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Intraspecific variability in heat resistance of fungal conidia

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ABSTRACT

Microbial species are inherently variable, which is reflected in intraspecies genotypic and phenotypic differences. Strain-to-strain variation gives rise to variability in stress resistance and plays a crucial role in food safety and food quality. Here, strain variability in heat resistance of asexual spores (conidia) of the fungal species *Aspergillus niger*, *Penicillium roqueforti* and *Paecilomyces variotii* was quantified and compared to bacterial variability found in the literature. After heat treatment, a 5.4- to 8.6-fold difference in inactivation rate was found between individual strains within each species, while the strain variability of the three fungal species was not statistically different. We evaluated whether the degree of intraspecies variability is uniform, not only within the fungal kingdom, but also amongst different bacterial species. Comparison with three spore-forming bacteria and two non-spore-forming bacteria revealed that the variability of the different species was indeed in the same order of magnitude, which hints to a microbial signature of variation that exceeds kingdom boundaries.

1. Introduction

Diversity of microbial species is key to adapt to environmental changes and to thrive in different niches. Intraspecies variability includes all variation within a species, including genotypic and phenotypic differences. Unravelling drivers for intraspecies variability has been a broadly studied subject the past decade including elucidating mechanistic differences between strains (Andersson, 2009; Choudoir, Panke-Buisse, Andam, & Buckley, 2017; Papke & Ward, 2004) and sources of phenotypic heterogeneity of genetic identical cell populations (Avery, 2006; Ackermann, 2015). Strain diversity can have huge consequences on diagnostics, virulence and antimicrobial treatments in clinical microbiology, or on the efficacy of food preservation methods (Davies et al., 2021; Den Besten, Wells-Bennik, & Zwietering, 2018; Lianou & Koutsoumanis, 2013b; Stratford et al., 2013; Lianou, Nychas, & Koutsoumanis, 2020).

As microbial species are inherently variable, strains of the same species may differ in their response to environmental and food preservation stresses. Indeed, large differences in stress robustness have been reported in bacterial species (Den Besten et al., 2018). Recently, strain variability in heat resistance has been quantified for bacterial vegetative cells of the pathogen *Listeria monocytogenes* (Aryani, Den Besten, Hazeleger, & Zwietering, 2015) and the food-borne organism *Lactiplantibacillus plantarum* (Aryani, Den Besten, & Zwietering, 2016) [previously known as *Lactobacillus plantarum* (Zheng et al., 2020)] and for bacterial spores of the pathogen *Bacillus cereus* (Den Besten et al., 2018), and the food spoilers *Bacillus subtilis* (Den Besten, Berendsen, Wells-Bennik, Straatsma, & Zwietering, 2017) and *Geobacillus stearothermophilus* (Wells-Bennik et al., 2019). Notably, the quantified strain variability was high for the tested microorganisms and inactivation rates of the most heat-sensitive and most heat-resistant strains of the same species could differ a factor ten. This means that when a similar temperature/time regime is applied, the most heat-resistant strain will be reduced with a factor 10, while the most heat-sensitive strains will be reduced with a factor 10^{10}. This results in huge differences in heat treatment efficacies, depending on the heat stress robustness of the microbial contaminant (Den Besten et al., 2018; Zwietering, Garre, & Den Besten, 2021).

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Spores of bacteria and fungi are considered more stress resistant than vegetative cells (Wyatt, Wösten, & Dijksterhuis, 2013). The stress resistance of fungal spores varies strongly, ranging from spores that display stress resistance similar to that of vegetative cells to very high stress resistance that can be comparable to bacterial spores (Beuchat, 1986; Van Leeuwen, Van Doorn, Golovina, Stark, & Dijksterhuis, 2010; Wyatt et al., 2013). Filamentous Ascomycete fungi that belong to the order Eurotiales produce asexual spores called conidia. Airborne conidia are resistant to various environmental stresses including ultra violet (UV) radiation, desiccation, cold and heat stress (Wyatt et al., 2013). Conidia are an integral part of the fungal life cycle, and can be distributed in space by air, wind or other vectors and are abundantly present in the environment. For example, *Penicillium chrysogenum* conidia are so widespread that they are considered being cosmopolitan (Henk et al., 2011) and these airborne conidia are found in the air and soil in many different habitats (Amend, Seifert, Samson, & Bruns, 2010). Studies have shown that conidia can travel large distances. For instance, conidia of *Aspergillus sydowii* have been suggested to be transported over thousands of kilometres from the Sahara desert to the Caribbean reefs (Shinn et al., 2000). Being airborne and widely present, fungal airborne conidia are often related to food spoilage, leading to considerable losses of food and feed (Dijksterhuis, 2017). In food industry, heat treatments are routinely used to inactivate or reduce the microbial count in a product, and quantitative data on the heat resistance of microorganisms is needed to predict treatment efficacy and product shelf life.

Recently, conidial heat resistance of various strains of the food spoilage fungus *Paecilomyces variotii* was reported to be highly variable. Decimal reduction times of thermal treatments at 60 °C (D_{60}-values) ranged from 3.5 to 27.6 min when different strains were used of the same species (Van den Brule, Lee, et al., 2020). This prompted us to study a larger selection of isolates and to quantify variability in conidial heat resistance among strains of *P. variotii* in detail. Furthermore, we also assessed strain variability in conidial heat resistance of the fungus *Penicillium roqueforti,* known as fermentation organism, and for *Aspergillus niger*, which is commonly used as model fungus. Both species are also known as food spoilers. The quantified strain variabilities were compared between the fungal species, and also compared to strain variability in heat resistance of bacterial vegetative cells and spores, to assess strain variability across kingdom borders.

