], Jennifer Zur[3], Elena Beny[2], Steffen Rietz[3], Amine Abbadi[3], Birgit Wassermann[1,2,#] and Gabriele Berg[2,4,5,#]

[1]Austrian Centre of Industrial Biotechnology (ACIB), Graz, Austria; [2]Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria; [3]NPZ Innovation GmbH, Holtsee, Germany; [4]Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Potsdam, Germany and [5]Institute for Biochemistry and Biology, Potsdam University, Potsdam, Germany

**Research Paper**

*Current address: Department of Biosystems, Division of Crop Biotechnics, University of Leuven, Willem de Croylaan 42, 3001 Leuven, Belgium.
#These authors contributed equally to the work.

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**Corresponding author:** Birgit Wassermann; Email: birgit.wassermann@tugraz.at

## Abstract

Healthy seeds are an important component of global food security, and their microbiome was recently identified as crucial for plant growth, resilience and health. Seed vigour is highly affected by storage conditions and aging. To study the impact of seed aging on the *Brassica napus* seed bacterial community, we conducted accelerated aging tests (45°C, humidity > 95%) with seed lots of four genotypes originating from two field sites in Germany. We found a strong effect of accelerated aging on germination, seedling phenotypes, as well as the seed bacterial community. Control seeds developed mainly into normal seedlings and were characterized by diverse bacterial communities comprising typical core seed microbes. Accelerated aging resulted in abnormal germination and reduced total germination. Furthermore, accelerated aging reduced diversity and evenness of the seed bacterial community and contributed to a shift from Gram-negative to Gram-positive bacteria. This effect, especially the enrichment of Firmicutes, was found irrespective of the genotype and field site; however, the way stress affected bacterial taxa varied, depended on both factors. *Tumebacillus* and *Bacillus* showed a significant negative correlation with germination phenotype, whereas alpha diversity correlated positively with a high total germination. At the functional level, the majority of isolated bacteria demonstrated plant-beneficial characteristics, showing a greater beneficial potential in the aged seeds. Our results show that accelerated aging tests affect the seed bacterial community structure and diversity, and correlate with the presence of certain taxa, which might have an effect on germination and seedling phenotype.

## Introduction

The seed microbiome is the primary microbial inoculum for the plant microbiota (Barret et al., 2015). These plant-associated microbes are acquired horizontally from the environment or vertically across generations (Shade et al., 2017; Abdelfattah et al., 2021). Some seed microbes were found to colonize the emerging seedling and adult plants efficiently, and exert lasting beneficial impacts on plant fitness by promoting seed germination and root symbiosis, producing phytohormones and essential nutrients, and increasing plant tolerance to biotic and abiotic stress (Bergna et al., 2018; Rodríguez et al., 2020; Matsumoto et al., 2021; Walsh et al., 2021). Therefore, seed-borne microbes have been identified as key organisms for biotechnological applications, including their use as biostimulants or biocontrol agents (Berg and Raaijmakers, 2018; Matsumoto et al., 2021; Lobato et al., 2024). Recent studies suggested their consideration in microbiome-assisted breeding strategies (Adam et al. 2018; Wassermann et al., 2019). Healthy seeds containing a beneficial microbiome are important for sustainable plant production to ensure global food security. Therefore, it is important to understand the main drivers impacting the seed microbiome composition. A variety of factors have been identified, e.g., the plant genotype (Rybakova et al., 2017; Chen et al., 2020; Bziuk et al., 2021), microbial inheritance from parental breeding lines (Fort et al., 2021; Wassermann et al., 2022), seed origin (Lobato et al., 2024), agricultural management (Christian et al., 2016; Wassermann et al., 2023), and storage conditions (Chandel et al., 2021). Further, vertically transmitted endophytes, as persistent and prevalent components, can successfully colonize plant tissues (Sanz-Puente et al., 2025) and most likely play a significant role in influencing host phenotype and immunity (Matsumoto et al., 2021; Tariq et al., 2025). However, the impact of seed aging on the seed microbiome is still poorly understood.

Seed aging involves deterioration processes and loss of protection from damage, such as inactivation of antioxidant enzymes, elevated presence of reactive oxygen species, and membrane and genetic damage (Yin et al., 2014; Ebone et al., 2019). Therefore, if seeds have to be stored for long periods, i.e., in breeding companies and seed banks, they are kept under controlled dry and cold conditions to conserve seed vigour. Nonetheless, storage conditions affect aging mechanisms and hence seed vigour, resulting, amongst others, in lower total germination and higher abnormal germination (Finch-Savage and Bassel, 2016). Artificial aging treatments are used to investigate seed vigour and storability, which are critical to ex situ seed conservation, e.g., in genebanks (Hay and Whitehouse, 2017). Different artificial aging tests are used, such as controlled deterioration (Powell and Matthews, 2005) or accelerated aging. The artificial aging tests mimic seed aging by short-term exposure to high temperature and moisture (Yin et al., 2015; Fenollosa et al., 2020; Malviya et al., 2023). They are conducted to estimate the storability of seed lots, where seed lots with a high percentage of germination are considered as high quality, and those with a low germination percentage are considered as low quality (Delouche and Baskin, 2021). Artificial aging treatments were reported to result in symptoms typical exhibited by naturally aged seeds (Yin et al., 2015; Finch-Savage and Bassel, 2016), where natural seed aging refers to the gradual, irreversible loss of viability and vigour caused by cumulative physiological and molecular damage, such as oxidative stress, lipid peroxidation and protein/DNA degradation (Pirredda et al., 2024). However, it is important to note that not all molecular responses are equivalent between artificial aging and seed bank storage conditions (Gianella et al., 2022). Generally, accelerated aging experiments are used to understand plant-related processes during seed aging and have been shown to, e.g., impact the endophyte viability in grass–*Epichloë* species (Gundel et al., 2009, 2010). However, their application to the broader associated seed microbiome remains largely unexplored.

In the present study, we have analysed the impact of accelerated aging stress on the seed bacterial community of oilseed rape (*Brassica napus* L.), one of the most important oilseed crops worldwide. Previous research on *B. napus* has shown that accelerated aging treatments can mimic the molecular changes observed in naturally aged seeds, particularly in terms of physio-chemical alterations (Yin et al., 2015). Here, we hypothesize that the stress imposed by accelerated aging will result in microbial diversity loss and taxonomic shifts towards stress-tolerant taxa. We exposed seeds of four different genotypes produced at two different field sites to the accelerated aging stress test and analysed the bacterial community after germination, on normal and abnormal seedlings, as well as on ungerminated seeds. Further, we aimed to identify taxa associated with the aging process and to find possible correlations with the reduced total germination.

## Materials and methods

### Experimental design and sample processing

Winter oilseed rape seeds of four different genotypes (named G1–G4) produced at two different field sites in Germany (Hohenlieth [HOH] and Hovedissen [HOV]), both sandy loam soil types, were used to investigate the effect of accelerated aging (Figure 1). Seeds were supplied by the NPZ Innovation GmbH (Germany). The seeds were harvested in July 2022 at both field sites (Supplementary Figure 1), and a subset of each accession received accelerated aging treatment in August 2022 at high temperature (45°C) and high humidity (>95%) for 48 h in a humidity-controlled incubator (Memmert, Germany). Seeds that were not exposed to accelerated aging treatment served as a control. Immediately after accelerated aging tests, total germination was evaluated under controlled conditions in two replicates per accession, where each replicate consisted of 100 seeds. Seeds were germinated for 6 days at 23°C and a 16/8-h photoperiod. Seeds were classified according to their germination phenotype: (i) normal or (ii) abnormal seedlings, following ISTA rules (ISTA, 2022), or (iii) ungerminated seeds.

In addition, directly after accelerated aging, a subset of treated and untreated seeds was sent to Graz University of Technology where unsterilized seeds were germinated under sterile conditions on wet folded seed testing paper (surface weight 160 g/m$^2$, width 110 mm, fold depth 20 mm, ROTILABO® Carl Roth GmbH, Germany) in plastic boxes (150 mm × 150 mm) with five seeds per fold. For the microbiome analysis, we aimed to obtain 7 replicates of 10 individual seedlings/ungerminated seeds per condition (genotype × field site × treatment × germination phenotype). However, most of the untreated control seeds germinated normally. Thus, we were not able to collect 7 replicates of 10 abnormal seedlings for all the control treatments. One control sample of ungerminated seeds was only available for G4, which exhibited a low total germination overall. Accelerated aging resulted in lower numbers of normal seedlings (HOH G2 and G4, 2 replicates; HOH G3, 4 replicates; HOV G2, 6 replicates; HOV G3, 3 replicates; and HOV G4, 4 replicates), as well as abnormal seedlings (HOH G3, 6 replicates; HOH G4, 2 replicates; HOV G1, 4 replicates; HOV G3, 6 replicates; and HOV G4, 2 replicates), thus, less replicates. Also here, all possible replicates for ungerminated seeds were included (HOH G1, 1 replicate; G2, 3 replicates; G3, 2 replicates; G4, 7 replicates; and HOV G4, 7 replicates). In total, we collected 197 samples (4 genotypes × 2 field sites × 2 treatments [accelerated aging and control] × 3 germination phenotypes × 1–7 replicates, with each replicate consisting of 10 individuals).

