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The impact of inter- and intra-species spore density on germination of the food spoilage fungus *Aspergillus niger*

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ABSTRACT

Aspergilli can be used to produce food but can spoil it as well. Both food production and spoilage are initiated by germination of the conidia of these fungi that have been introduced by inoculation and contamination, respectively. Germination of these spores includes activation, swelling, establishment of cell polarity, and formation of a germ tube. So far, only quantitative single-species germination studies of fungal spores have been performed. Here, spore germination of the food spoilage fungus *Aspergillus niger* was studied quantitatively in mono-culture or when mixed with other food-relevant aspergilli (*Aspergillus nidulans, Aspergillus terreus, Aspergillus clavatus,* and *Aspergillus oryzae*). In the presence of the germination inducing amino acids proline or alanine, but not in the case of the lowly inducing amino acid arginine, the incidence of swelling and germ tube formation was reduced when 35,000 extra conidia of *Aspergillus niger* were added to wells containing 5000 of these spores. Adding 35,000 spores of one of the other aspergilli also did not have an effect on germination in the presence of arginine, but the germination inhibition was stronger when compared to the extra *A. niger* spores in the case of alanine. A similar effect was obtained with proline. Together, results show that the germination of *A. niger* conidia is impacted by the density of its own spores and that of other aspergilli under favorable nutritional conditions. These results increase our understanding of food spoilage by fungi and can be used to optimize food production with fungi.

1. Introduction

Aspergillus species are commonly found in food. For instance, *Aspergillus niger* can be isolated from most stored commodities, while *Aspergillus nidulans, Aspergillus terreus,* and *Aspergillus clavatus* are found on, for instance, cereals and cereal products (Pitt and Hocking, 1997). Aspergilli can also be responsible for food spoilage. For instance, *A. clavatus* can grow massively during malting of barley, during which mycotoxins may be produced (Flannigan et al., 1986), while *Aspergillus niger* can spoil fresh fruit and onions (Snowdon, 1990, 1991). On the other hand, aspergilli are used for food production. For instance, *Aspergillus oryzae* is used to produce fermented foods such as soy sauce and miso (Kusumoto et al., 2021). In addition, aspergilli are widely used as cell factories for the production of enzymes and small molecules that are used in food processing (Meyer et al., 2020; Wösten, 2019).

Food is contaminated or inoculated with conidia of aspergilli. These asexual spores are produced in high numbers and are dispersed by wind, water, and animals such as insects (Krijgsheld et al., 2013). Resting conidia have low metabolic activity (Novodvorska et al., 2016; Teertstra et al., 2017) and are resistant to various stresses such as drought, high and low temperatures, high salt, reactive oxygen species, and UV (Beauvais and Latgé, 2018). Germination of conidia in the food substrate includes activation, swelling, establishment of cell polarity, and formation of a germ tube (d'Enfert, 1997; Wendland, 2001). The swelling and germ tube formation stages can be distinguished by an increase in the volume of the spores and a decrease in their circularity, respectively (Ijadpanahsaravi et al., 2021, 2022). In contrast to resting spores, germinating spores are highly sensitive to environmental stress conditions. Therefore, aspergilli implement a bet hedging strategy by initiating germination of only part of the spores even under favorable conditions (Ijadpanahsaravi et al., 2021, 2022).

Germination of *Aspergillus* spores is influenced by various environmental factors, including temperature (Anderson and Smith, 1972), water availability (Ayerst, 1969; Nanguy et al., 2010), light (Röhrig

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et al., 2013), pH (Stevenson et al., 2017), and CO_2 (Vakil et al., 1961). Nutritional triggers such as the presence of particular (in)organic carbon and nitrogen sources also induce germination of *Aspergillus* (Hayer et al., 2013, 2014; Ijadpanahsaravi et al., 2021, 2022). Conidia of different aspergilli have different germination dynamics when exposed to different nutritional conditions (Ijadpanahsaravi et al., 2022). Consequently, *Aspergillus* species have different competitive potential when grown in different media together with other aspergilli. Spore density can also affect the germination incidence of aspergilli (Barrios-González et al., 1989; Araujo and Rodrigues, 2004). Increasing the spore concentration of *Aspergillus niger* or *Aspergillus fumigatus* results in a lower germination percentage. However, the effect of spore density on the germination dynamics of *Aspergillus* spores in mixed-species cultures has not been assessed so far. Here, it is shown that germination of *A. niger* conidia is impacted not only by the density of its own spores but also by those of other aspergilli. This effect contributes to our understanding of fungal growth in nature but may also provide leads to prevent food spoilage, to optimize food fermentation processes, and to produce enzymes and small molecules when using aspergilli as a cell factory.

2. Methods and materials

2.1. Strains and culture conditions

A. niger N402 (Bos et al., 1988), *A. oryzae* RIB40 (Machida et al., 2005), *A. clavatus* NRRL1 (Arnaud et al., 2012), *A. nidulans* FGSC A4 (Arnaud et al., 2012), and *A. terreus* NHI 2624 (Arnaud et al., 2012) were used in this study. For the production of conidia, these strains were grown for seven days at 30 °C on minimal medium (MM) with 1 % glucose and 1.5 % agar (Ijadpanahsaravi et al., 2021). Spores were collected from the colonies with a water-wetted cotton swab and hyphae were removed by filtering the spore suspension through cotton wool (Ijadpanahsaravi et al., 2021, 2022). This was followed by centrifugation at 4 °C at 4000*g* for 5 min, after which the pellet was washed with ice-cold water followed by centrifugation. Spores were kept on ice until the moment of inoculation. Conidia were counted with a Buerker Tuerk counter and diluted to a final density of 2.7 10^5 ml^{-1}. Different numbers of spores were inoculated in wells of a 96–well suspension culture plate (Greiner bio-one, Cellstar 655,185, www.gbo.com) containing 150 µl 2 mM $MgSO_4$, 25 mM $NaPO_4$ buffer, pH 6, as well as 10 mM alanine, proline, or arginine. The process of swelling and germination was monitored at 30 °C during a period of 24 h by using an oCelloScope (Biosense Solutions, www.biosensesolutions.dk) (Aunsbjerg et al., 2015; Ijadpanahsaravi et al., 2021, 2022) with UniExplorer software version 8.1.0.7682-RL2.

2.2. Germination analysis

Germination analysis was done according to Ijadpanahsaravi et al. (2021, 2022). A maximum number of 1000 spores within a total scan length of ≥405 µm were scanned every hour during a 24 h period. Scanning was started 1 h after inoculation until the conidia settled at the bottom of the plate. Neighboring or aggregated spores were manually removed at $t = 1$ to obtain one conidium per object area of 40–700 pixels (a pixel is approx. 0.55 × 0.55 µm). The circularity and surface area of each object (each spore having its own object ID) in the time interval t = 1 to $t = 24$ were used as input to classify the objects as resting conidia, swollen conidia, and germinated conidia by custom R scripts (Ijadpanahsaravi et al., 2021, 2022).