2. Material and methods

2.1. Strain selection and identification

All strains (21 *A. niger*, 20 *P. roqueforti* and 20 *P. variotii* strains) were selected and obtained from the CBS culture collection and the working collection of the Food and Indoor Mycology (DTO) group, both housed at the Westerdijk Fungal Biodiversity Institute (Table S1). Strains included the previously studied *A. niger* N402 (Bos et al., 1988), *P. roqueforti* DTO 377-G3 (Punt et al., 2020) and *P. variotii* DTO 032-I3, DTO 212-C5, DTO 217-A2 and DTO 280-E5 (CBS 101075) (Urquhart et al., 2018; Van den Brule, Punt, et al., 2020). The other strains were selected from both food and non-food sources. Identity of strains was confirmed by sequencing the partial calmodulin (*caM*) gene for *A. niger* and the partial beta-tubulin (*benA*) gene for *P. roqueforti* and *P. variotii* that can be used as identification marker (Samson, Houbraken, Varga, & Frisvad, 2009; Varga et al., 2011; Visagie et al., 2014). After alignment using MUSCLE, a maximum likelihood tree of each species was computed with 1,000 bootstrap replications using MEGA7 (Kumar, Stecher, & Tamura, 2016). Reference sequences of closely related species and the type strain were included in the phylograms (Houbraken et al., 2020). For each tree, the model with the lowest Bayesian Information Criterion (BIC) score was used. The Kimura 2-parameter model including gamma distribution (K2+G) was used for *A. niger*, and the Jukes-Cantor (JC) model and the Kimura 2-parameter model including invariant sites (K2+I) model were used for *P. roqueforti* and *P. variotii*, respectively.

2.2. Growth conditions and harvesting conidia

Culturing and harvesting of conidia were performed as described previously (Van den Brule, Punt, et al., 2020). In short, fungal strains stored in 30% (w/v) glycerol at −20 °C were spot-inoculated on malt extract agar (MEA, Oxoid, Hampshire, UK) and incubated for 7 days at 25 °C. *A. niger* strains DTO 028-I3 and DTO 058-I1 were cultured at 30 °C because sporulation was not sufficient at 25 °C. Freshly harvested conidia were used to spread-inoculate a new MEA plate to minimize differences in age within the conidia population. Conidia were harvested after 7 days of incubation in ACES buffer [10 mM N(2-acetamido)-2-aminoethanesulfonic acid, 0.02% Tween 80, pH 6.8, $a_w > 0.995$] and filtered using either sterile glass wool in a syringe or sterilized Amplitude EcoCloth wipes (Contec Europe, Vannes, France). Subsequently, conidia were washed two times in ACES buffer. The concentration of conidia in suspension was determined using a Coulter Counter Multisizer 3 (Beckman Coulter, Life Sciences, Indianapolis, USA) (Van den Brule, Lee, et al., 2020), a Bio-Rad TC20 Automated Cell Counter (Bio-Rad Laboratories, Lunteren, The Netherlands), or a Bürker-Türk heamocytometer (VWR, Amsterdam, The Netherlands) and the conidia suspension was set at 8.3 $\log_{10}$ CFU mL^{-1}.

2.3. Thermal treatments

A volume of 0.2 to 1 mL of conidia suspension was added to pre-heated ACES buffer to a total volume of 20 mL in Erlenmeyer flasks in a water bath. Conidia of *A. niger*, *P. roqueforti* and *P. variotii* were treated at 54 °C, 56 °C and 60 °C, respectively. At various time points, 1 mL samples were taken, immediately chilled on ice, and decimally diluted. An aliquot (0.1 mL) was surface-inoculated on MEA and plates were incubated at 25 °C. The non-heated conidia suspension was also decimally diluted and subsequently plated to determine the initial viable concentration of conidia at $t = 0$ min. Colonies of *P. variotii* were counted after three days of incubation, while *A. niger* and *P. roqueforti* colonies were counted after 7 days. The $\log_{10}$ colony forming units (CFU) mL^{-1} were calculated for each sampling time point.

2.4. Quantification of heat resistance

The reparameterised Weibull model (Eq. (1)) (Metselaar, Den Besten, Abee, Moezelaar, & Zwietering, 2013) was fitted to the $\log_{10}$ CFU mL^{-1} data of each inactivation experiment with the R package Growthrates using the Levenberg-Marquardt algorithm (Hall, Acar, Nandipati, & Barlow, 2013). The Weibull model allows fitting linear, downward concave, and upward concave inactivation curves and was able to fit the different thermal inactivation curves of the strains.

$$\mathrm{Log}_{10}(N_t) = \mathrm{Log}_{10}(N_0) - \Delta \bullet \left(\frac{t}{t_{\Delta D}}\right)^{\beta} \quad (1)$$

where N_0 is the initial concentration of conidia (CFU mL^{-1}), N_t is the number of surviving conidia (CFU mL^{-1}) at time point t, Δ is the reference number of decimal reductions, $t_{\Delta D}$ represents the time needed to reduce the initial number of conidia with Δ decimals, and β the shape parameter where $\beta > 1$ gives a downward concave and $\beta < 1$ an upward concave behaviour. When β was significantly different from 1, the average D-value was estimated as $\frac{t_{\Delta D}}{\Delta}$. If not, the negative reciprocal of the linear regression slope, $\frac{-1}{slope}$, was used to estimate the D-value as described (Van den Brule, Punt, et al., 2020).