Here, it is important to mention that accelerated aging was chosen over the controlled deterioration method, which is recommended by ISTA. Preliminary trials on *B. napus* seeds confirmed a significant positive correlation between accelerated aging and controlled deterioration in terms of both final seed moisture content and post-treatment germination. Accelerated aging was therefore considered a robust and physiologically relevant stress treatment for assessing microbiome dynamics in response to seed aging.

### DNA extraction and 16S rRNA gene library preparation

Immediately after 6 days of germination, whole seedlings/seeds were homogenized by crushing them with a mortar in 0.85% NaCl under sterile conditions. Total DNA from seedling samples was extracted using the FastDNA SpinKit for Soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions, with the following modifications: three bead-beating steps were performed at the beginning. After the addition of protein precipitation solution, the samples were kept on ice for 5 min, and elution included an additional step at 55°C for 5 min after adding DES solution. 16S rRNA gene libraries were created in a one-step PCR targeting 16S rRNA gene region V4 (Caporaso et al., 2011) (515F [5′-GTGYCAGCMGCCGCGGTAA-3′] and 806R [5′-GGACTACHVGGGTWTCTAAT-3′]), including sample-specific Illumina barcodes with the Taq-&Go master mix for PCR (MP Biomedicals, Illkirch, France). To lower the amount

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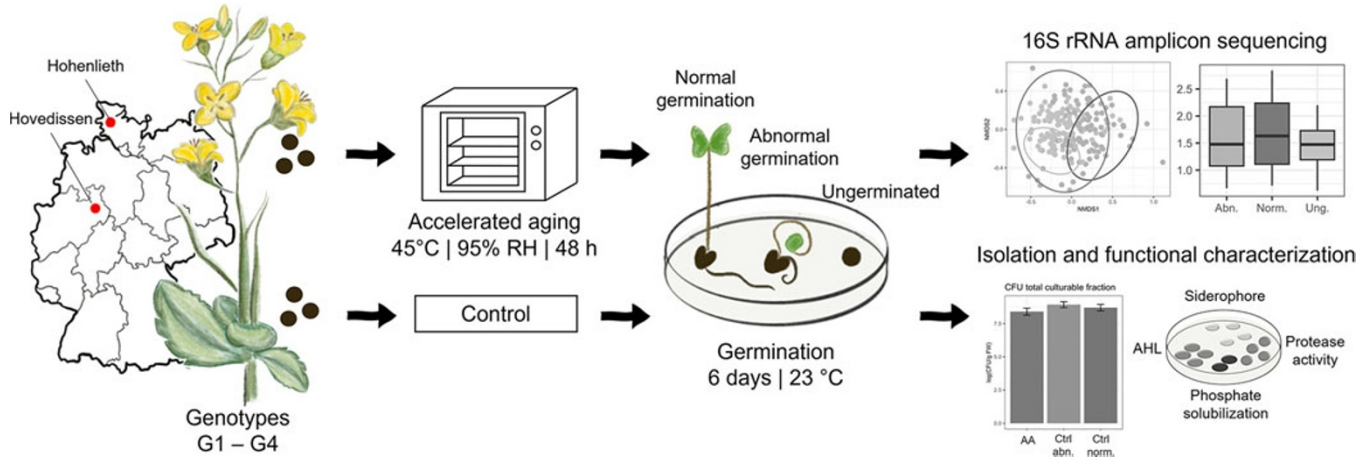


**Figure 1.** Experimental design to investigate accelerated aging in *B. napus* seeds. Seeds from four genotypes (G1–G4) originating from two field sites in Germany (Hohenlieth and Hovedissen) were left untreated or exposed to accelerated aging (45°C, 95% relative humidity (RH) for 48 h). Following treatment, seeds were germinated. Germination phenotype was categorized as normal germination, abnormal germination, or ungerminated. Total DNA of seedlings from all germination types, field sites, and genotypes was extracted and subsequently processed for 16S rRNA gene amplicon sequencing. Additionally, bacteria were isolated from seedlings and tested for plant growth-promoting activities, including the production of AHLs, siderophore production, protease activity, and phosphate solubilization.

of amplification of mitochondrial and plastidial PCR products, peptide nucleic acid (mPNA and pPNA) oligomers were added to the reaction (PNA Bio, Newbury Park, CA, USA). These PNA clamps bind to the targeted region of chloroplasts and mitochondria of the plant DNA and therefore reduce its amplification during the PCR (Kawasaki and Ryan, 2021). For each sample, undiluted DNA was used in duplicates. The final PCR master mix contained 1× Taq-&GO master mix, 0.2 mM of each primer, 1.5 µM activated (55°C for 5 min) mPNA and pPNA, respectively, and 1 µL of template DNA. Amplification was performed with the following steps: 5 min at 96°C, 30 cycles at 96°C for 60 s, 78°C for 5 s, 54°C for 60 s, 74°C for 60 s, and 10 min at 72°C for final extension. The success of PCR amplification was verified through agarose gel electrophoresis.

After pooling the duplicates for each sample, the PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). DNA concentrations were estimated based on gel picture analysis (ImageJ, Fiji), and a random subset of samples was additionally measured via Qubit 4 Fluorometer (Invitrogen, Waltham, MA, USA). The combined results allowed for the analysis of the absolute quantity of DNA using equimolar amounts for all samples. 16S rRNA gene amplicon sequencing was conducted on an Illumina NovaSeq 6000 (PE250) at Novogene Company Limited (Cambridge, UK).

## Bioinformatic analysis and statistics

Paired-end raw reads were demultiplexed, and primer sequences were removed using cutadapt (Martin, 2011). The sequences were quality filtered, trimmed, denoised and merged, and chimeric sequences were removed using the DADA2 (Callahan et al., 2016) plugin for QIIME2 (Bolyen et al., 2019). The generated amplicon sequence variants (ASVs) were classified using the VSEARCH algorithm (Rognes et al., 2016) with the SILVA database version 138.1 (Quast et al., 2013). The resulting ASV table was further cleaned using phyloseq (McMurdie et al., 2013) in RStudio version 4.2.3 (RStudio, Boston, MA, USA). Eukarya, unassigned ASVs, chloroplast, and mitochondria sequences were removed from the dataset (0.003%, 0.0007%, 2.27%, and 0.03%, respectively). DECONTAM (Davis et al., 2018) revealed no true contaminants based on the presence of ASVs in negative controls. ASVs were further filtered to a length between 230 and 270 bp.

Richness and Shannon diversity were estimated using phyloseq (random subsampling to the lowest amount of reads) and tested for significance with ANOVA and post hoc Tukey using the vegan package (Oksanen et al., 2019). Bacterial community composition was based on cumulative sum scaling normalization using metagMisc (Mikryukov, 2023) and Bray–Curtis dissimilarities. PERMANOVA was conducted to test for significant effects of the different sample groups with vegan (Wagner, 2019). Beta dispersion test of all samples based on Bray–Curtis dissimilarities and the specific permutation test (999 permutations) was performed using vegan. Relative abundances were obtained by normalization to compositional data using the microbiome package (Lahti and Shetty, 2017), and the most abundant ASVs were filtered using MicEco (Russel, 2024) and grouped on the genus level. Differential abundance analysis of taxa was conducted using DAtest with the accelerated aging as the predictor and field site and genotype as covariates (Russel et al., 2018), and resulted in the implementation of DeSeq2 as the best representative method to determine differentially abundant taxa. Correlation analysis was performed using stats (R Core Team, 2023), effects (Fox and Weisberg, 2019), and MicroViz (Barnett et al., 2021). Plots were visualized using ggplot2 (Wickham et al., 2023).

Germination data were analysed with the dplyr, tidyr, ggplot2, ggpubr and FSA. Percentages of normal and abnormal germination and ungerminated seeds were compared between treatments using the Wilcoxon rank-sum test, and visualized as boxplots with jittered individual data points. Pairwise comparisons for genotype effects across all three phenotypes were conducted using Dunn's test with Bonferroni correction for multiple testing.