A. niger conidia were either used in mono-cultures or co-cultured with other *Aspergillus* spores. Resting conidia of *A. niger* had a surface area ≤ 150 pixels and a circularity >0.97; swollen conidia had a surface area > 150 pixels and a circularity >0.97; conidia with germ tubes had a surface area > 150 pixels and a circularity ≤0.97. Data was fitted in an asymmetric model (Dantigny et al., 2011) using the GrowthRates R package and the Levenberg-Marquardt algorithm (Ijadpanahsaravi et al., 2021, 2022). The model estimated P_{max}, τ, and d, which represent the maximal percentage of swollen or germinated conidia, the time where $P = 0.5\ P_{max}$, and the degree of heterogeneity in the germination response (a higher d representing lower heterogeneity), respectively. Parameters were limited to $P \geq 0$ and ≤ 120 %, $\tau \geq 1$ h and ≤ 18 h, $d \geq 1$ and ≤ 30 when fitting the model, and data from t = 1 h to t = 24 h were used for modeling (Ijadpanahsaravi et al., 2021). Confidence intervals (95 %) were determined using the standard error of the parameter estimates, were Y represents the estimated P, τ or d value; SEy is the standard error obtained for P, τ or d; α is 0.05; df is the degrees of freedom; *qt* represents the Students T distribution function (Eq. (1)).

$$CI_y = SE_y \pm qt\left(1 - \frac{\alpha}{2}, df\right) \quad (1)$$

The germination metrics R package was used for visualization of swelling and germ tube formation dynamics in spores.

2.3. Classification of Aspergillus spores in co-culture

A subset of approximately 1000 random spores of each *Aspergillus* species was selected from the monoculture datasets. The Euclidean distance in a two-dimensional space was calculated between each spore in the co-culture and the monoculture dataset based on the values of "area" and "contrast" (for script see https://github.com/Ijadpanahsaravi/Supplemental-Material). Each spore was assigned to the species with the shortest Euclidean distance and scored 100 to 0. To assess spore overlap between *A. niger* and other *Aspergillus* species in co-culture, we defined a range of spore scores <50 for each species in co-culture. Then the overlap percentage was calculated by dividing the number of spores falling within this range by the total number of spores in the co-culture. Only spores of *A. niger* were selected for swelling and germ tube formation analysis with a score > 90 to ensure a lack of misclassification with the other species.

2.4. Culture media analysis

The quantification of amino acids in the culture medium was done using the ninhydrin colorimetric method (Li et al., 2011). To this end, culture media were transferred to cellulose acetate membrane filters with a pore size of 0.22 µm (Corning Costar Spin-X centrifuge tube filters, CLS8161, www.sigmaaldrich.com) and centrifuged at 4000 *g* for 1 min. Ninhydrin reagent was added to a 10-fold diluted culture medium, and tubes were incubated at 100 °C for 10 min in a water bath. The absorbance of proline was measured at 440 nm, while that of alanine and arginine was measured at 570 nm. This was done in a 96-well flat-bottom microplate with a BioTek Synergy H1 multi-mode Reader (www.biotek.com). Alanine, proline, or arginine solutions (0.1 to 1 mM) served as standards.

3. Results

3.1. Effect of spore density on germination of A. niger

To assess the effect of spore density on germination, we first had to assess which spore numbers should be used. To this end, swelling and germ tube formation of conidia of *A. niger* were monitored in wells containing 5000, 10,000, 20,000 and 40,000 conidia with 25 mM Na-phosphate buffer, 2 mM $MgSO_4$, and 10 mM alanine, proline, or arginine as carbon and nitrogen source. Proline and alanine strongly induce swelling and germ tube formation, while arginine is only a weak trigger (Ijadpanahsaravi et al., 2021). The maximum number of spores that had swollen (P_{max} swelling) or formed germ tubes (P_{max} germ tube formation) was estimated based on the asymmetrical model (Dantigny et al., 2011). Also, the time (τ) to reach 0.5 P_{max} swelling and 0.5 P_{max} germ tube formation was estimated as well as the degree of heterogeneity in the germination response (d). From this experiment it was concluded

that 5000 and 40,000 spores are representative for use for quantitative analysis of swelling and germ tube formation at low and high densities (Supplemental Text 1). Thus, these spores numbers were used in the follow up experiments.

The impact of density of *A. niger* spores on the d value was relatively low because it only affected germ tube formation in proline (Supplemental Text 2 A). P_{max} and τ of swelling were not significantly different at low (25.28 % and 18 h) and high (12.05 % and 18 h) spore density in the presence of arginine (Table 1; Fig. 1C). P_{max} of germ tube formation was also not different at low and high density (5.5 % vs 5.61 %), while τ was higher at high density (18 h) when compared to low density (9.91 h). Notably, P_{max} of swelling in alanine was significantly higher at low density (93.28 %) than at high density (76.71 %), but their τ was similar (5.27 h and 5.43 h, respectively) (Table 1; Fig. 1A). P_{max} of germ tube formation was also different at low (80.47 %) and high (56.89 %) density in the presence of alanine, while τ was shorter at low density (10.06 h vs 10.67 h). Similarly, P_{max} of swelling and germ tube formation in proline was significantly higher at low density (95.5 % and 80.09 %, respectively) than at high density (89.42 % and 63.59 %, respectively), while τ was shorter at low density (3.13 h and 7.84 h, respectively) than at high density (3.41 h and 7.92 h, respectively). Together, higher spore density of *A. niger* reduces the incidence of swelling and germ tube formation in the presence of the inducing amino acids alanine and proline, while these amino acids do not highly, if at all, affect the rate of swelling and germ tube formation.

3.2. Identification of *A. niger* spores in co-cultures

In the previous section, germination dynamics was compared at low (5000 spores) and high (40,000 spores) density. Next, it was assessed whether replacing the 35,000 extra *A. niger* spores with those of other aspergilli (*A. clavatus, A. nidulans, A. terreus,* and *A. oryzae*) has the same effect on the incidence and rate of swelling and germ tube formation of *A. niger* spores. To do this, we first had to assess whether conidia of *A. niger* can be discriminated by oCelloScope analysis when present in a mixture with another *Aspergillus*. To this end, differences in circularity, surface area, and contrast of the different conidia were determined at $t = 1$ h. There was no detectable difference between the circularity of the spores of the different *Aspergillus* species (all ranging between 1.0 and 1.1). In contrast, surface area and contrast did differ between the spores of the aspergilli (Fig. 2). Yet, they partly overlapped with a spore percentage of 0.5 %, 3.8 %, 33.9 %, and 34.7 % when *A. niger* was mixed with *A. terreus, A. nidulans*, *A. oryzae,* and *A. clavatus*, respectively. To overcome the overlap problem, especially in the case of co–cultures with *A. clavatus* and *A. oryzae*, the Euclidean distance was calculated for each spore in the co-culture with those of the monoculture datasets (see Material & Methods). We only selected the *A. niger* spores with scores >90 (i.e., the dark blue region in Fig. 3 having no overlap with the other *Aspergillus* species) for germination analysis in co-culture. The selected spore sets of *A. niger* depended on the contrast and surface area of the other *Aspergillus* species in co-culture, and therefore they were different for each co-culture (from now on collectively called subpopulations).