2.5. Quantification of variability

Experimental, biological and strain variabilities were quantified per species using the method described by Aryani et al. (2015). For each

strain, three biologically independent batches of conidial spores were prepared, and conidial heat resistance was tested in duplicate for each batch of conidial spores. Experimental variability (σ_e) was defined as the variability between parallel experimental replicates using the same batch of conidial spores, and expressed by the root mean square error ($\sqrt{MSE_e}$) of Eq. (2)

$$MSE_e = \frac{RSS_e}{DF_e} = \frac{\sum_{S=1}^{i}\sum_{B=1}^{3}\sum_{E=1}^{2}(X_{EBS} - X_{BS})^2}{n-p} \quad (2)$$

where MSE_e is mean square error, RSS_e is Residual Sum of Squares, DF_e is Degrees of Freedom, X_{EBS} is the $\log_{10}$ D-value of each experiment 'E' of biological replicate 'B' and strain 'S', X_{BS} is the average of the $\log_{10}$ D-value of the experimental duplicates of each biological replicate 'B' of strain 'S', i is the number of strains used per species and DF_e is the number of data points ($n = 2*3*i$) minus the number of parameters ($p = 3*i$).

Biological variability (σ_b) was expressed by $\sqrt{MSE_b}$ of Eq. (3)

$$MSE_b = \frac{RSS_b}{DF_b} = \frac{\sum_{S=1}^{i}\sum_{B=1}^{3}(X_{BS} - X_S)^2}{n-p} \quad (3)$$

where X_S is the average of X_{BS} from the biological triplicates of strain 'S' and DF_b is the number of data points ($n = 3*i$) minus the number of parameters ($p = 1*i$).

Strain variability (σ_s) was expressed by $\sqrt{MSE_s}$ of Eq. (4)

$$MSE_s = \frac{RSS_s}{DF_s} = \frac{\sum_{S=1}^{i}(X_S - X)^2}{n-p} \quad (4)$$

where X is the average of X_S of all i strains and DF_s is the number of data points ($n = i$) minus the number of parameters ($p = 1$).

The 95% confidence intervals of σ_e, σ_b and σ_s were calculated according to Eq. (5)

$$\sqrt{\frac{RSS}{\chi^2_{DF;\alpha/2}}} \le \sigma \le \sqrt{\frac{RSS}{\chi^2_{DF;1-\alpha/2}}} \quad (5)$$

where χ^2 is the critical Chi-square value at $\alpha/2$ and $1-\alpha/2$ with $\alpha = 0.05$, using the same RSS and DF definitions as in Eqs. (2)–(4).

2.6. D-values from literature

Data describing the inactivation kinetics of conidia of *A. niger* (Baggerman, 1981; Ballestra & Cuq, 1998; Belbahi et al., 2017; Esbelin, Mallea, Ram, & Carlin, 2013; Fujikawa, Morozumi, Smerage, & Teixeira, 2000; Rege & Pai, 1999; Reveron, Barreiro, & Sandoval, 2005; Shearer, Mazzotta, Chuyate, & Gombas, 2002) and *P. roqueforti* (Blank, Yang, & Scanlon, 1998; Bröker Spicher & Ahrens, 1987a, 1987b; Kunz, 1981; Punt et al., 2020; Shearer et al., 2002) were collected from literature. The obtained D-values were $\log_{10}$ transformed and the mean $\log_{10}$ D-value of each strain tested in this study were added to the data set, resulting in 48 and 148 data points for *A. niger* and *P. roqueforti*, respectively. The $\log_{10}$ D-values versus the temperature were used to calculate the z-value for each species, being the negative reciprocal of the linear regression slope, $\frac{-1}{slope}$. Subsequently, the 95% prediction interval of the linear regression was calculated using Eq. (6)

$$\mathrm{Log}_{10}D_{ref} \pm t_{DF;1-0.5\alpha}\sqrt{\frac{RSS}{DF}} \quad (6)$$

where D_{ref} is the $\log_{10}$ D-value at the reference temperature, t is the Student t-value with degrees of freedom (DF) $n-2$ and $\alpha = 0.05$, RSS is the residual sum of squares calculated from the deviation of the data to the linear regression line. The overall variability (σ_t) was defined as the deviation of the data to the linear regression line, $\sqrt{\frac{RSS}{DF}}$, with RSS the residual sum of squares calculated from the deviation of the data to the linear regression line, and $n-2$.

3. Results

3.1. Verification of strain identity

In total, 21 *A. niger*, 20 *P. roqueforti* and 20 *P. variotii* strains were selected (Table S1) and their identity was verified by sequencing genetic marker genes according to the phylogenetic standards (Houbraken et al., 2020). Based on the partial sequences of *caM*, all *A. niger* strains grouped together with type strain *A. niger* NRRL 326, with *Aspergillus welwitschiae*, the most closely related species, being the sister clade (Fig. 1a). Similarly, the partial *benA* sequences of the *P. roqueforti* strains grouped with type strain CBS 221.30 and segregated from the closely related *Penicillium mediterraneum* (Fig. 1b). In agreement with previous studies (Houbraken, Varga, Rico-Munoz, Johnson, & Samson, 2008; Van den Brule, Punt, et al., 2020), we found more intraspecies variation in the partial *benA* sequences of *P. variotii* compared to *A. niger* and *P. roqueforti*. However, all *P. variotii* strains clustered with type strain CBS 102.74, while *Paecilomyces brunneolus* was sister to this cluster (Fig. 1c).

3.2. Quantification of heat resistance

Strains of *A. niger*, *P. roqueforti*, and *P. variotii* were heat-treated using biologically independent batches of conidia and technical duplicates. The differences between the technical replicates were rather small for all strains of the three species (Fig. 2a, d, g). The differences between the biological replicates were clearly higher than those of the experimental duplicates (Fig. 2b, e, h), but much higher differences were found between the individual strains per species (Fig. 2c, f, i). Most inactivation kinetics, i.e. 237 out of 366, did not show a significant tailing or a shoulder curvature and the shape parameter β was not significant different from 1 (see Table S2 with the 95% confidence interval of the shape parameter β), and a linear model was used to calculate the D-value. For the other data sets the reparameterized Weibull model (Eq. (1)) was used to calculate the average D-value (Table S2).