## Isolation and functional characterization of bacteria

To investigate the culturable bacterial fraction of the seedling microbiome, bacterial isolates were gained from genotype G4 (field site Hovedissen). Seeds of the control and accelerated aging treatments were germinated as described above. For each

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treatment, four replicates were taken from normal and abnormal seedlings. Due to poor germination after accelerated aging, only three replicates of abnormal seedlings were obtained, and no normal germinated seedlings. Each replicate consisted of 10 seedlings which were placed into Whirl-Paks (Carl Roth GmbH, Karlsruhe, Germany) to isolate epi- and endophytes. The samples were smashed carefully with 1.5 mL sterile NaCl solution (0.9%) using a mortar and pestle until homogenized. The suspension was transferred into 2 mL reaction tubes and a serial dilution until $10^{-5}$ was plated on Reasoner's 2 Agar supplemented with 25 µg/mL Nystatin to inhibit fungal growth. Plates were incubated initially at 30°C for 24 h and an additional 48 h at 20°C. For specific isolation of *Bacillus* species, the homogenized seedling suspension was heated for 10 min at 80°C. A serial dilution of up to $10^{-3}$ was plated on Nutrient Agar II supplemented with 25 µg/mL Nystatin and incubated at 30°C for 24 h. Colony-forming units (CFUs) were determined per gram fresh mass (FM) of seedlings.

Per replicate of the total culturable fraction, 10 randomly picked colonies were further cultured on Nutrient Agar II to create a culture collection. As the *Bacillus* plates revealed fewer colonies, five colonies per replicate were used for further cultivation. DNA from the isolated bacteria was extracted using the Monarch Genomic DNA Purification Kit (New England Biolabs GmbH, Frankfurt, Germany). BOX-PCR was performed to generate species-specific fingerprints (Martin et al., 1992). One representative of each fingerprint was used to amplify the partial 16S rRNA gene (Heuer et al., 2009) and sent for Sanger sequencing (LGC Genomics GmbH, Berlin, Germany). Sequences were quality-checked with MEGA 11 (Tamura et al., 2021), and if quality allowed, sequences were processed, and forward and reverse sequences were assembled using SeaView (Gouy et al., 2010). The consensus sequences were used for taxonomic assignment using the basic local alignment search tool for nucleotides (NCBI, Bethesda, MD, USA). The first hit was used for the final taxonomic assignment. In case the second or third hit had the same percent identity as the first hit, it was noted as well.

Functional characterization of the isolates included assessment of protease activity (Weinert et al., 2010), siderophore production (Schwyn and Neilands, 1987) and phosphate solubilization (Nautiyal, 1999). A subset of isolates (from control seedlings; as these were assumed to have a higher proportion of Gram-negative strains) was used to determine the production of *N*-acyl homoserine lactones (AHLs) in a cross-streak assay using *Chromobacterium violaceum* cv026 for short-chain detection of C4–C8 AHLs (Durán et al., 2016). All plate assays were incubated at 30°C for 24 to 48 h, and an additional time at room temperature if needed. Emerged clearing zones were categorized as follows: − = no activity, + = low activity, ++ = moderate activity, and +++ = high activity.

## Results

### Accelerated aging reduced the total germination

Accelerated aging significantly affected the proportions of different germination phenotypes compared to control seeds (Figure 2). Normal seedling development was reduced (median: 60.4% vs. 86.9%, $p = 0.001$), while both abnormal seedling rates (26.1% vs. 11.6%, $p = 0.003$) and the proportion of ungerminated seeds (13.5% vs. 1.6%, $p = 0.03$) increased significantly. Supporting data can be found in Supplementary Table 1. No significant effect of the field site was observed, while the impact of the genotype was significant for all germination phenotypes (normal germination: $p = 0.01$, abnormal germination: $p = 0.007$, and ungerminated seeds: $p = 0.002$), which is mainly due to differences between G1 and G2 and G2 and G3 (Supplementary Table 2). Genotype G4 was particularly sensitive to accelerated aging, showing a mean of 28.7% abnormal seedlings and 48.5% ungerminated seeds across both locations, while its performance under control conditions was comparable to other genotypes. However, the difference between G4 and the other genotypes was insignificant. In some cases, fungal infections were observed in seedlings following accelerated aging (Supplementary Table 1).

### Structure of the B. napus seedling bacterial community

16S rRNA gene amplicon sequencing resulted in a final dataset containing 5,230,972 reads, assigned to 1484 ASVs. The main phyla inhabiting the oilseed rape seedlings were assigned to Proteobacteria, Firmicutes, and Actinobacteria, whereby accelerated aging increased the proportion of Firmicutes compared to the control. Generally, the most abundant genera (above 1% relative abundance; here averaged) belonged to *Tumebacillus* (HOV, 72%; HOH, 0.76%), *Pseudomonas* (HOV, 6.5%; HOH, 8.6%), *Pantoea* (HOV, 37.5%; HOH, 40.2%), *Stenotrophomonas* (HOV, 2.5%; HOH, 7.5%), *Curtobacterium* (HOV, 0.7%; HOH, 4.3%), *Paenibacillus* (HOV, 21.8%; HOH, 22.2%) and *Bacillus* (HOV, 17.4%; HOH, 2.7%) (Figure 3). While different genotypes and germination phenotypes resulted in similar bacterial communities for both field sites in control seedlings, the relative abundance of the genera changed after accelerated aging and was dependent on the genotype. For example, in Hohenlieth, genotype G4 exhibited a higher relative abundance of *Paenibacillus*, whereas in Hovedissen, G2 and G4 correlated with a higher relative abundance of *Bacillus*. G1 and G3 exhibited a higher relative abundance of *Paenibacillus*, whilst G2 and G4 showed a higher relative abundance of *Tumebacillus*.

### Seed aging affected bacterial diversity and community composition

Accelerated aging stress exhibited the highest impact on the bacterial community structure ($R^2 = 0.08$, $p \leq 0.001$), followed by field site ($R^2 = 0.03$, $p \leq 0.001$), genotype ($R^2 = 0.03$, $p \leq 0.001$), and germination phenotype ($R^2 = 0.01$, $p \leq 0.001$; Figure 4A–D). However, the impact of accelerated aging on the seedling bacterial community was higher for seedlings from the field site Hohenlieth ($R^2 = 0.11$, $p \leq 0.001$; Supplementary Figure 2) compared to Hovedissen ($R^2 = 0.10$, $p \leq 0.001$; Supplementary Figure 2). Moreover, the influence of the oilseed rape genotype on the bacterial community structure was also higher for seedlings from Hohenlieth compared to Hovedissen (Supplementary Figure 2). In contrast, the impact of the germination phenotype (normal/abnormal/ungerminated) was similar at both sites (Supplementary Figure 2). Considering accelerated aging and control seedlings separately (Supplementary Figure 3), the bacterial community revealed a significant impact for both the field site and the genotype. In detail, for seedlings from the control, a clear separation of the seedling bacterial community between the two field sites was visible ($R^2 = 0.06$, $p \leq 0.001$; Supplementary Figure 3) and less pronounced in the accelerated aging seedlings ($R^2 = 0.04$, $p \leq 0.001$; Supplementary Figure 3). The genotype effect was higher for the control seedlings compared to the accelerated-aged seedlings (Supplementary Figure 3). Overall, a higher variation in beta diversity was visible for the bacterial community composition of the accelerated-aged seedlings for each of the four genotypes

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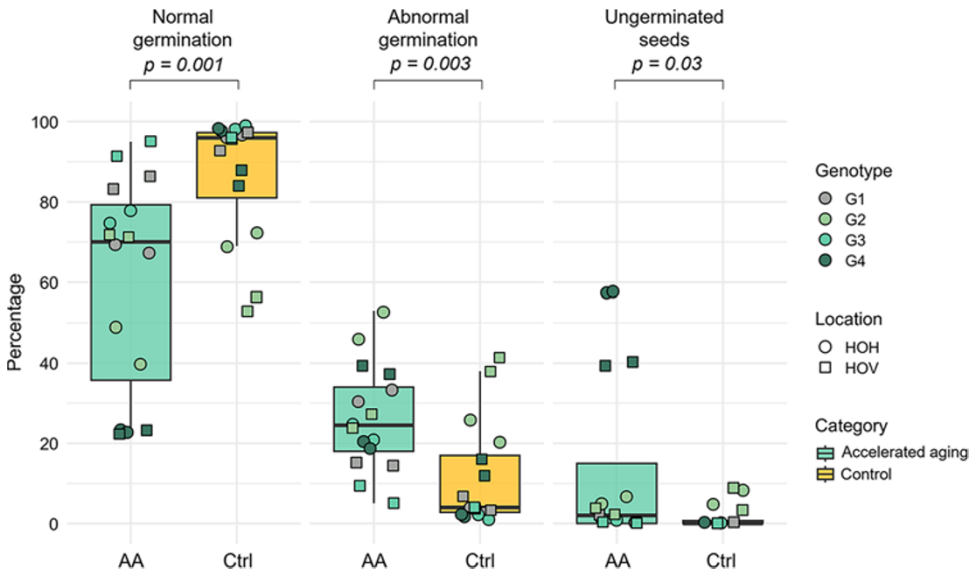


**Figure 2.** Boxplots illustrate the percentage of normal germination, abnormal germination and ungerminated seeds under accelerated aging (AA) and control conditions (Ctrl). Each point represents a biological replicate ($n = 2$ per genotype × location), with point colour corresponding to genotype (G1–G4) and point shape indicating field location (circle = Hohenlieth [HOH], square = Hovedissen [HOV]). The boxes represent the interquartile range (IQR) from the 25th to 75th percentile, the bold line within each box shows the median, and the whiskers extend to 1.5× the IQR. Statistical differences between treatments (AA vs. Ctrl) were assessed for each phenotype using the Wilcoxon rank-sum test, with exact *p*-values displayed above each comparison. All differences were statistically significant ($p < 0.05$). Y-axis values are germination percentages.