Next, it was assessed whether the *A. niger* subpopulations were representative of the whole population of its spores. To this end, the dynamics of swelling and germ tube formation in the subpopulations were compared to the total *A. niger* spore population. In the presence of arginine, P_{max} and τ of swelling and germ tube formation of all subpopulations were similar to those of the whole 40,000 *A. niger* conidia population, except for the subpopulation selected for co-culturing with *A. oryzae* (Table 2). The P_{max} and τ of swelling and germ tube formation of the *A. oryzae* subpopulation were 3.17 % and 2.5 % and 11.71 h and 14.22 h, respectively, while these values were 12.05 % and 5.61 % and 18 h and 18 h for the whole population. Thus, the subpopulation selected for the *A. oryzae* co-culture germinated less when compared to the whole population. In the presence of alanine, the P_{max} of swelling of the selection of *A. niger* spores for co-culturing with *A. oryzae* (72.56 %) and *A. clavatus* (83.75 %) was also different when compared to the whole population (76.71 %), while τ was only different for the selected subpopulation for co-culturing with *A. clavatus* (4.42 h) and *A. nidulans* (4.91 h) when compared to the whole population (5.43 h) (Table 2). In the case of germ tube formation, only the P_{max} of the *A. niger* selection for *A. clavatus* co-culture was different (61.46 % vs 56.89 %), while τ was the same for all cases. In the presence of proline, the P_{max} of swelling of the *A. niger* selection for co-culturing with *A. clavatus* was different from that of the whole population (92.41 % vs 89.42 %), while τ of swelling was different in all subpopulations for co-culturing (2.88 h–3.33 h), except for *A. terreus* (3.39) when compared to the whole population of *A. niger* (3.41) (Table 2). The P_{max} and τ of germ tube formation were all different (64.86 %–68.47 % and 7.61 h–7.81 h), except for the selection for *A. terreus* co-culture (63.72 % and 7.91 h) when compared to the whole population (63.59 % and 7.92 h). The subpopulations of *A. niger* spores that were selected to distinguish these spores from those of *A. oryzae* or, to a minor extent, *A. clavatus* also showed a deviating heterogeneity in germination response when compared to the whole population (Supplemental Text 2B). Together, the subpopulation of *A. niger* spores for *A. terreus* co-culture behaves identically with respect to germination parameters when compared to the whole *A. niger* population, while the other subpopulations behave

Table 1
Parameter estimates of the asymmetrical model describing swelling and germ tube formation of *Aspergillus niger* conidia in monoculture at low (5000 spores) and high (40000) density. P_{max}, τ, and d represent the maximal percentage of swollen or germinated conidia, the time where $P = 0.5\ P_{max}$, and the degree of heterogeneity in the germination response, respectively. Confidence intervals are indicated between brackets, N represents the number of objects at $t = 1$ h, while M represents the number of objects that were no longer detected because the hypha had become too long or the object was obscured by hyphae of other objects. RMSE represents the root mean square error of the modelled data and is a measure for the goodness of fit (Dantigny et al., 2011; Ratkowsky, 2004).

Species	AA	P_{max} (%)	τ (h)	d (−)	RMSE	M	N
Swelling							
A. niger (5000)	Ala	93.28 [92.05;94.51] a	5.27 [5.15;5.39] a	2.83 [2.66;3.00] a	0.26	7	190
A. niger (40000)	Ala	76.71 [75.85;77.57] b	5.43 [5.33;5.53] a	3.08 [2.91;3.25] a	0.19	190	1181
A. niger (5000)	Arg	25.28 [12.87;37.69] a	18.00 [3.31;32.69] a	1.21 [0.84;1.57] a	0.13	7	214
A. niger (40000)	Arg	12.05 [5.57;18.52] a	18.00 [4.70;31.30] a	1.47 [0.98;1.97] a	0.08	36	1282
A. niger (5000)	Pro	95.50 [95.22;95.78] a	3.13 [3.10;3.15] a	4.08 [3.96;4.20] a	0.11	16	389
A. niger (40000)	Pro	89.42 [88.97;89.87] b	3.41 [3.36;3.45] b	3.99 [3.80;4.17] a	0.16	110	1216
Germ tube formation							
A. niger (5000)	Ala	80.47 [78.71;82.23] a	10.06 [9.88;10.24] a	7.11 [6.28;7.94] a	0.43	7	190
A. niger (40000)	Ala	56.89 [55.67;58.11] b	10.67 [10.49;10.84] b	6.93 [6.21;7.65] a	0.27	190	1181
A. niger (5000)	Arg	5.50 [4.89;6.12] a	9.91 [8.94;10.88] a	4.89 [2.77;7.00] a	0.11	7	214
A. niger (40000)	Arg	5.61 [3.71;7.50] a	18.00 [13.68;22.32] b	3.05 [2.26;3.84] a	0.03	36	1282
A. niger (5000)	Pro	80.09 [78.13;82.05] a	7.84 [7.62;8.06] a	5.45 [4.68;6.21] a	0.52	16	389
A. niger (40000)	Pro	63.59 [63.35;63.84] b	7.92 [7.89;7.96] a	8.19 [7.94;8.43] b	0.08	110	1216

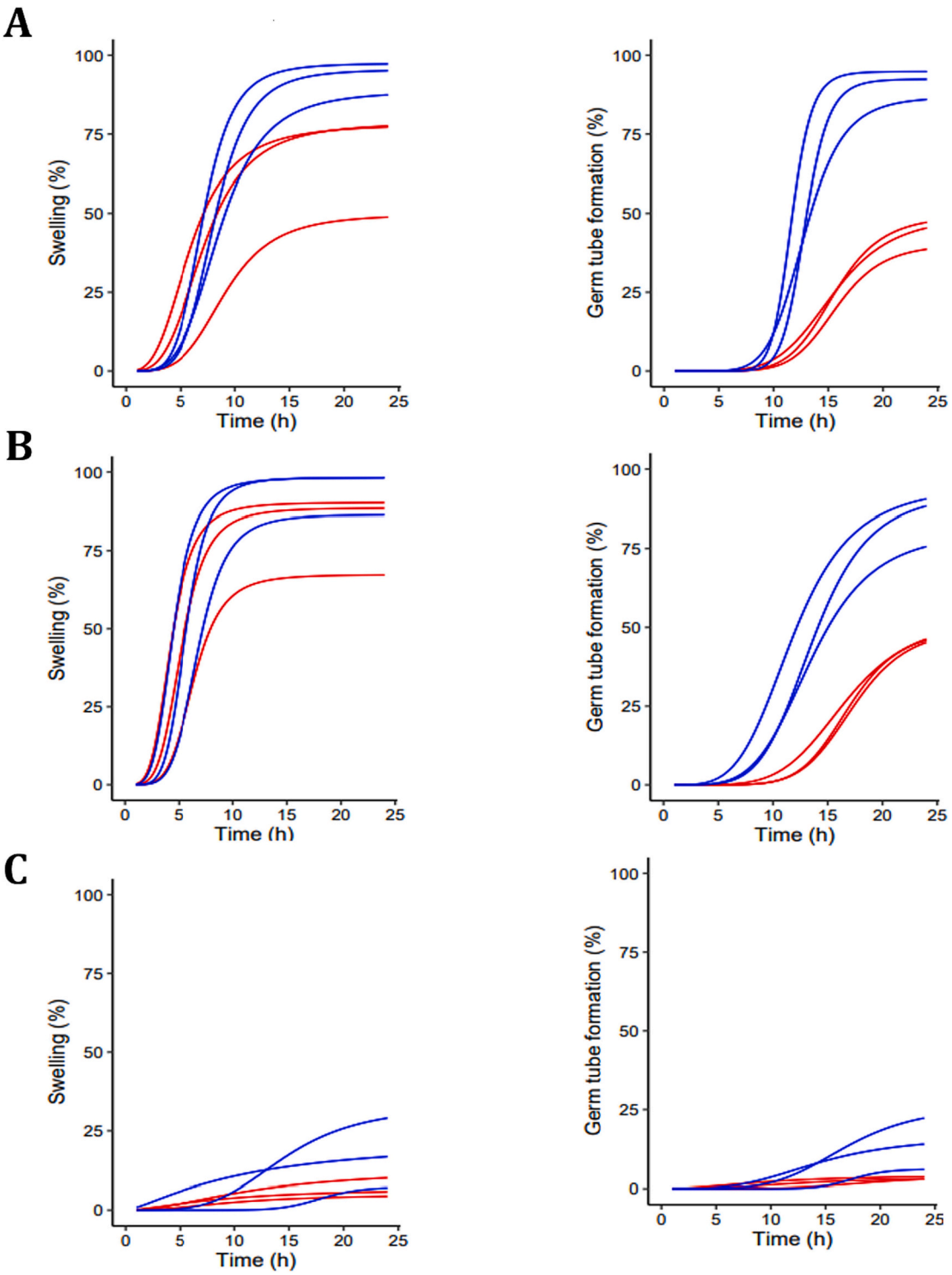

Fig. 1. Plots of biological triplicates of swelling and germ tube formation of *Aspergillus niger* conidia at low (5000 spores; indicated in blue) and high (40,000 spores; indicated in red) density in alanine (A), proline (B), and arginine (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differently.