The most heat-resistant *P. roqueforti* strain was DTO 013-F5, while the most heat-sensitive strain was DTO 130-C1 with D_{56}-values of 13.6 ± 3.0 and 1.6 ± 0.38 min, respectively. Similar to *P. roqueforti*, about an eight-fold difference was found between the most heat-resistant *P. variotii* strain DTO 195-F2 and the most heat-sensitive strain DTO 212-C5, with corresponding D_{60}-values of 26.6 ± 3.4 and 3.5 ± 0.30 min, respectively. This indicates that for this specific heat treatment, one out of ten cells will survive for the most resistant strain, while only one out of 10^8 cells will survive for the most sensitive strain. Three out of 21 *A. niger* strains, DTO 028-I3, DTO 029-B1 and DTO 058-I1, did not sporulate well after 7 days growth on MEA at 25 °C. A better sporulation was achieved when cultivating at 30 °C instead of 25 °C, and therefore this temperature was used to culture conidia. Interestingly, these three strains belonged to the most heat sensitive strains, with D_{54}-values of 12.6 ± 1.7, 3.7 ± 0.60 and 9.9 ± 2.2 min, respectively. Impeded sporulation can be a sign of degeneration of a strain (Li et al., 2014). Indeed, these three strains were deposited more than six decades ago into the CBS culture collection and it cannot be excluded that the strains degenerated over the years or arrived in a degenerated state when deposited. Because growing cultures at higher temperatures can significantly enhance heat resistance in the case of *Aspergillus fumigatus* (Hagiwara et al., 2017) and *P. roqueforti* conidia (Punt et al., 2020), it was decided to exclude DTO 028-I3, DTO 029-B1 and DTO 058-I1 for further analysis. This made DTO 367-D1 the most sensitive and DTO 326-A2 the most resistant *A. niger* strain with D_{54}-values of 9.4 ± 0.85 and 50.4 ± 11.9 min, respectively.

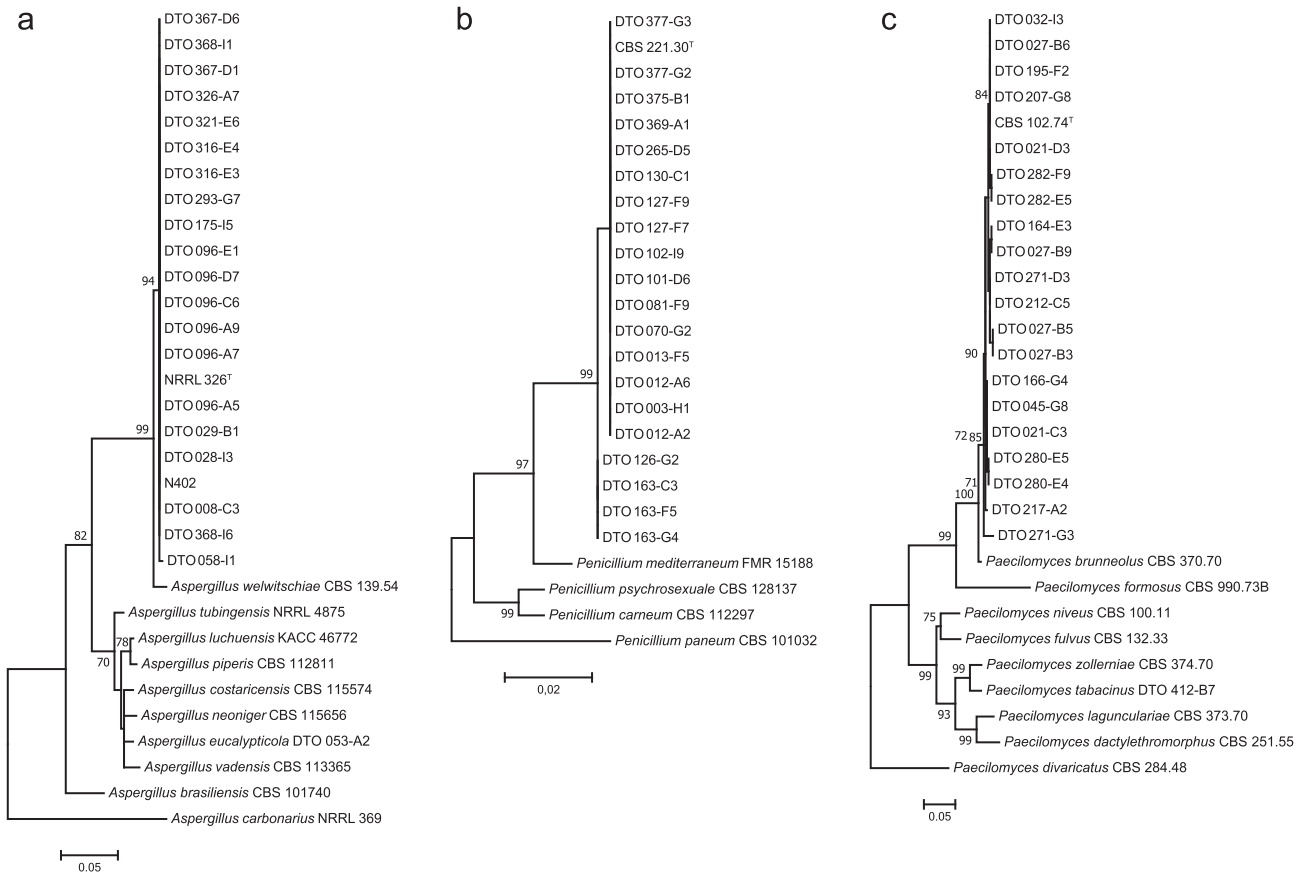

Fig. 1. Maximum likelihood trees for strain identification. (a) Phylogram based on partial *caM* sequence of studied *A. niger* strains, including type strain NRRL 326 and other closely related *Aspergillus* species with *Aspergillus carbonarius* used as outgroup. (b) Phylogram based on partial *benA* sequences of *P. roqueforti* strains, including type strain CBS 221.30 and other closely related *Penicillium* species with *Penicillium paneum* as outgroup. (c) Phylogram based on partial *benA* gene sequences of *P.. variotii* strains, including type strain CBS 102.74 and other closely related *Paecilomyces* species with *Paecilomyces divaricatus* as outgroup.