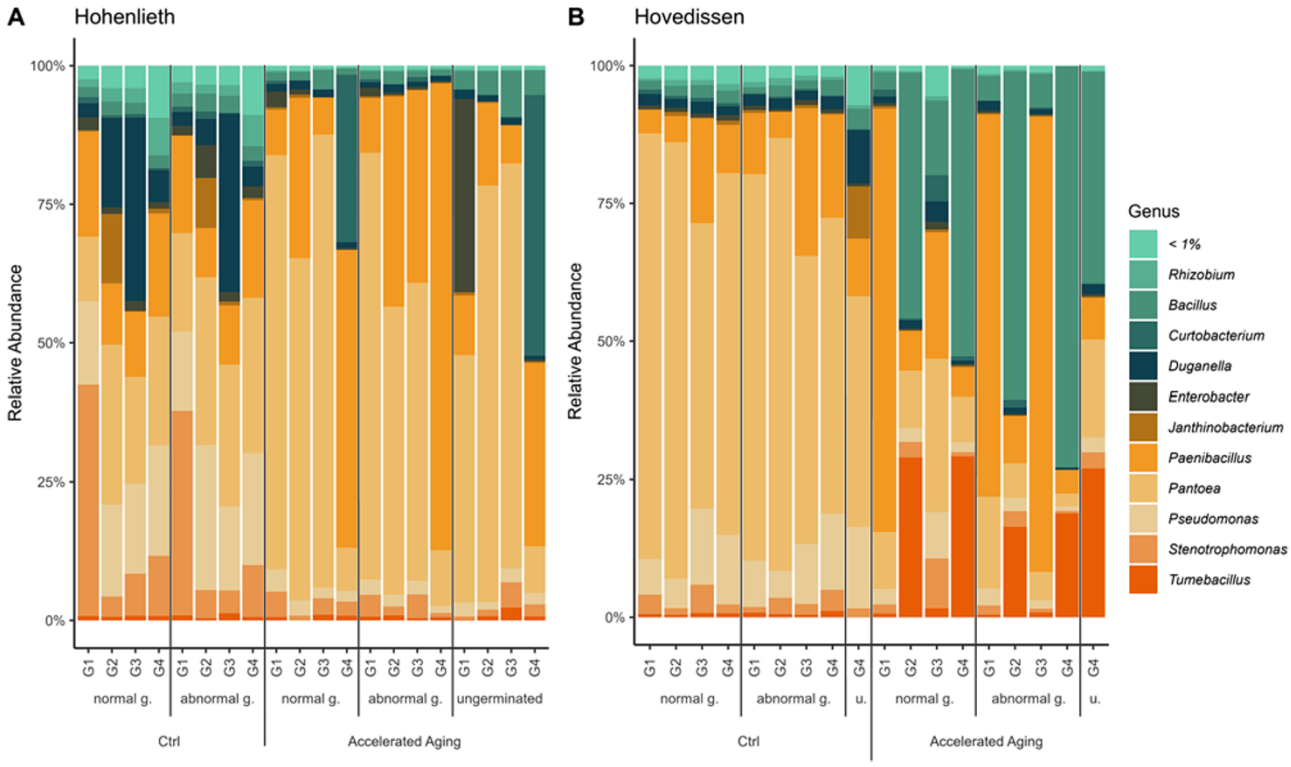


**Figure 3.** Relative abundance of the most abundant bacterial genera (>1%) in accelerated aging (AA) and control (Ctrl) seedlings of the two field sites, Hohenlieth (A) and Hovedissen (B). Three different germination (g.) phenotypes were analysed (normal, abnormal, and ungerminated [u.]).

(Figure 4E–H). This trend was validated by a beta dispersion analysis (Figure 5) showing a significant difference between the two treatments independent of the genotype, field site, or germination phenotype ($p \leq 0.01$).

Accelerated aging significantly reduced bacterial alpha diversity ($p \leq 0.001$). Field site ($p \leq 0.01$) and genotype ($p \leq 0.01$) showed a significant but lower impact, while the effect of germination phenotype was insignificant ($p = 0.262$; Figure 6). The bacterial diversity was analysed separately for the two field sites as well. For field site Hohenlieth, accelerated-aged seedlings showed significantly reduced bacterial diversity compared to the control seedlings, whereas for field site Hovedissen, the impact of accelerated aging

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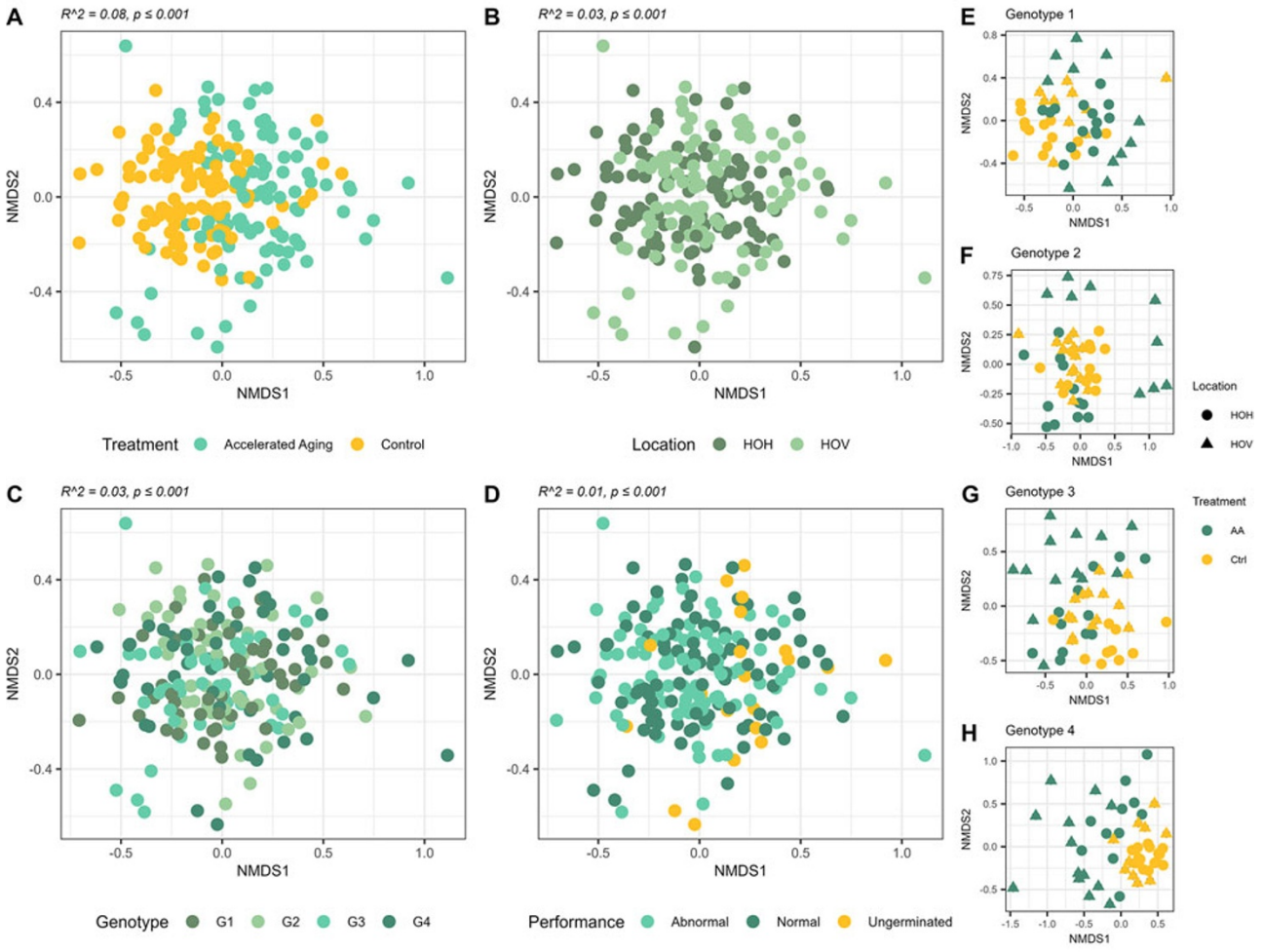


**Figure 4.** Non-metric multidimensional scaling (NMDS) of the bacterial community of oilseed rape seedlings based on Bray–Curtis dissimilarities. Panels (A), (B), (C), and (D) visualize the samples grouped by treatment, field site (Hohenlieth, HOH; Hovedissen, HOV), genotype (G1–G4), and germination phenotype, respectively. Statistical results based on PERMANOVA are reported at the top of each panel. NMDS stress: 0.2246. Panels (E–H) visualize the samples of each genotype separately, distinguishing between accelerated aging (AA) and control (Ctrl). NMDS stress (E) 0.2142, (F) 0.2132, (G) 0.2134, and (H) 0.2248.

had no significant effect due to high variation within replicates (Supplementary Figure 4). Regarding the impact of the genotype, G3 and G4 showed a higher bacterial diversity than G1 and G2 in both field sites for both normal and abnormal germinated control seedlings (Supplementary Figure 4).