3.3. Effect of the presence of other aspergilli conidia on A. niger germination

A total of 5000 spores of *A. niger* was mixed with 35,000 spores of *A. clavatus, A. nidulans, A. terreus,* or *A. oryzae.* Swelling and germ tube formation of the *A. niger* subpopulations were compared to the respective subpopulations of the 40,000 spores *A. niger* monoculture (see 3.2). The d of germ tube formation was reduced in proline and alanine, but not in arginine, in the presence of another *Aspergillus*, implying germ tube formation of *A. niger* becomes more heterogeneous in the presence of other aspergilli when exposed to an inducing amino acid (Supplemental Text 2C). This phenomenon was also observed in proline in the case of swelling.

Adding other *Aspergillus* spores had the same effect as adding extra

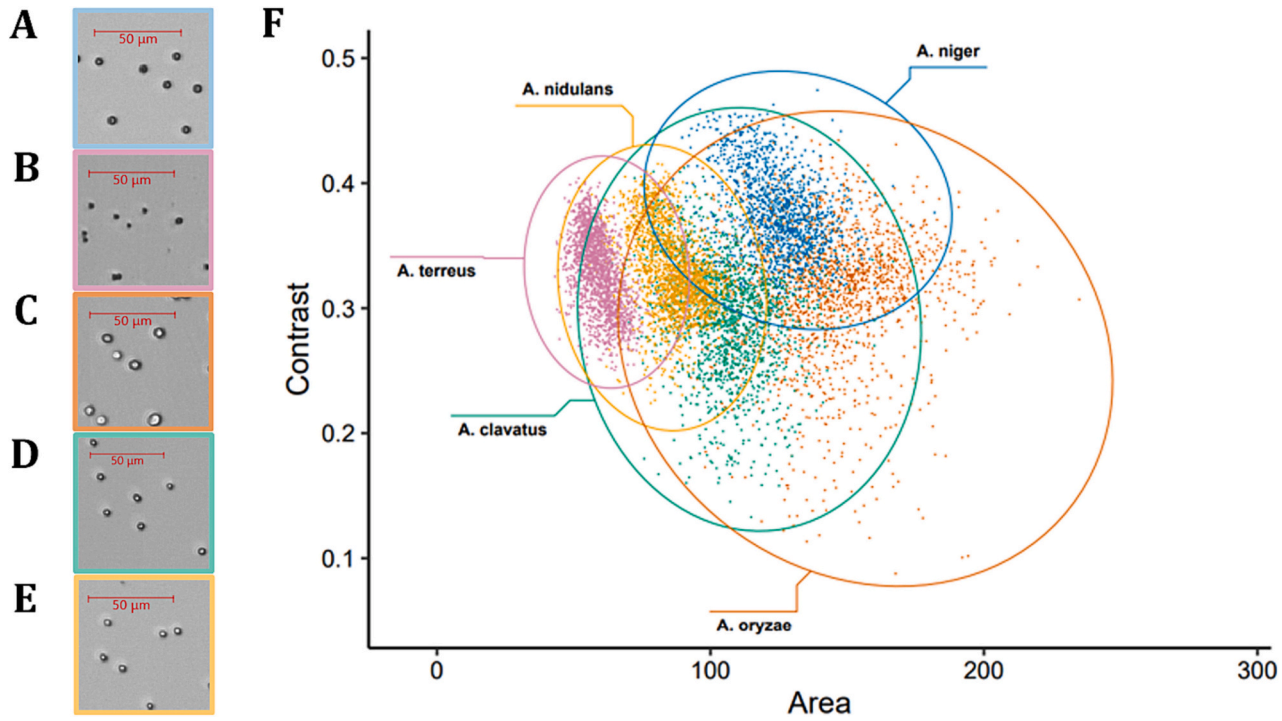

Fig. 2. Images of conidia of *A. niger* (A), *A. terreus* (B), *A. oryzae* (C), *A. clavatus* (D), and *A. nidulans* (E) and scatter plot of area and contrast of Aspergillus conidia (F) at t = 1 h. Sample size in F was ≥1200 conidia for each species.

A. niger spores to the wells containing arginine. P_{max} and τ of swelling and germ tube formation varied between 3.17 %–18.47 % and 7.85 h–18 h and between 2.43 %–8.42 % and 14.22 h–18 h (Table 3). In contrast, when the spores were incubated in alanine, P_{max} of swelling was always lower in the presence of other aspergilli (55.15–61.57 %) when compared to adding extra *A. niger* spores (72.56 %–83.75 %), while the τ was always higher (6.11 h–7.34 h versus 4.42 h–5.53 h). Also, P_{max} of germ tube formation was always lower in the presence of other aspergilli (34.17 %–42.96 %) when compared to adding extra *A. niger* spores (56.36 %–61.46 %), while τ was always higher (18 h vs 10.56 h–10.66 h). Together, the spores of other aspergilli have a higher inhibiting effect on swelling and germ tube formation in alanine than the higher density of *A. niger* itself. A similar effect was observed in the case of proline. P_{max} of swelling was always lower in the presence of other aspergilli (77.60 %–81.97 %) when compared to adding extra *A. niger* spores (89.56 %–92.41 %), while the τ was always higher (3.44 h–4.47 h versus 2.88 h–3.39 h). The P_{max} of germ tube formation was lower in the presence of *A. clavatus* and *A. oryzae* (49.87 %–49.44 %) but similar in the cases of *A. terreus* and *A. nidulans* (58.92 %–64.86 %). The τ was always higher (15.36 h–16.69 h vs 7.61 h–7.91 h). Together, in most cases, spores of other aspergilli have a higher inhibiting effect on swelling and germ tube formation than adding additional *A. niger* conidia.

3.4. Analysis of the culture medium of different spore densities and co-cultures

The ninhydrin colorimetric method was performed to determine proline, alanine, and arginine concentrations in the culture medium during incubation of mono- (5000 or 40,000 spores) or co-cultures (5000 *A. niger* + 35,000 spores of other aspergilli) of *A. niger*. This revealed that the concentration of amino acids in the culture medium did not change during a 24 h period (Fig. 4). These data indicate that the amino acids are not taken up but rather activate sensors at the plasma membrane.

4. Discussion

Aspergilli are abundant in nature as well as in food and will therefore compete with each other for substrates. The fact that the germination response of the different aspergilli depends on the medium conditions implies that their competitive potential differ depending on the substrate (Ijadpanahsaravi et al., 2022). A high germination incidence within a certain substrate will give it higher competitiveness when compared to a lower germination incidence. We here show that spores of other aspergilli can suppress the germination incidence of *A. niger*. In fact, this inhibition is stronger than observed by increasing the density of *A. niger* conidia itself. This inter-species germination inhibition thereby adds another layer of competitiveness in co-cultures.