3.3. Quantification of variability

The experimental, biological and strain variability of the three species was quantitatively expressed in $\sqrt{MSE}$ (i.e. the standard deviation, σ) of the $\log_{10}$ *D*-values, which is a measure of variability (Fig. 3). Indeed, as observed in Fig. 2, experimental variability was the lowest variability factor with σ_e values of 0.045, 0.044 and 0.033 for *A. niger*, *P. roqueforti* and *P. variotii*, respectively. Biological variability values were larger with a σ_b of 0.092, 0.096 and 0.084 for *A. niger*, *P. roqueforti* and *P. variotii*, respectively. Strain variability was clearly higher, with σ_s of 0.20, 0.18 and 0.23 for the three fungi, respectively. Interestingly, the 95% confidence intervals of the three species were overlapping for each of the variability factors. This indicates that there were no significant differences in the magnitude of the variability between the species. However, the variability factors were clearly different, with strain variability being higher than biological variability, and both being higher than experimental variability.

3.4. D-values from literature

The conidial heat resistance of *A. niger* and *P. roqueforti* strains presented in this study was compared with available data from the literature (Table S3). Only recently, two studies described the heat resistance for *P. variotii* conidia (Van den Brule, Lee, et al., 2020; Van den Brule, Punt, et al., 2020) and therefore this fungus was excluded for the meta-analysis. The *D*-values from literature for *A. niger* and *P. roqueforti* and the *D*-values collected in the current study are shown in Fig. 4a and 4b, respectively. The linear correlation between the $\log_{10}$ *D*-values and temperature allowed to calculate the global *z*-value, indicating the temperature increase needed to decrease *D*-values 10-fold. The *z*-values were 8.9 °C for *A. niger* and 7.8 °C for *P. roqueforti*, respectively, which is comparable to global *z*-values found for multiple bacterial species (Van Asselt & Zwietering, 2006). Note that the *z*-value of the individual fungal isolates might be slightly different, and estimation of a strain specific *z*-value would require *D*-value determination at multiple temperatures per fungal isolate. The deviation of each data point to the linear regression between $\log_{10}$ *D*-value and temperature was used to quantify the overall variability σ_t, which was 0.432 for *A. niger* and 0.413 for *P. roqueforti*. With σ_s values 46% and 43% of the σ_t values for *A. niger* and *P. roqueforti*, respectively, these results indicate that strain variability is a substantial source of variability in the overall variability reported in literature.

3.5. Comparison with variability of bacterial species

It is well known that heat resistance of bacterial spores and vegetative cells differs enormously among species, and consequently the *D*-values are very different when determined at the same temperature. Interestingly, contrary to the magnitude, the intraspecies variability of bacterial species inactivation rates were in the same order of magnitude when five different bacterial species were compared (Den Besten et al., 2018), including three spore-forming bacteria, *B. subtilis*, *B. cereus*, *G. stearothermophilus*, and two non-spore-forming bacteria, *L. monocytogenes* and *L. plantarum*. Because the current study used a similar experimental set up to determine heat resistance between fungal strains, taking into account the variability between biologically independent replicates and variability between technical duplicates, we could compare the σ_e, σ_b and σ_s values of the three fungal species to