Indeed, seedlings and seeds are different physiological states and comparing them directly can bias the results. Therefore, we repeated the alpha and beta diversity analysis without the samples of ungerminated seeds (Supplementary Figure 5). Those data revealed the same statistical significances as reported above, where ungerminated seeds are included.

## Distribution and occurrence of highly abundant taxa were influenced by accelerated aging and field site

DeSeq2 was used to identify which bacterial genera showed differential abundance between the control and the accelerated aging-treated seedlings ($p$-value $\leq 0.05$ and log2-fold change $\geq 2$). In general, *Tumebacillus, Paenibacillus* and *Bacillus* were highly enriched in accelerated-aged seedlings, as well as one ASV each affiliated to *Ralstonia, Burkholderia* and *Variovorax. Sphingomonas, Pedobacter, Rhizobium, Methylobacterium, Duganella* and *Burkholderiales* were more abundant in control seedlings (Figure 7). For the field site Hohenlieth, mainly *Pantoea, Paenibacillus, Bacillus and Curtobacterium* were enriched in accelerated-aged seedlings, whereas in control seedlings, mainly *Rhizobium, Pedobacter* and *Chryseobacterium* were enriched compared to accelerated aging (Supplementary Figure 6A). For the field site Hovedissen, *Pantoea, Pseudomonas, Rhizobium* and *Sphinogmonas* were significantly more abundant in control seedlings, whereas mainly *Bacillus* and *Tumebacillus* were significantly more abundant after accelerated aging (Supplementary Figure 6B). Whereas different genotypes and germination phenotypes resulted in similar bacterial communities for both field sites in control seedlings, the relative abundance of the genera changed after the accelerated aging and was dependent on the genotype.

## Correlation of highly abundant taxa with seed germination potential

We detected an increased abnormal germination after accelerated aging, as well as a reduced total germination (Supplementary Table 1). Correlations between the germination potential (in percent)

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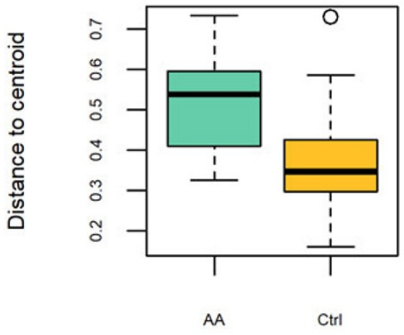


**Figure 5.** Beta dispersion test of accelerated aging (AA) and control (Ctrl) *B. napus* seed samples showing the sample variance based on Bray–Curtis dissimilarities ($p \leq 0.01$). The average distance to the median is 0.5142 for the accelerated aging group and 0.3653 for the control group. Samples represent the whole dataset.

and ASV richness, Shannon diversity, and highly abundant genera (*Pantoea, Paenibacillus, Bacillus*, and *Tumebacillus*) were calculated (Figure 8). Both Shannon diversity and ASV richness showed a positive correlation ($p \leq 0.001$) with the oilseed rape germination potential. For the tested highly abundant genera, only *Tumebacillus* and *Bacillus* showed a significant negative correlation with the germination potential ($p \leq 0.001$).

### *Taxonomic and functional profile of bacterial isolates*

As seeds of the genotype G4 showed the lowest total germination after accelerated aging (Supplementary Table 1), the bacterial communities of seeds that still germinated after accelerated aging were also characterized with culture-dependent approaches. Therefore, bacteria were isolated from emerged seedlings from the control and accelerated aging. In addition to the total culturable fraction, *Bacillus* species were specifically isolated, as the high abundance of *Bacillus* was common for both field sites (Figure 3). The CFUs per gram FM of the total culturable fraction from oilseed rape seedlings revealed similar values for each treatment ($8 \times 10^8$ logCFU/g FM for control and $4 \times 10^8$ logCFU/g FM for accelerated aging; Figure 9). The number of *Bacillus* isolates was much lower for the control ($2 \times 10^4$ logCFU/g FM) compared to accelerated aging ($2 \times 10^8$ logCFU/g FM; Figure 9). Among the 109 randomly isolated bacteria from the total culturable fraction, the majority were assigned to the genera *Bacillus, Curtobacterium, Frigoribacterium, Pantoea, Pseudomonas*, and *Pseudarthrobacter* (Table 1). The functional profile revealed that isolates from the control showed fewer phosphate solubilization traits and siderophore production than isolates from the accelerated aging samples (Table 1). For the specific *Bacillus* isolates (51), 10 different BOX fingerprints (Supplementary Figure 7) were selected and identified as members of *Bacillaceae* with different functional abilities (Table 1). BOX fingerprints served as a DNA fingerprinting technique and enabled, e.g., distinguishing isolates of *Bacillus* based on highly conserved repetitive sequences in their genome. A partial 16S rRNA gene sequence of one representative of each BOX fingerprint was sequenced and used to identify the members of the culture collection.

## Discussion

Our study provides new insights into the accelerated aging test, a technique that has been relied upon for more than 50 years to estimate the storability of seed lots (Delouche and Baskin, 2021), by introducing the microbiome as a mostly overlooked component. Although molecular studies on plants have shown distinct differences between artificial aging and long-term seed bank storage conditions (Gianella et al., 2022), *B. napus* has been reported to exhibit certain similarities in physio-chemical properties under both accelerated and natural aging (Yin et al., 2015). The present study indicates a shift in the composition of the bacterial community towards reduced bacterial diversity and specificity, and an increase in potentially heat-resistant strains, such as *Bacillus* species. Our study did not consider the fungal component of the seed microbiome, although fungi can also be vertically transmitted, influence seed and seedling performance, and interact with bacterial communities (Hodgson et al., 2014; Bastías et al., 2022).

In the present study, we proved that accelerated aging affected bacterial community structure, composition and diversity in *B. napus* seeds (Figures 3, 4 and 6). For all parameters characterizing the bacterial community, accelerated aging was identified as the main driver. We assume that the observed taxonomic shifts (Figures 3 and 7) are largely due to the hot and moist conditions of accelerated aging. In addition to those external stressors, the plant response also plays a role, e.g., reduction of enzyme activities, such as superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidase (Yin et al., 2014; Ebone et al., 2019). Amongst others, they can lead to the enrichment of reactive oxygen species (Bailly, 2004; Gill and Tuteja, 2010), which might impact the seed microbiome. Reactive oxygen species can have a dual role in the seed, releasing from dormancy, as well as causing oxidative damage. However, in the study by Yin et al. (2015), reactive oxygen species were not increased in accelerated-aged seeds of *B. napus*; instead, increased abscisic acid levels were observed.

Interestingly, the observed differences in the bacterial communities caused by accelerated aging were highly dependent on the field site where the seeds were produced, and slightly, on the genotype (Supplementary Figure 3). The impact of the field site reflects the location-specific bacterial fingerprint of the soil, meaning that since seeds were not surface sterilized, our dataset also includes seed epiphytes derived from the environment, known to influence the seed microbiome composition of *B. napus* (Morales Moreira et al., 2021). The plant genotype, in contrast, significantly determines the seed endophyte communities (Rybakova et al., 2017; Chen et al., 2020; Bziuk et al., 2021; Wassermann et al., 2022), where the host genotype most likely contributes to the vertically transmitted microbiome inside the seeds (Newcombe et al., 2018; Abdelfattah et al., 2023). Gram-positive bacteria, particularly *Bacillus* species, were enriched after accelerated aging (Figures 3 and 9), as expected due to heat-induced spore formation (Gould, 2006). The effect of accelerated aging on the bacterial community was found to be field site dependent, but it also led to an overall higher variability in the bacterial community structure (Figure 5). Microbial survival in seeds can be governed by diverse strategies. Certain bacteria may persist through dormancy, spore formation, or protective associations with seed tissues, while