Germination of conidia was quantitatively assessed in the presence of the amino acids proline, alanine, and arginine. The former two amino acids induce germination highly in *A. niger, A. oryzae, A. clavatus,* and *A. nidulans* but not in the case of *A. terreus* (Ijadpanahsaravi et al., 2022). On the other hand, arginine highly induces germination in *A. oryzae, A. clavatus,* and *A. nidulans* but not in *A. niger* and *A. terreus*. To assess the germination of *A. niger* in co-cultures, its spores have to be distinguished from the spores of other aspergilli. The circularity of conidia was similar between the aspergilli, but surface area and contrast were different. Yet, the scatter plots of these parameters partially overlapped. The overlap with *A. terreus* and *A. nidulans* was minimal (0.5 % and 3.8 %, respectively), while it was relatively high (33.9 % and 34.7 %) in the cases of *A. oryzae* and *A. clavatus*, respectively. The latter can be explained by the large heterogeneity in size and contrast of the conidia of the latter two species (Fig. 2). It is not known why these fungi produce such heterogeneous spore populations.

To overcome the overlap in the scatter plots, sub-populations of *A. niger* conidia were defined for the combination with each *Aspergillus* species. To this end, *A. niger* spores were selected that were outside the region of overlap in contrast and surface area. Previously, we showed that sub-populations of *Aspergillus* spores differing in size or contrast can behave differently within a spore population (Ijadpanahsaravi et al., 2022). Differences in dynamics of swelling and germ tube formation

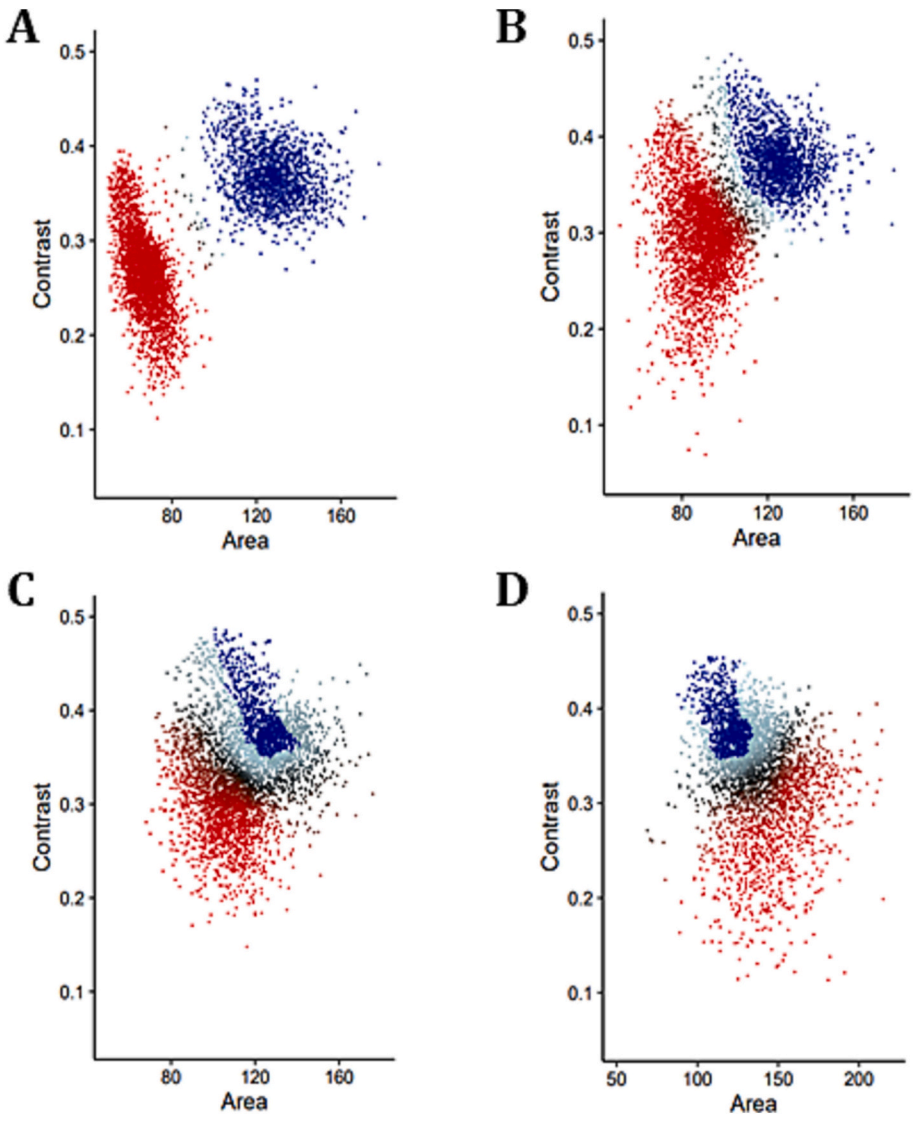

Fig. 3. Simulated co-cultures of spores of Aspergillus niger and Aspergillus terreus (A), Aspergillus nidulans (B), Aspergillus clavatus (C) and Aspergillus oryzae (D). Light (Eucladian distance score 50–90) and dark (Eucladian distance score > 90) blue dots represent *A. niger* spores that are not overlapping with the population of one of the other aspergilli. The dark blue dots are selected for analysis of swelling and germ tube formation. The black dots (Eucladian distance score < 50) represent the overlap, while the red dots (Eucladian distance score ≥ 50) represent spores of the other aspergilli that a*re not part of the overlap.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were observed in the subpopulations of small and large conidia of *A. niger* and between the conidia with low and high contrast. Similar results were found for *A. oryzae, A. clavatus,* and *A. nidulans,* but not in the case of *A. terreus.* Therefore, it was assessed whether the swelling and germ tube formation of the subpopulation of *A. niger* spores for co-culturing with each of the other aspergilli were representative of the whole spore population. Results showed that the subpopulation of *A. niger* spores for the co-culture with *A. terreus* behaved identically to the whole *A. niger* population, but the other subpopulations behaved differently. This may be explained by the fact that the overlap in contrast and size was smallest in the case of *A. terreus*, but also the overlap with *A. nidulans* was small. Yet, it should be noted that the selection criteria for the identification of *A. niger* spores were strict. Making these criteria less strict would create a better representation of the sub-populations for the whole population, but at the same time, the incidence of misidentification would increase.

Germination analysis showed that increasing the spore density of *A. niger* conidia in culture medium with arginine did not impact the maximum number of spores (P_{max}) that started to swell or that formed germ tubes, nor the rate of germination. In contrast, higher spore density of *A. niger* reduced the incidence of swelling and germ tube formation in the presence of the inducing amino acids alanine and proline, while the rate of swelling and germ tube formation was not highly, if at all, affected. These data are in line with the results of Barrios-González et al. (1989). However, we show that the density effect depends on the medium composition and that particularly the percentage of germinating spores is affected but not the rate of germination. Spore density not only impacts *Aspergillus* germination but also the differentiation processes and the production of secondary metabolites. For instance, high spore density results in increased production of the mycotoxins ochratoxin A and aflatoxin in *Aspergillus ochraceus* and *Aspergillus flavus,* respectively (Li et al., 2017; Yan et al., 2012), while sclerotia formation is also promoted at high spore density in the latter fungus (Horowitz Brown et al., 2008).