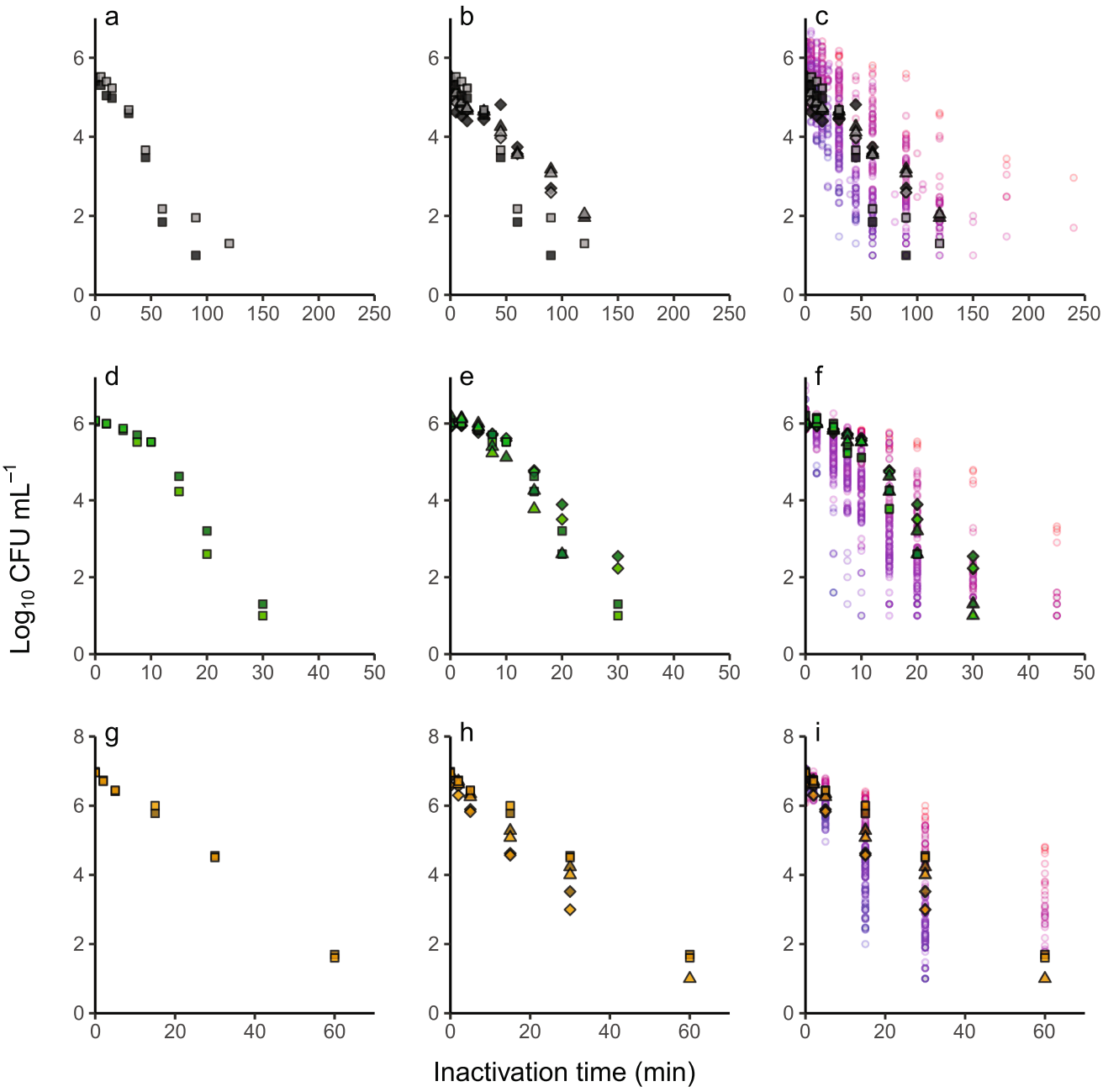

Fig. 2. Variability in thermal inactivation of species. Thermal inactivation was performed at 54 °C for *A. niger* (a-c), 56 °C for *P. roqueforti* (d-f) and 60 °C for *P. variotii* (g-i). Experimental variability (a, d, g), biological variability (b, e, h) and strain variability (c, f, i) is depicted by the $\log_{10}$ CFU mL^{-1} data of each experiment. The strains *A. niger* DTO 316-E3 (black; a-c), *P. roqueforti* DTO 163-C3 (green; d-f) and *P. variotii* DTO 166-G4 (orange; g-i) are highlighted, showing two experimental replicates (dark fill, light fill) of three biological replicates (□, ◇ and △). All other strains (○; c, f, i) are coloured using a gradient from blue (heat-sensitive) to red (heat-resistant) based on the mean *D*-values presented in Table S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

those of the five bacterial species (Fig. 5). Note that the *B. subtilis* strains were grouped in a high-level heat resistant group and a low-level heat resistant group for quantification of strain variability. The *B. subtilis* strains that produced high-level heat resistant spores proved to harbour a mobile genetic element, *spoVA*^{2mob}, that confers high-level heat resistance to spores (Berendsen, Boekhorst, Kuipers, & Wells-Bennik, 2016), giving genetic evidence for clustering the corresponding strains into two groups. Interestingly, for all microbial species, strain variability was larger than biological and experimental variabilities. Even though the difference was small, the experimental variability of the two *Bacillus* species was slightly higher than the rest of the species, although the variability of *B. cereus* was not significantly larger compared to *A. niger* and *P. roqueforti*. Both *Bacillus* species showed a significant higher biological variability than the other species, while the vegetative cells of *L. monocytogenes* and *L. plantarum* showed a significant lower biological variability compared to the bacterial and fungal spores. Less significant differences were found between strain variability of the different species. Altogether, these data suggest that the different levels of variability in heat resistance of fungal conidia are rather similar to those of bacterial spores and cells.

4. Discussion

Intraspecies variability is inherent in microbial species. We

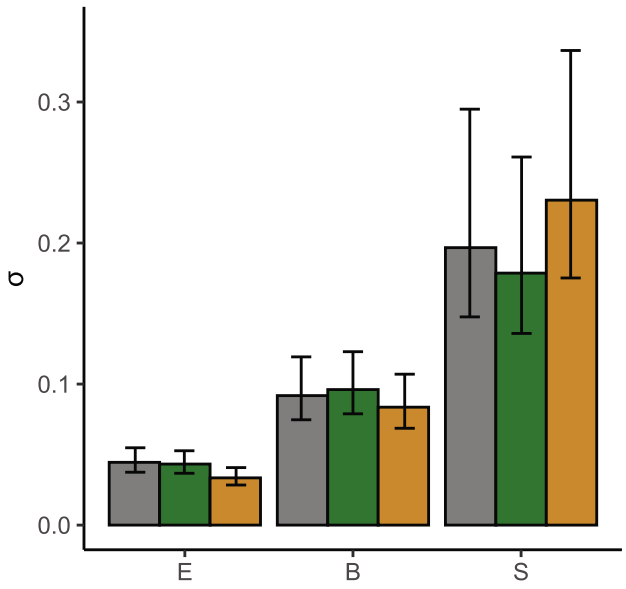

Fig. 3. Quantification of variability. Experimental variability (E), biological variability (B) and strain variability (S) of *A. niger* (grey), *P. roqueforti* (green) and *P. variotii* (orange). For strain variability 18 *A. niger*, 20*P. roqueforti* and 20*P. variotii* strains were used to determine σ_s values. Error bars represent the 95% confidence interval of the σ values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

scrutinized conidial heat resistance of three fungal species and quantified variability at experimental, biological and strain levels. In total, 18 *A. niger*, 20 *P. roqueforti* and 20 *P. variotii* strains were used to quantify strain variability. Although some reference strains were included in the strain selection it is of importance to select the strains randomly in order to represent variability found in nature. In mycological research, it can be challenging to identify fungal isolates to species level as some are cryptic species. For instance, *A. niger* is difficult to distinguish from *Aspergillus luchuensis* and *Aspergillus welwitschiae* based on morphology (Samson, Houbraken, Thrane, Frisvad, & Andersen, 2019). Even identification by sequencing of genetic marker genes can be puzzling since databases can contain sequences of previously misidentified isolates (Hofstetter, Buyck, Eyssartier, Schnee, & Gindro, 2019; Lücking et al., 2020). Identification of the strains used in this study by phylogenetic analysis of marker genes sequences of reference strains provided a robust identification of the current species. Therefore, the selection of fungal strains and the genetic locus used for comparison indicate that the observed variation in conidial heat resistance is truly intraspecies.