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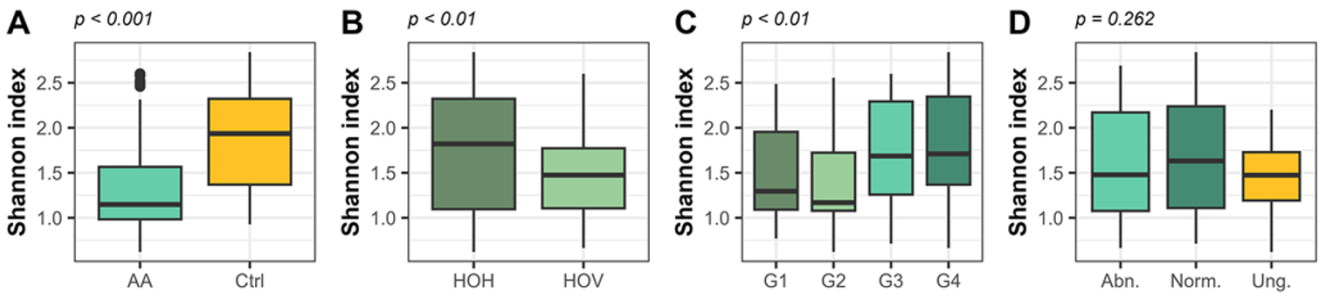


**Figure 6.** Bacterial alpha diversity in oilseed rape seedlings, presented as the Shannon diversity index. Panels (A), (B), (C) and (D) represent the samples grouped by treatment (AA: accelerated aging; Ctrl: control), field site (Hohenlieth, HOH; Hovedissen, HOV), genotype (G1–G4) and germination phenotype (abnormal/normal/ungerminated), respectively. Results of statistical significance tests, based on one-way ANOVA, are presented on top of each panel.

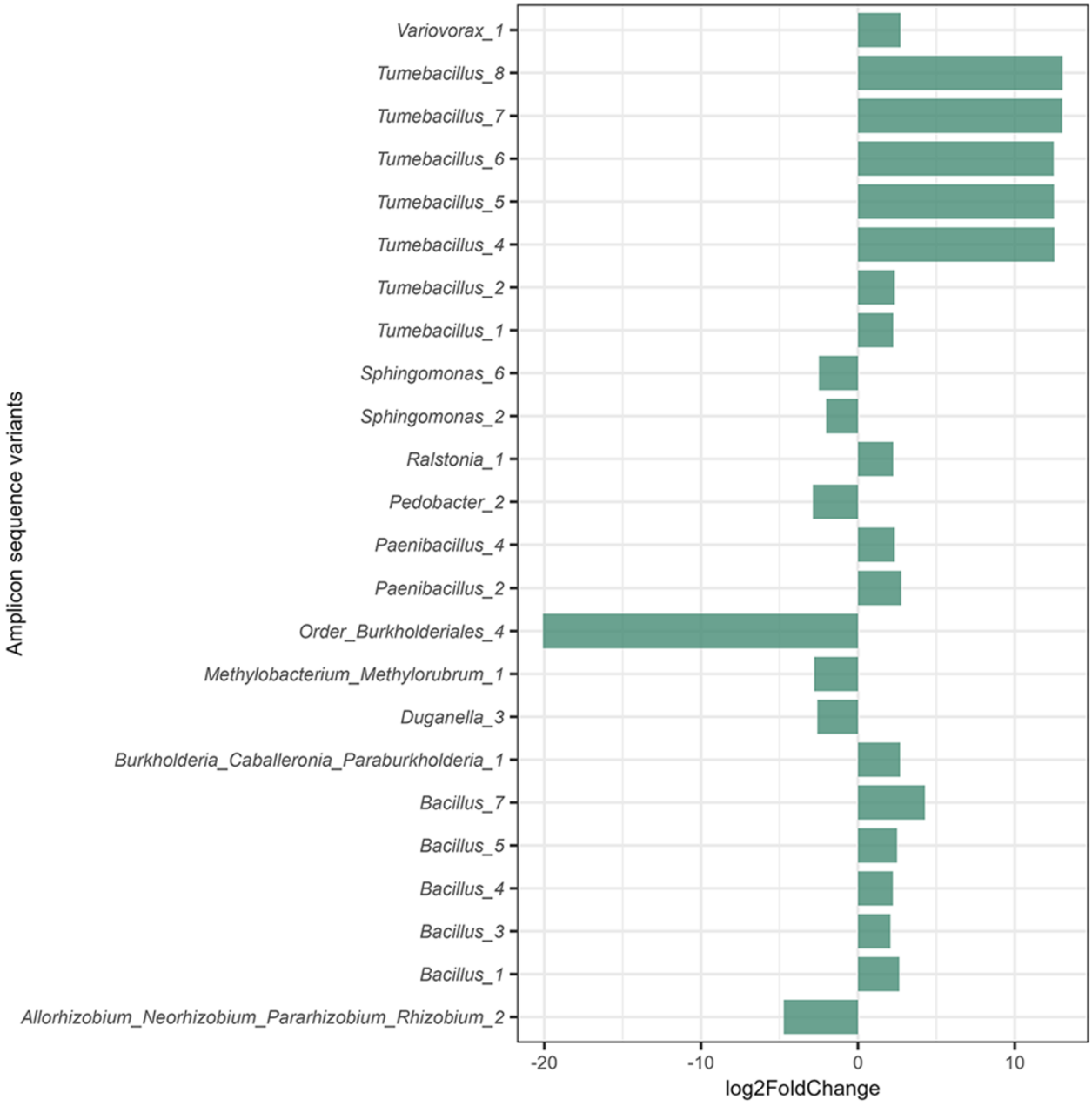


**Figure 7.** Significantly enriched or depleted amplicon sequence variants (ASVs) in accelerated aging samples versus control according to DeSeq2 for the complete dataset (field site and genotype as covariates). The dataset was filtered based on a $p$-value $\leq 0.05$ and a log2-fold change $\geq 2$.

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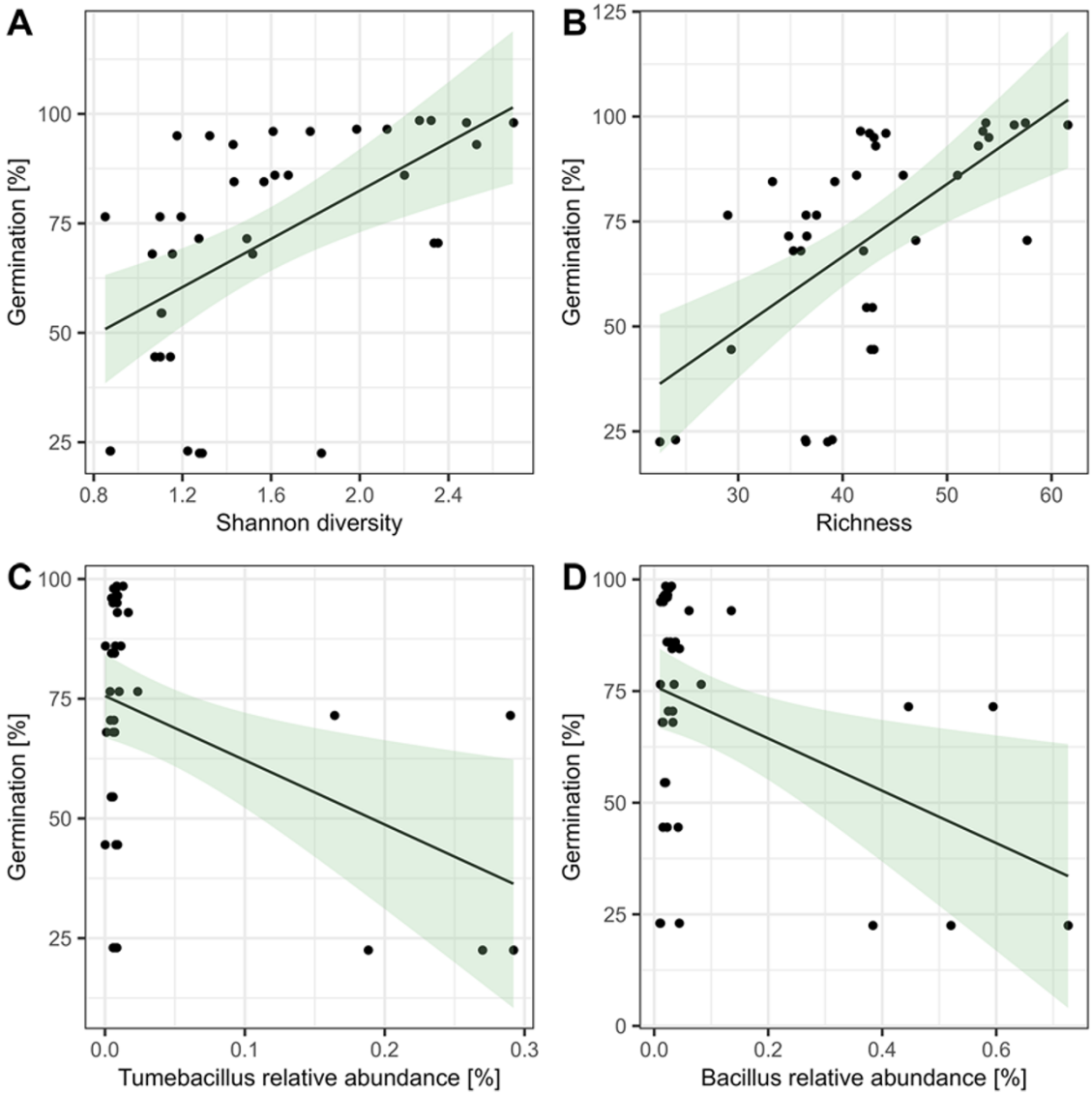