Adding other *Aspergillus* spores had the same effect on swelling and germ tube formation as adding extra *A. niger* spores when culturing in arginine. In contrast, spores of other aspergilli had a higher inhibiting effect on the incidence and rate of swelling and germ tube formation

Table 2
Parameter estimates of the asymmetrical model describing swelling and germ tube formation of *Aspergillus niger* conidia in monoculture in high density (all 40,000 spores) and the subsets of spores after applying the selection criteria to distinguish the *A. niger* spores with each of the other aspergilli. P_{max}, τ, and d represent the maximal percentage of swollen or germinated conidia, the time where $P = 0.5\ P_{max}$, and the degree of heterogeneity in the germination response, respectively. Confidence intervals are indicated between brackets, N represents the number of objects at t = 1 h, while M represents the number of objects that were no longer detected because the hypha had become too long or the object was obscured by hyphae of other objects. RMSE represents the root mean square error of the modelled data and is a measure for the goodness of fit (Dantigny et al., 2011; Ratkowsky, 2004).

	AA	P_{max} (%)	τ (h)	d (−)	RMSE	M	N
Swelling							
A. niger	Ala	76.71 [75.85;77.57] a	5.43 [5.33;5.53] a	3.08 [2.91;3.25] a	0.19	190	1181
A. niger selection with *A. clavatus*	Ala	83.75 [82.90;84.59] b	4.42 [4.33;4.52] b	3.28 [3.07;3.49] a	0.24	24	223
A. niger selection with *A. nidulans*	Ala	77.88 [76.94;78.82] a	4.91 [4.80;5.01] c	3.16 [2.95;3.37] a	0.24	145	965
A. niger selection with *A. oryzae*	Ala	72.56 [71.64;73.48] c	5.53 [5.42;5.65] a	4.06 [3.74;4.39] b	0.26	82	492
A. niger selection with *A. terreus*	Ala	76.68 [75.78;77.57] a	5.37 [5.26;5.47] a	3.12 [2.93;3.30] a	0.21	186	1161
A. niger	Arg	12.05 [5.57;18.52] a	18.00 [4.70;31.30] a	1.47 [0.98;1.97] a	0.08	36	1282
A. niger selection with *A. clavatus*	Arg	10.40 [5.89;14.92] a	18.00 [9.98;26.02] a	2.01 [1.43;2.60] a	0.06	10	284
A. niger selection with *A. nidulans*	Arg	13.89 [6.18;21.60] a	18.00 [4.04;31.96] a	1.45 [0.95;1.95] a	0.09	30	1077
A. niger selection with *A. oryzae*	Arg	3.17 [3.01;3.34] b	11.71 [11.23;12.18] a	3.90 [3.43;4.37] b	0.02	19	632
A. niger selection with *A. terreus*	Arg	11.95 [5.49;18.41] a	18.00 [4.51;31.49] a	1.46 [0.97;1.95] a	0.08	36	1276
A. niger	Pro	89.42 [88.97;89.87] a	3.41 [3.36;3.45] a	3.99 [3.80;4.17] a	0.16	110	1216
A. niger selection with *A. clavatus*	Pro	92.41 [92.16;92.66] b	2.88 [2.86;2.90] b	4.67 [4.53;4.81] b	0.1	20	296
A. niger selection with *A. nidulans*	Pro	90.12 [89.68;90.56] a	3.11 [3.07;3.15] c	4.38 [4.16;4.60] a	0.17	86	1012
A. niger selection with *A. oryzae*	Pro	90.00 [89.67;90.33] a	3.33 [3.30;3.36] a	5.22 [5.00;5.44] c	0.13	45	541
A. niger selection with *A. terreus*	Pro	89.56 [89.08;90.03] a	3.39 [3.34;3.43] a	4.04 [3.84;4.24] a	0.17	107	1204
Germ tube formation							
A. niger	Ala	56.89 [55.67;58.11] a	10.67 [10.49;10.84] a	6.93 [6.21;7.65] a	0.27	190	1181
A. niger selection with *A. clavatus*	Ala	61.46 [59.57;63.34] b	10.60 [10.35;10.85] a	7.31 [6.17;8.45] a	0.44	24	223
A. niger selection with *A. nidulans*	Ala	57.57 [56.28;58.86] a	10.56 [10.38;10.75] a	6.88 [6.13;7.63] a	0.29	145	965
A. niger selection with *A. oryzae*	Ala	56.36 [55.05;57.67] a	10.56 [10.37;10.75] a	7.46 [6.56;8.36] a	0.31	82	492
A. niger selection with *A. terreus*	Ala	56.89 [55.68;58.11] a	10.66 [10.49;10.84] a	6.96 [6.24;7.68] a	0.27	186	1161
A. niger	Arg	5.61 [3.71;7.50] a	18.00 [13.68;22.32] a	3.05 [2.26;3.84] a	0.03	36	1282
A. niger selection with *A. clavatus*	Arg	3.47 [1.48;5.46] a	18 0.00 [12.34;23.66] a	4.16 [1.98;6.33] a	0.05	10	284
A. niger selection with *A. nidulans*	Arg	6.23 [4.00;8.45] a	18.00 [13.54;22.46] a	3.14 [2.26;4.02] a	0.04	30	1077
A. niger selection with *A. oryzae*	Arg	2.50 [2.09;2.90] b	14.22 [12.55;15.89] a	3.56 [2.73;4.40] a	0.02	19	632
A. niger selection with *A. terreus*	Arg	5.48 [3.56;7.41] a	18.00 [13.55;22.45] a	3.07 [2.24;3.91] a	0.04	36	1276
A. niger	Pro	63.59 [63.35;63.84] a	7.92 [7.89;7.96] a	8.19 [7.94;8.43] a	0.08	110	1216
A. niger selection with *A. clavatus*	Pro	65.25 [65.00;65.50] b	7.61 [7.58;7.65] b	8.57 [8.30;8.84] a	0.08	20	296
A. niger selection with *A. nidulans*	Pro	64.86 [64.60;65.12] b	7.79 [7.76;7.83] c	8.23 [7.97;8.49] a	0.08	86	1012
A. niger selection with *A. oryzae*	Pro	68.71 [68.50;68.91] c	7.81 [7.78;7.83] c	9.04 [8.82;9.26] b	0.07	45	541
A. niger selection with *A. terreus*	Pro	63.72 [63.46;63.97] a	7.91 [7.88;7.94] a	8.19 [7.94;8.45] a	0.08	107	1204

when the spores were cultured in alanine. A similar effect was observed in the case of proline. In the latter amino acid, τ of swelling and germ tube formation was always higher in the presence of other aspergilli when compared to adding extra *A. niger* spores. P_{max} of swelling was always lower in the presence of other aspergilli, but this was only true for *A. clavatus* and *A. oryzae* for germ tube formation. Adding other *Aspergillus* spores also increased heterogeneity of germ tube formation in the case of these inducing amino acids evidenced by a reduced d value.

Together, in most cases, the spores of other aspergilli have a higher inhibiting effect on swelling and germ tube formation than the higher density of *A. niger* itself. How can we explain this phenomenon? This is most probably not explained by a faster germination response of other aspergilli when compared to *A. niger*. *A. oryzae, A. clavatus,* and *A. nidulans* show a higher germination response on arginine when compared to *A. niger*, while *A. terreus* shows a lower response (Ijadpanahsaravi et al., 2022). Yet, no increased effect of other aspergilli was observed on germination (see above). On alanine, only *A. oryzae* shows a much higher germination when compared to *A. niger* (Ijadpanahsaravi et al., 2022) but all aspergilli showed a higher germination inhibitory effect on *A. niger* in the presence of this amino acid. A similar effect was observed in the case of proline. Release of primary and/or secondary metabolites may be the cause of stronger germination inhibition of the presence of other aspergilli when compared to a higher *A. niger* density. These compounds should be released by the dormant spores in the case of alanine and proline since they generally do not germinate faster than *A. niger* in the presence of these amino acids. In contrast, these metabolites may also be released by germlings of *A. oryzae, A. clavatus,* and *A. nidulans* in arginine because they do show a higher germination response on this amino acid.