Differences in heat resistance are not only caused by variation in genetic background. Heterogeneity in genetically uniform cells can contribute to survival against environmental stress in yeast species (Holland, Reader, Dyer, & Avery, 2014). Some strains of *P. variotii* can produce conidia populations that are heterogeneous in size (Van den Brule, Lee, et al., 2020). The same study stated that strains producing conidia with a larger mean size tend to be more heat resistant; hinting that the larger conidia within spore populations could be more heat resistant compared to small size conidia. In addition to these examples of phenotypic heterogeneity, environmental growth conditions can have a significant effect on heat resistance. Besides cultivation temperature (Hagiwara et al., 2017; Punt et al., 2020), the maturation of conidia also plays a role in the development of heat resistance as conidia from older colonies of *A. niger* and *P. roqueforti* showed higher robustness to heat treatments (Teertstra et al., 2017; Punt et al., 2020). On the other hand, environmental conditions during conidiation can also reduce heat resistance of fungal conidia. Growth at pH 4.6 resulted in sensitive conidia compared to the more optimal conditions at pH 8.0 of the insect-pathogenic fungus *Metarhizium robertsii* (Rangel et al., 2015). Intracellular compatible solutes and protective proteins are known to provide heat robustness to fungal species (Van Leeuwen et al., 2016; Wyatt et al., 2013). Conidia of *A. niger* contain large amounts of Hsp70 transcripts (Van Leeuwen et al., 2013) and mannitol (Teertstra et al., 2017), while arabitol, the hydrophilins con-6 and con-10 and 17 predicted proteins with unknown function were implied to play a role in heat resistance of *P. roqueforti* conidia (Punt et al., 2020). On the other hand, *P. variotii* conidia contain predominantly trehalose as compatible solute (Van den Brule, Punt, et al., 2020) and in higher amount than *A. niger* (Teertstra et al., 2017) and *P. roqueforti* (Punt et al., 2020), which might explain, at least in part, the higher heat resistance of this species.

Our experimental set up was aimed to reduce environmental varia-

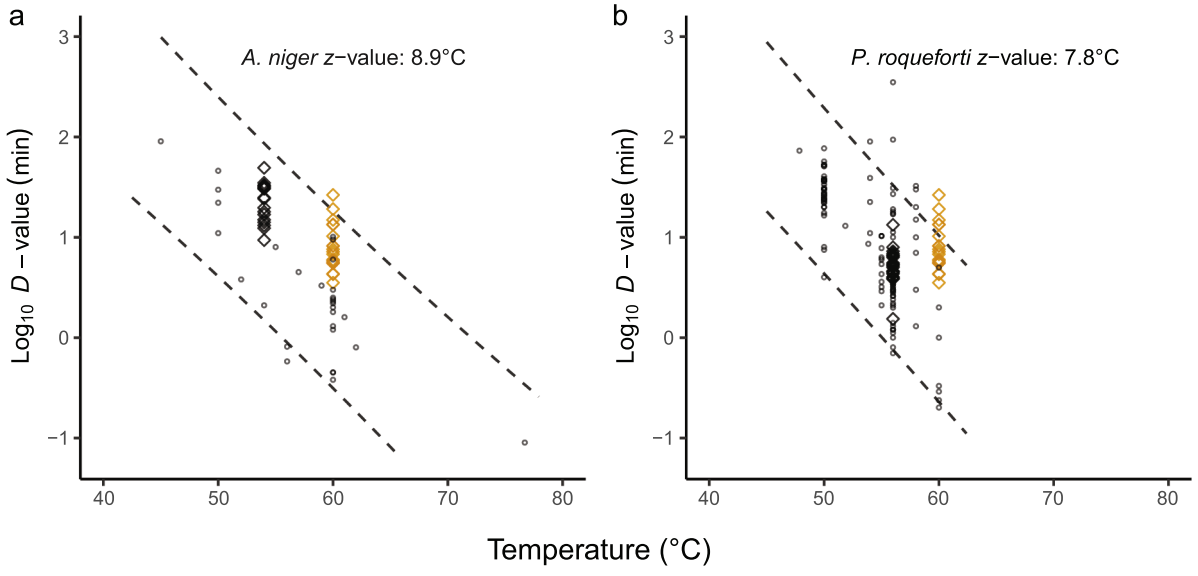

Fig. 4. *A. niger* and *P. roqueforti* *D*-values. Log_{10} *D*-values from literature (○) and mean $\log_{10}$ *D*-values per strain presented in this study (◇) were combined to determine *z*-values and overall variability for *A. niger* (a) and *P. roqueforti* (b). Mean $\log_{10}$ *D*-values of *P. variotii* strains presented in this study are depicted as orange ◇ in both panels. The 95% prediction intervals of the linear regression analysis are depicted as dashed lines in both panels. All data from literature is summarized in Table S3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