**Figure 8.** Correlation of total germination of *B. napus* seeds with (A) Shannon diversity (estimate +27.54, $p \leq 0.001$), (B) ASV richness (estimate +1.73, $p \leq 0.001$), and relative abundance of (C) *Tumebacillus* (estimate −134.303, $p \leq 0.01$) and (D) *Bacillus* (estimate −58.611, $p \leq 0.05$).

others may be more vulnerable to oxidative stress, desiccation, or nutrient depletion during storage (Khan et al., 2025). These differential survival mechanisms likely contribute to the observed shifts in community structure in accelerated-aged seedlings. In addition, this observation supports the 'Anna Karenina Principle' for microbiomes, which suggests that microbiome responses to perturbations are often stochastic, leading to transitions from stable to unstable community states. As a result, dysbiotic individuals tend to exhibit greater variability in microbial composition than healthy ones, mirroring Tolstoy's dictum that 'all happy families are alike; each unhappy family is unhappy in its own way (Zanefeld et al., 2017). Moreover, under stress conditions, the microbiome can contribute to the holobiont's resilience by deterministic selection of beneficial microorganisms (Arnault et al., 2023). This was shown in our study as well by enrichment of potentially beneficial members of Firmicutes, e.g., *Bacillus* and *Tumebacillus*.

However, *Bacillus* and *Tumebacillus* were furthermore found to negatively correlate with oilseed rape germination phenotype (Figure 8). *Bacillus* is a versatile genus containing strains that produce a broad range of antimicrobial compounds and are highly competitive in the plant microbiome (Boro et al., 2022). Usually, members of *Bacillus* are known for their plant-beneficial characteristics, increasing plant health (Hauschild et al., 2024), including enhanced seed germination under abiotic stress (Li et al., 2021). Also in seeds, *Bacillus* species are frequently detected (Figueiredo Dos Santos et al., 2021). The presence of *Bacillus* in our dataset is, thus, expected. Not much is known about *Tumebacillus* as a member of the plant microbiome; it was mainly isolated from water systems or soils (Her et al., 2015; Zhang et al., 2023). The loss of seed microbial diversity and evenness and the dominance of *Tumebacillus* might lead to reduced growth of seedlings after accelerated aging. However, both *Tumebacillus* and *Bacillus* were found to be enriched in plants receiving salt stress (Mukhtar et al.,

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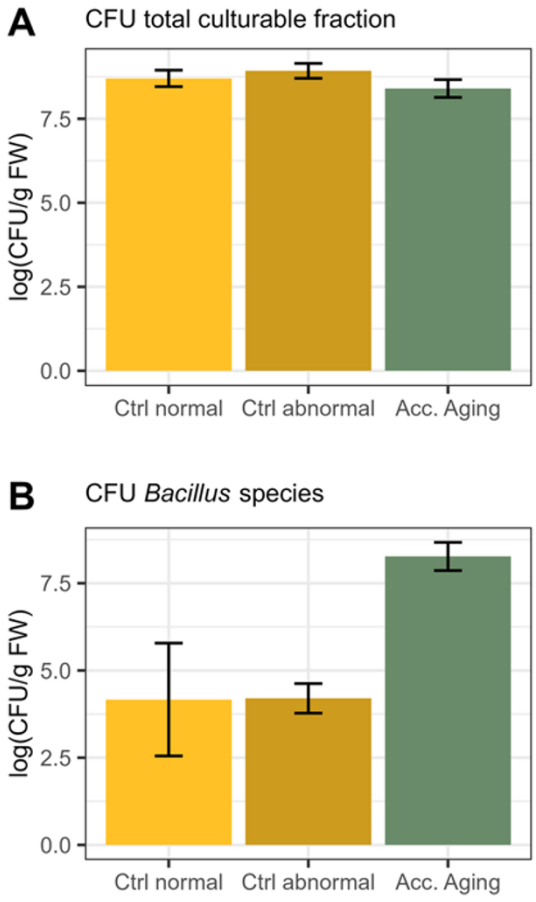


**Figure 9.** Log-transformed colony-forming units (CFUs) per gram of fresh weight (FW) for seedlings of *B. napus* genotype G4 (field site Hohenlieth), of (A) total culturable fraction and (B) *Bacillus* species of different germination phenotypes (normal, abnormal germination), for both control (Ctrl) and accelerated aging (Acc. Aging). Error bars represent standard deviations.

2018; Han et al., 2023). Seed germination phenotype of accelerated-aged seeds was also found to correlate with microbial diversity (Figure 8), underlining the importance of microbial diversity for plant health (Berg et al., 2017).

In contrast to accelerated aging samples, in total 26 bacterial ASVs were significantly more abundant in the control samples, with seven ASVs up to two times more abundant (Figure 7). Simonin et al. (2022) conducted a large-scale meta-study on the seed microbiome, including 50 plant species from agricultural and natural environments. In this study, the seed core microbiome consisted of 13 ASVs that were assigned to *Pantoea, Pseudomonas, Rhizobium, Sphingomonas, Methylobacterium*, and *Paenibacillus*, in descending abundance. Except for *Pantoea* and *Paenibacillus*, all of them were also identified as core taxa for control samples in our dataset. Particularly, strains from the genera *Pseudomonas, Rhizobium*, and *Sphingomonas* have been described to possess beneficial traits for plant health (Zamioudis and Pieterse, 2012; Matsumoto et al., 2021; Tanveer et al., 2023; Hauschild et al., 2024), which were detected in lower abundance after accelerated aging (Figure 7; Supplementary Figure 6). On that basis, accelerated aging caused a reduction of specific, and presumably plant-beneficial, microbiomes that are natively associated with seeds. Further, there is a complex relationship between microbial diversity and its ability to increase resilience (Shade et al., 2012), suggesting that the accelerated aging stress and the connected loss of diversity are reducing the plant's resilience to its environment.

In the cultivation-dependent approach, we confirmed a significantly higher abundance of *Bacillus* species in accelerated-aged seedlings (Figure 9). The reduced microbial diversity of accelerated-aged seedlings in the amplicon dataset (Figure 6) was reflected among the isolated bacteria (Table 1), affecting both the total culturable bacteria and specifically *Bacillus* species. Isolated bacteria from the accelerated-aged seedlings, presenting the same genus reflected by BOX fingerprints, possessed a higher plant beneficial potential with a more diverse functional profile compared to the control (Table 1), suggesting a different functional profile influenced by the accelerated aging. As the sampled oilseed rape seedlings were still able to germinate, although germinating abnormally, bacteria associated with them should be explored as potential plant biostimulants. Both groups of microorganisms are important to ensure either health or resilience. In general, the accelerated aging stress shifted the microbiome lifestyle, as more copiotrophic bacteria were isolated from seedlings that received the accelerated aging stress compared to the control (e.g., *Bacillus, Pseudomonas*; Table 1). Interestingly, a higher functional variation was found for bacteria isolated from accelerated-aging seedlings, again confirming the Anna Karenina Principle for the plant microbiome, recently reviewed by Arnault et al. (2023). It seems that the disruption caused by accelerated aging leads to more variable and less predictable microbiomes.

While we observed significant and clear responses of the seed bacterial community to accelerated aging, our study lacks a comparison to naturally aged seeds. Such experiments are of great importance to get deeper insights into the effect of seed aging on the microbiome. Overall, more mechanistic studies are needed to better understand the communication and interaction between the plant and its microbiome and the impacts of stress conditions, including long-term storage. In addition, modelling approaches that combine plant origins, genetics, the microbiome, and storage conditions could help to predict the storability of seed lots.