The concentration of amino acids did not change in the culture media during the time of incubation of the mono- and co-cultures. Therefore, uptake of this carbon and nitrogen source by the competing aspergilli does not explain the strong inhibiting effect of the other aspergilli on *A. niger* germination. Possibly, it can be explained by competition for O_2 or CO_2. In the case of *A. nidulans,* experimental evidence indicates that the germination of conidia at high density was inhibited due to an insufficient supply of carbon dioxide (Trinci and Whittaker, 1968). Another possibility is the involvement of a quorum sensing-like mechanism in the release of inhibitory molecules. *A. niger* conidia contain self-inhibitors that diffuse out of the spores. This would result in a higher concentration of the inhibitors in the culture medium at high spore density (Barrios-González et al., 1989). When washing the spores, the auto-inhibitors are removed and can therefore no longer inhibit spores germination. 1-octen-3-ol, 3-octanone, and 3-octanol reversibly inhibit the germination of *A. nidulans* at high conidia density, with the former molecule being the most active inhibitor (Herrero-Garcia et al., 2011). Similarly, 1-octen-3-ol blocks the germination process in *Penicillium paneum* (Chitarra et al., 2004). However, our preliminary data indicate that inhibition of spore germination is not due to auto-inhibitors. Culture media from different stages of incubation ($t = 1$, 6, 12, and 24 h) of high density spore *A. niger* monocultures were not able to inhibit germination when added to low density conidia of this fungus. This difference with previous findings (Barrios-González et al., 1989; Herrero-Garcia et al., 2011; Chitarra et al., 2004) may be explained by the fact that we used water-washed spores in our experiments, while the auto-inhibitors are produced during sporulation and not during

Table 3
Parameter estimates of the asymmetrical model describing swelling and germ tube formation of the subpopulations of 5000 *Aspergillus niger* conidia when co-cultured with 35,000 conidia of one of the other aspergilli (indicated in rows with species description *A. niger* co-cultured with one of the other aspergilli) using as control the respective selected subsets of 40,000 *A. niger* spores in monoculture (indicated in rows with species description *A. niger* selection). All control data was taken from Table 1. P_{max}, τ, and d represent the maximal percentage of swollen or germinated conidia, the time where $P = 0.5\ P_{max}$, and the degree of heterogeneity in the germination response, respectively. Confidence intervals are indicated between brackets, N represents the number of objects at t = 1 h, while M represents the number of objects that were no longer detected because the hypha had become too long or the object was obscured by hyphae of other objects. RMSE represents the root mean square error of the modelled data and is a measure for the goodness of fit (Dantigny et al., 2011, Ratkowsky, 2004).

Species	AA	P_{max} (%)	τ (h)	d (−)	RMSE	M	N
Swelling							
A. niger co-cultured with *A. clavatus*	Ala	56.52 [55.82;57.22] a	6.38 [6.27;6.50] a	4.46 [4.13;4.80] a	0.19	43	670
A. niger selection	Ala	83.75 [82.90;84.59] b	4.42 [4.33;4.52] b	3.28 [3.07;3.49] b	0.24	24	223
A. niger co-cultured with *A. nidulans*	Ala	61.57 [59.30;63.84] a	6.11 [5.79;6.44] a	2.81 [2.40;3.22] a	0.4	132	1474
A. niger selection	Ala	77.88 [76.94;78.82] b	4.91 [4.80;5.01] b	3.16 [2.95;3.37] a	0.24	145	965
A. niger co-cultured with *A. oryzae*	Ala	55.15 [54.41;55.88] a	7.34 [7.22;7.47] a	4.42 [4.12;4.72] a	0.18	44	750
A. niger selection	Ala	72.56 [71.64;73.48] b	5.53 [5.42;5.65] b	4.06 [3.74;4.39] a	0.26	82	492
A. niger co-cultured with *A. terreus*	Ala	60.33 [58.06;62.61] a	6.39 [6.06;6.72] a	2.75 [2.37;3.13] a	0.37	157	1624
A. niger selection	Ala	76.68 [75.78;77.57] b	5.37 [5.26;5.47] b	3.12 [2.93;3.30] a	0.21	186	1161
A. niger co-cultured with *A. clavatus*	Arg	7.46 [−2.30;17.22] a	18.00 [8.16;27.84] a	5.91 [−3.24;15.05] a	0.33	134	565
A. niger selection	Arg	10.40 [5.89;14.92] a	18.00 [9.98;26.02] a	2.01 [1.43;2.60] a	0.06	10	284
A. niger co-cultured with *A. nidulans*	Arg	18.33 [−0.04;36.71] a	7.85 [−9.86;25.56] a	1.00 [−0.20;2.20] a	0.48	256	1314
A. niger selection	Arg	13.89 [6.18;21.60] a	18.00 [4.04;31.96] a	1.45 [0.95;1.95] a	0.09	30	1077
A. niger co-cultured with *A. oryzae*	Arg	4.52 [0.07;8.98] a	17.93 [12.99;22.86] a	11.49[−14.83;37.80] a	0.35	165	601
A. niger selection	Arg	3.17 [3.01;3.34] a	11.71 [11.23;12.18] a	3.90 [3.43;4.37] a	0.02	19	632
A. niger co-cultured with *A. terreus*	Arg	18.47 [−4.86;41.80] a	9.62 [−17.11;36.34] a	1.00 [−0.29;2.29] a	0.49	285	1429
A. niger selection	Arg	11.95 [5.49;18.41] a	18.00 [4.51;31.49] a	1.46 [0.97;1.95] a	0.08	36	1276
A. niger co-cultured with *A. clavatus*	Pro	79.95 [79.34;80.55] a	3.88 [3.82;3.95] a	3.66 [3.44;3.87] a	0.2	33	459
A. niger selection	Pro	92.41 [92.16;92.66] b	2.88 [2.86;2.90] b	4.67 [4.53;4.81] b	0.1	20	296
A. niger co-cultured with *A. nidulans*	Pro	81.97 [80.68;83.27] a	3.44 [3.31;3.58] a	2.61 [2.35;2.87] a	0.35	94	1255
A. niger selection	Pro	90.12 [89.68;90.56] b	3.11 [3.07;3.15] b	4.38 [4.16;4.60] b	0.17	86	1012
A. niger co-cultured with *A. oryzae*	Pro	77.60 [76.79;78.41] a	4.47 [4.38;4.57] a	3.54 [3.29;3.79] a	0.24	41	458
A. niger selection	Pro	90.00 [89.67;90.33] b	3.33 [3.30;3.36] b	5.22 [5.00;5.44] b	0.13	45	541
A. niger co-cultured with *A. terreus*	Pro	81.21 [79.87;82.56] a	3.73 [3.59;3.87] a	2.42 [2.20;2.64] a	0.31	114	1408
A. niger selection	Pro	89.56 [89.08;90.03] b	3.39 [3.34;3.43] b	4.04 [3.84;4.24] b	0.17	107	1204
Germ tube formation							
A. niger co-cultured with *A. clavatus*	Ala	40.29 [34.96;45.62] a	18.00 [16.76;19.24] a	4.44 [3.88;5.00] a	0.13	43	670
A. niger selection	Ala	61.46 [59.57;63.34] b	10.60 [10.35;10.85] b	7.31 [6.17;8.45] b	0.44	24	223
A. niger co-cultured with *A. nidulans*	Ala	42.69 [35.44;49.93] a	18.00 [16.30;19.70] a	4.09 [3.46;4.71] a	0.17	132	1474
A. niger selection	Ala	57.57 [56.28;58.86] b	10.56 [10.38;10.75] b	6.88 [6.13;7.63] b	0.29	145	965
A. niger co-cultured with *A. oryzae*	Ala	34.17 [29.73;38.60] a	18.00 [16.84;19.16] a	4.70 [4.09;5.30] a	0.12	44	750
A. niger selection	Ala	56.36 [55.05;57.67] b	10.56 [10.37;10.75] b	7.46 [6.56;8.36] b	0.31	82	492
A. niger co-cultured with *A. terreus*	Ala	40.97 [33.53;48.41] a	18.00 [16.20;19.80] a	4.13 [3.45;4.81] a	0.17	157	1624
A. niger selection	Ala	56.89 [55.68;58.11] b	10.66 [10.49;10.84] b	6.96 [6.24;7.68] b	0.27	186	1161
A. niger co-cultured with *A. clavatus*	Arg	4.62 [−0.43;9.67] a	18.00 [9.46;26.54] a	5.62 [−1.36;12.60] a	0.16	134	565
A. niger selection	Arg	3.47 [1.48;5.46] a	18.00 [12.34;23.66] a	4.16 [1.98;6.33] a	0.05	10	284
A. niger co-cultured with *A. nidulans*	Arg	8.42 [−0.82;17.67] a	18.00 [−0.92;36.92] a	2.18 [0.55;3.80] a	0.13	256	1314
A. niger selection	Arg	6.23 [4.00;8.45] a	18.00 [13.54;22.46] a	3.14 [2.26;4.02] a	0.04	30	1077
A. niger co-cultured with *A. oryzae*	Arg	2.43 [−0.27;5.14] a	18.00 [11.88;24.12] a	9.49 [−10.21;29.19] a	0.16	165	601
A. niger selection	Arg	2.50 [2.09;2.90] a	14.22 [12.55;15.89] a	3.56 [2.73;4.40] a	0.02	19	632
A. niger co-cultured with *A. terreus*	Arg	7.97 [−1.37;17.31] a	18.00 [−1.25;37.25] a	2.30 [0.44;4.16] a	0.14	285	1429
A. niger selection	Arg	5.48 [3.56;7.41] a	18.00 [13.55;22.45] a	3.07 [2.24;3.91] a	0.04	36	1276
A. niger co-cultured with *A. clavatus*	Pro	49.87 [46.83;52.90] a	15.36 [14.77;15.94] a	4.02 [3.70;4.35] a	0.13	33	459
A. niger selection	Pro	65.25 [65.00;65.50] b	7.61 [7.58;7.65] b	8.57 [8.30;8.84] b	0.08	20	296
A. niger co-cultured with *A. nidulans*	Pro	59.91 [53.88;65.94] a	16.58 [15.42;17.74] a	3.31 [2.98;3.63] a	0.16	94	1255
A. niger selection	Pro	64.86 [64.60;65.12] a	7.79 [7.76;7.83] b	8.23 [7.97;8.49] b	0.08	86	1012
A. niger co-cultured with *A. oryzae*	Pro	49.44 [45.34;53.54] a	15.81 [14.96;16.67] a	3.74 [3.37;4.10] a	0.15	41	458
A. niger selection	Pro	68.71 [68.50;68.91] b	7.81 [7.78;7.83] b	9.04 [8.82;9.26] b	0.07	45	541
A. niger co-cultured with *A. terreus*	Pro	58.92 [53.18;64.66] a	16.69 [15.59;17.8] a	3.39 [3.07;3.71] a	0.15	114	1408
A. niger selection	Pro	63.72 [63.46;63.97] a	7.91 [7.88;7.94] b	8.19 [7.94;8.45] b	0.08	107	1204