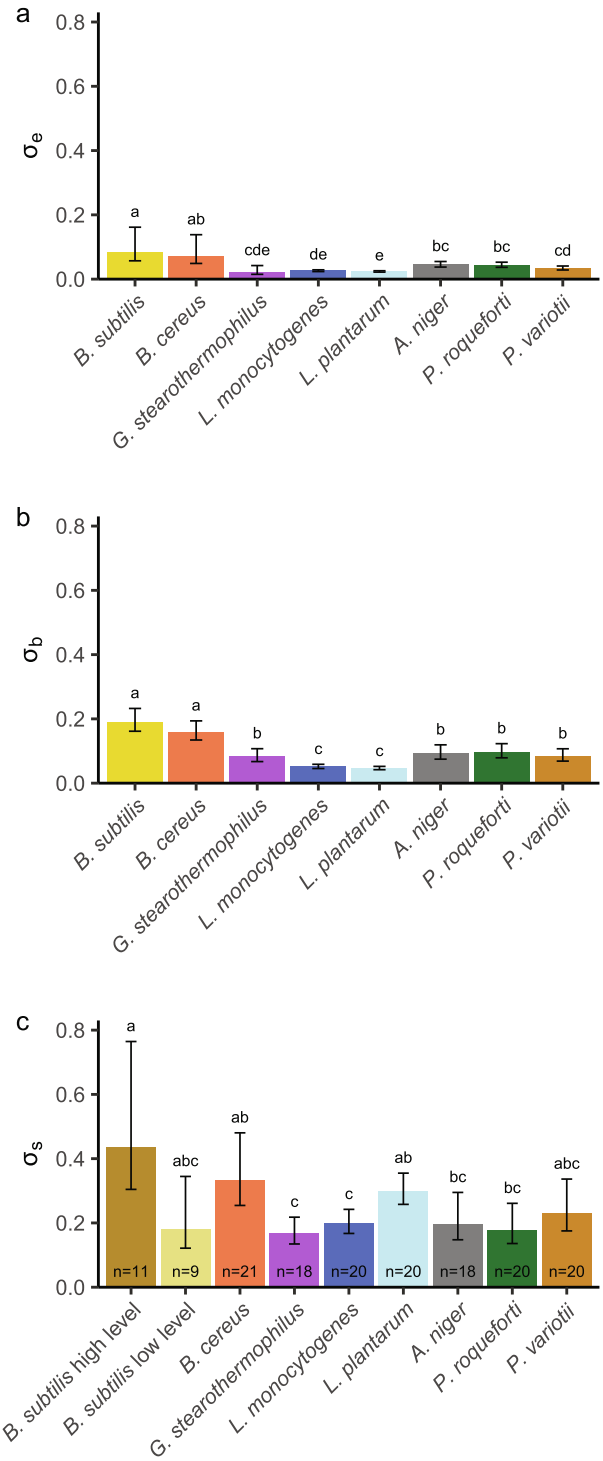

Fig. 5. Comparison of variability in heat resistance between bacterial spores, vegetative cells and fungal conidia. Experimental (a), biological (b) and strain variability (c) in heat resistance of bacterial spores of *B. subtilis*, *B. cereus* and *G. stearothermophilus*, bacterial vegetative cells of *L. monocytogenes* and *L. plantarum* and fungal conidia of *A. niger*, *P. roqueforti* and *P. variotii*. For strain variability, *n* represents the number of strains used to determine σ_s values. Error bars represent the 95% confidence interval of the σ values. At each level of variability, significance groups are indicated above the error bars and different letters between species indicate no overlap of the 95% confidence intervals. Data of bacterial species was adapted from Den Besten et al. (2018).

tion as much as possible by spreading many conidia over one plate for inoculation. This way, we anticipated to differences due to colony age, which could be interpreted as environmental variation. In the meta-analysis, we compared our data with data available in literature, where different growth conditions and heating menstrua were applied, to visualize this overall variability and to estimate a global *z*-value per species. This demonstrated that strain variability is a large, if not the largest source of the overall variability. This is consistent with bacterial species, where σ_s values are typically 40% to 75% of the overall variability found in literature (Den Besten et al., 2018). For two other bacterial species, *Escherichia coli* O157:H7 and *Staphylococcus aureus*, *D*-values have been obtained for a large number of strains at one or two temperatures, allowing us also to quantify the strain variability for these two species using our approach (Eq. (4)). This showed that the strain variability of *Escherichia coli* O157:H7 was 0.206 (n = 17) as calculated by the $\log_{10}$ *D*-value at 55 °C and 60 °C, whereas for *S. aureus* the strain variability was 0.360 (n = 15) as calculated by the $\log_{10}$ *D*-value at 58 °C. Interestingly, these values are in the same order of magnitude as presented in Fig. 4c. To the best of our knowledge, for one species, namely *Salmonella* spp., reported differences in heat resistance among strains isolated from various sources tend to be much smaller with σ_s values of 0.07 and 0.09 (Den Besten et al., 2018; Gurtler et al., 2015; Lianou & Koutsoumanis, 2013a), and this supports the relevance to quantify the strain variability of this species in more detail. Also quantitative information on strain variability of zygomycetes and fungal ascospores is not available yet, and would be of relevance to quantify in following up studies. Quantified microbial variability is crucial information to be included in risk assessments to realistically predict microbial behaviour.

In conclusion, strain variability in conidial heat resistance of the three fungal species was in the same order of magnitude as for several bacterial species, which hints to a natural diversity that stretches beyond kingdoms. In other fields of research, intraspecies variability also occurs in virulence, growth and biofilm formation (Lianou et al., 2020), and an intriguing question is whether the impact of strain variability is also comparable for other microbial traits.

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Authors contributions

TvdB, MP, SJS, JD and HMWdB devised the study; experiments were performed by TvdB, MP, SJS, FJJS and WCH. Phylogram construction was performed by TvdB and JH. The manuscript was drafted by TvdB and revised by HABW, MHZ, JD, HMWdB and AFJR. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111302.

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