## Conclusion

This study demonstrates that accelerated aging affects the seed-associated bacterial community in *B. napus*. In particular, we observed an increase in Gram-positive bacteria, which correlated with reduced germination rates. These microbial shifts are likely driven by the elevated temperature and humidity conditions characteristic of the accelerated aging protocol. While this test induces physiological symptoms similar to those observed in naturally aged seeds, it may not fully replicate the microbiome changes associated with natural seed aging. Future research should directly compare the microbial dynamics of naturally aged seeds with those subjected to various artificial aging methods. Since the seed microbiome is crucial for germination and plant health, integrating microbial assessments into seed storage tests is increasingly important. Moreover, identifying storage practices that preserve both seed viability and microbial integrity will

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**Table 1** Isolated bacteria and their functional profile (genotype G4, Hovedissen) from (A) the total culturable fraction of control (Ctrl) and accelerated aging (AA) B. napus seedlings and (B) isolates from the targeted *Bacillus* isolation. The hit with the highest percent identity revealed by the nblast algorithm of NCBI was used for taxonomic identification; in case several genera showed the same percent identity, they are indicated by /. Functional profile is categorized as follows: − indicates no activity, +, ++, and +++ indicate slight, moderate, and strong activity, respectively. / indicates differences in the isolates tested from the same genus/box fingerprint. Short-chain AHL production was only tested for selected isolates

| BOX fingerprint | Taxonomic affiliation according to NCBI | Percent identity | Number of isolates (source) | Protease | Phosphate solubilization | Siderophore | Short-cain AHLs |
|---|---|---|---|---|---|---|---|
| | | | A | | | | |
| 1 | *Actinotalea fermentans* | 79.53% | 1 (Ctrl) | − | − | − | |
| 2 | *Bacillus zhangzhouensis/pumilus/safensis* | 99.55% | 1 (Ctrl) | +++ | − | ++ | |
| 3 | *Bacillus zhangzhouensis/pumilus/safensis* | 99.85% | 2 (Ctrl) | +++ | − | + | |
| 4 | *Clavibacter phaseoli* | 97.89% | 1 (Ctrl) | +++ | − | − | − |
| 5 | *Curtobacterium allii/flaccumfaciens* | 99.85% | 2 (Ctrl) | ++/+++ | −/++ | − | |
| 6 | *Curtobacterium allii/flaccumfaciens* | 99.39% | 1 (Ctrl) | + | − | − | |
| 7 | *Curtobacterium allii/herbarum/flaccumfaciens* | 99.05% | 2 (Ctrl) | + | − | −/+ | − |
| 8 | *Curtobacterium allii/herbarum/flaccumfaciens* | 98.28% | 3 (Ctrl) | − | − | − | − |
| 9 | *Frigoribacterium faeni* | 98.69% | 7 (Ctrl) | − | − | − | − |
| 10 | *Frigoribacterium faeni* | 99.19% | 2 (Ctrl) | − | − | − | − |
| 11 | *Frigoribacterium faeni* | 97.88% | 1 (Ctrl) | − | − | − | − |
| 12 | *Frigoribacterium faeni* | 98.18% | 3 (Ctrl) | − | − | − | |
| 13 | *Niallia circulans* | 98.83% | 2 (Ctrl) | +/+++ | −/+ | −/+ | |
| 14 | *Niallia oryzisoli/alba/circulans* | 98.29% | 1 (Ctrl) | + | − | − | − |
| 15 | *Pantoea agglomerans/brenneri* | 98.32% | 19 (Ctrl) | − | ++/+++ | − | + |
| 16 | *Peribacillus acanthi/Bacillus zhangzhouensis/pumilus* | 98.27% | 2 (Ctrl) | +++ | −/+ | + | − |
| 17 | *Plantibacter auratus* | 97.75% | 1 (Ctrl) | − | ++ | − | − |
| 18 | *Priestia megaterium* | 99.85% | 1 (Ctrl) | +++ | ++ | ++ | |
| 19 | *Pseudarthrobacter equi* | 99.24% | 1 (Ctrl) | +++ | − | +++ | |
| 20 | *Pseudarthrobacter equi* | 99.83% | 2 (Ctrl) | + | −/+ | + | |
| 21 | *Pseudarthrobacter niigatensis* | 97.29% | 1 (Ctrl) | +/+++ | −/+ | −/+/++ | − |
| 22 | *Pseudarthrobacter phenanthrenivorans* | 88.69% | 1 (Ctrl) | + | − | + | |
| 23 | *Pseudomonas viridiflava* | 98.20% | 1 (Ctrl) | + | +++ | − | − |
| 24 | *Pseudomonas viridiflava* | 97.33% | 3 (Ctrl) | + | +++ | − | − |
| 25 | *Pseudomonas viridiflava* | 92.38% | 2 (Ctrl) | −/+ | +++ | − | |
| 26 | *Pseudomonas viridiflava* | 99.84% | 3 (Ctrl) | ++ | ++/+++ | − | |
| 27 | *Rathayibacter festucae* | 100.00% | 1 (Ctrl) | − | − | − | − |

(*Continued*)

**Table 1** (*Continued.*)

| BOX fingerprint | Taxonomic affiliation according to NCBI | Percent identity | Number of isolates (source) | Protease | Phosphate solubilization | Siderophore | Short-cain AHLs |
|---|---|---|---|---|---|---|---|
| | | | A | | | | |
| 28 | *Rathayibacter festucae/tritici/rathayi* | 98.30% | 1 (Ctrl) | − | + | − | − |
| 29 | *Rathayibacter tritici* | 99.84% | 1 (Ctrl) | − | − | − | |
| 30 | *Rhodococcus fascians* | 99.85% | 1 (Ctrl) | − | + | − | |
| 31 | *Saccharibacillus brassicae* | 99.24% | 1 (Ctrl) | − | − | − | |
| 32 | *Sanguibacter inulinus* | 99.64% | 1 (Ctrl) | − | − | − | |
| 33 | *Sphingomonas aerolata* | 99.84% | 1 (Ctrl) | +++ | − | − | |
| 34 | *Sphingomonas desiccabilis* | 100.00% | 1 (Ctrl) | − | − | − | |
| 35 | *Sphingomonas faeni/aurantiaca* | 96.01% | 3 (Ctrl) | − | − | − | − |
| 36 | *Bacillus aerius* | 97.95% | 6 (AA) | + | + | − | |
| 37 | *Bacillus altitudinis/aerius/stratosphericus* | 97.14% | 9 (AA) | + | −/+ | −/+/++ | |
| 38 | *Bacillus subtilis/stercoris/rugosus* | 98.54% | 7 (AA) | +/++/+++ | + | +++ | |
| 39 | *Bacillus zhangzhouensis/pumilus/safensis* | 94.91% | 2 (AA) | +/++ | − | ++ | |
| 40 | *Peribacillus acanthi/Bacillus zhangzhouensis* | 97.37% | 1 (AA) | ++ | + | + | |
| 41 | *Curtobacterium allii* | 98.99% | 3 (AA) | + | −/+ | −/+/++ | |
| 42 | *Moraxella osloensis* | 98.23% | 1 (AA) | − | − | − | |
| 43 | *Paenibacillus mobilis/xylanivorans/pabuli* | 97.71% | 1 (AA) | + | + | − | |
| BOX fingerprint | Taxonomic affiliation according to NCBI | Percent identity | Number of isolates | Protease | Phosphate solubilization | Siderophore | AHL |
| | | | B | | | | |
| 1 | *Bacillus zanthoxyli/Priestia megaterium* | 99.71% | 3 (Ctrl) | ++ | −/+ | +++ | |
| 2 | *Bacillus zhangzhouensis/pumilus/safensis* | 99.85% | 8 (Ctrl) | ++/+++ | −/+ | −/(+) | |
| 3 | *Bacillus zhangzhouensis/pumilus/safensis* | 99.71% | 13 (Ctrl) | +++ | −/+ | +/++ | |
| 4 | *Niallia circulans* | 98.89% | 1 (Ctrl) | + | − | − | |
| 5 | *Priestia filamentosa* | 99.45% | 1 (Ctrl) | − | + | − | |
| 6 | *Priestia megaterium* | 99.85% | 2 (Ctrl) | ++ | + | +++ | |
| 7 | *Pseudomonas viridiflava* | 97.69% | 1 (Ctrl) | − | +++ | − | |
| 8 | *Bacillus altitudinis/aerius/stratosphericus* | 99.00% | 10 (Ctrl/AA) | + | −/+ | +/++ | |
| 9 | *Bacillus cabrialesii/inaquosorum* | 100.00% | 10 (AA) | +++ | + | +++ | |
| 10 | *Bacillus altitudinis/aerius/stratosphericus* | 99.00% | 1 (AA) | − | + | + | |
| 11 | *Bacillus haynesii* | 91.62% | 1 (AA) | − | + | +++ | |

help maintain optimal plant performance even after long-term storage.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S0960258526100099.

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**Competing interests.** The authors declare no conflicts of interest.

**Data availability.** The 16S rRNA gene amplicon sequences are deposited in the EMBL Nucleotide Archive (ENA; ebi.ac.uk/ena) under the accession number PRJEB89949.

**Ethical standards.** The authors confirm that they have adhered to the ethical policies of the journal.

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