germination. The fact that we do observe germination inhibition at high spore density may therefore be explained by physical rather than chemical signaling.

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CRediT authorship contribution statement

Maryam Ijadpanahsaravi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization, Writing – original draft. **Basten L. Snoek:** Methodology, Software, Writing – review & editing. **Wieke R. Teertstra:** Supervision, Writing – review & editing. **Han A.B. Wösten:** Supervision, Writing – review & editing.

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.

Data availability

Data will be made available on request.

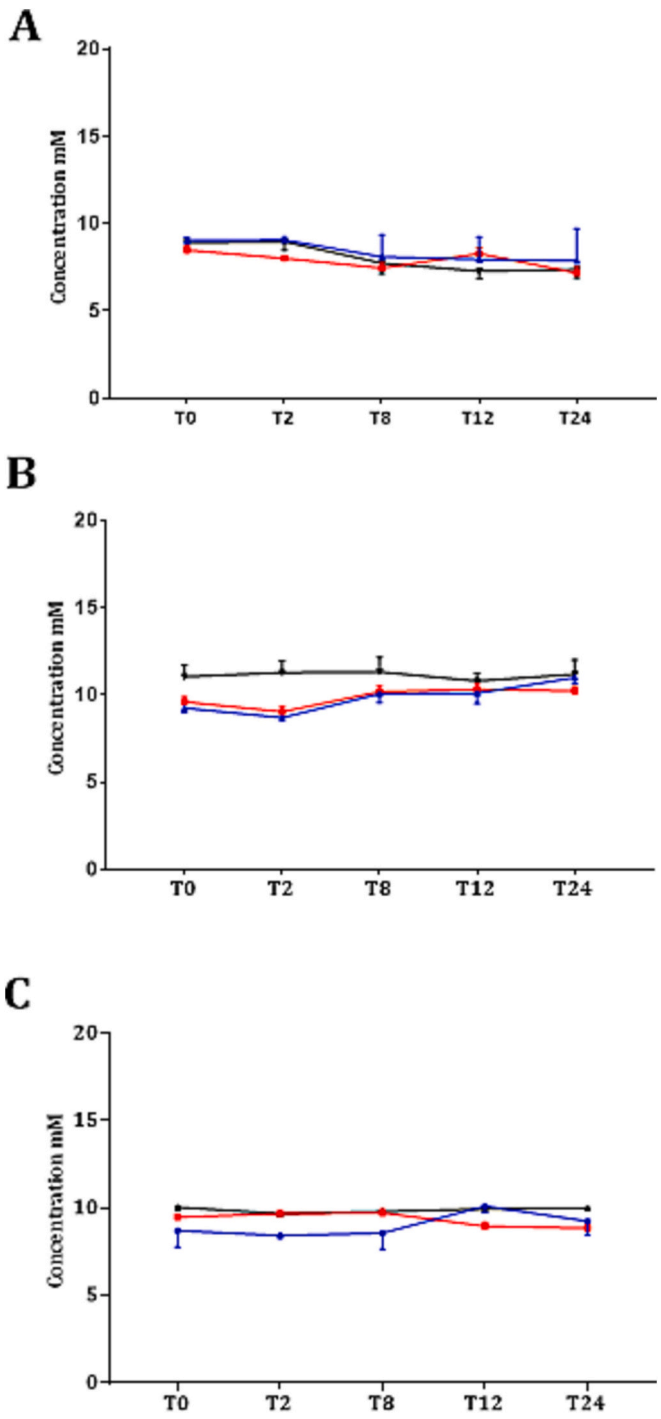

Fig. 4. Concentration of alanine (A), proline (B) and arginine (C) in culture media during a 2-day-incubation of low (blue lines) and high (red lines) *Aspergillus niger* spore density using biological triplicates. Error bars represent the standard error of the mean (SEM). Black lines represent the co-culture of *A. niger* conidia with spores of *Aspergillus oryzae*. Similar results were obtained with the other co-cultures (data not shown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110495.

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