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Comparative pathogenicity of opportunistic black yeasts in *Aureobasidium*

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Summary

Aureobasidium pullulans and *A. melanogenum* are black-yeast-like surface colonisers and are commonly encountered as contaminants in the hospital. The species are able to produce melanin which play a role in protection against environmental stress and irradiation. *Aureobasidium melanogenum* shows higher frequency in opportunistic infections compared to *A. pullulans*. Comparative pathogenicity of opportunistic black yeasts between *Aureobasidium pullulans* and *A. melanogenum* to explain the observed differences in frequency in infection. Degrees of melanisation and thermotolerance were measured, and virulence of strains from different sources was examined in *Galleria mellonela* and murine infection models. *Aureobasidium melanogenum* responds with increased melanisation to temperature stress and generally survives at 37°C, *A. pullulans* on average scored less on these parameters. In the murine model, differences between species were not significant, but the melanised *A. melanogenum* group showed the highest virulence. This result was not reproducible in *Galleria mellonella* larvae at 25°C. The *A. melanogenum* black group showed higher pathogenicity in murine model, indicating that the combination of melanisation and thermotolerance rather than species affiliation is instrumental. *Galleria* larvae did not survive very well at 37°C, and hence, this model is judged insufficient to detect the small virulence differences observed in *Aureobasidium*.

KEYWORDS
Aureobasidium melanogenum, *Aureobasidium pullulans*, *Galleria melonella*, melanin, murine model, thermotolerance, virulence

1 | INTRODUCTION

Aureobasidum is a black yeast genus in the order Dothideales (Dothideomycetes), particularly known for its biotechnological significance as a producer of the biodegradable extracellular polysaccharide pullulan.[1] Numerous other applications are known in medicine, pharmacy and food industry, and the fungus is also used as a biocontrol agent in agriculture.[2-4] It is regarded as safe for large-scale application. *Aureobasidium* species are ubiquitous in moist, oligotrophic environments with fluctuating water activities, particularly on the phyllosphere, but also on rock.[4] Natural habitats of *Aureobasidium* are osmotically stressed and often subjected to solar irradiation[5,6] and therefore the fungi are ecologically classified as extremotolerants.[7] In the human-dominated environment, they are common on damp indoor surfaces in bathrooms and saunas and may colonise medical instrumentation.[8,9] *Aureobasidium* also occurs as a commensal on healthy human skin,[10] but compared to black yeasts in the genus *Exophiala* (order Chaetothyriales, Eurotiomycetes), *Aureobasidium* is, compared to its common occurrence in clinical settings, extremely rarely involved in human infection; its pathogenic potential is judged to be very low.[11] Cases mostly concern superficial infections in healthy individuals, including onychomycosis or keratitis.[11,12] Systemic infections, for example, peritonitis or disseminated infections exclusively occur in patients with severe immune disorders, in patients undergoing peritoneal dialysis or in neonates, which is in line with the opportunistic nature of the fungus.[13-15]

Aureobasidium strains differ significantly in their production of melanised chlamydospores, colonies varying from pale pink to jet black. De Hoog & Yurlova[16] distinguished *A. pullulans* and *A. melanogenum* on the basis of melanin production and some differences in growth with galactitol, glucono-δ-lactone, creatine and creatinine, and in gelatin liquefaction. Separation of two groups is supported by some SNPs in the rDNA internal transcribed spacer region, partial translation elongation factor-1α, β-tubulin and RNA polymerase II sequences.[17] Gostinčar et al[18] found sufficient genomic support for maintenance of the taxa at species level.

In the present paper, we analysed a set of clinical strains that were identified phenotypically as *Aureobasidium pullulans* group. After molecular identification of strains in this data set, we noticed that *A. melanogenum* was much more prevalent than *A. pullulans*, which was nearly limited to environmental sources (Table 1). In an attempt to explain this difference between the two species, we compared melanisation and temperature relations, and tested virulence in a murine model and in an alternative model using *Galleria mellonella* larvae.

2 | MATERIALS AND METHODS

2.1 | Strains and culture conditions

Ninety-two strains from the reference collection of the Centraalbureau voor Schimmelcultures (housed at Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CBS), which included a large set of isolates from symptomatic clinical cases sent by D.A. Sutton (collection of University of Texas Health Science Center, San Antonio, USA; UTHSC), are listed in Table 1. Of these, 45 were identified as *A. pullulans* and 47 as *A. melanogenum* based on molecular data. For preservation and serial transfer, 5% Malt Extract Agar (MEA, Oxoid) in 8-cm culture plates were used and incubation was done at 24°C for max. 7 days. For measuring thermotolerance, strains were inoculated onto MEA and oatmeal agar (OA, Difco) plates at 24 and 37°C. Growth rates were determined by measuring diameters of colonies at 2, 5, 8, 11 and 15 days at both temperatures.

2.2 | Morphology

Plates on MEA incubated at 24°C were monitored daily to detect gross melanisation which was ranked on day 15 in five categories proportional to the percentage blackening of the colony: 0 = colony without darkening, 1 ≤ 20%, 2 = 21%-40%, 3 = 41%-60%, 4 = 61%-80% and 5 = 81%-100% (Figure 1). For microscopic examination, MEA blocks of about 1 × 1 cm^2 were cut out aseptically, placed on sterile microscope slides and inoculated at the upper four edges. Inoculated agar blocks were covered with sterile cover slips and incubated in moist chambers for 1, 2, 4 and 7 day at 24°C. The structure and branching pattern of the immersed hyphae were examined in intact slide cultures under the microscope without removing the cover slips from the agar blocks by magnifications of 100× and 40×.

2.3 | Molecular identification

DNA was extracted from 7-day-old colonies grown on MEA at 24°C and rDNA ITS and D1/2 of LSU were sequenced using methods and primers after Gerrits van den Ende & de Hoog.[19] Sequences for ITS and LSU were aligned in MEGA5 in the Laser gene software (DNASTAR). Iterative alignment was performed automatically in BIONUMERICS (Applied Maths), and diagnostic SNPs were selected. ITS sequences were diagnostic for the two species.

2.4 | Murine infection model

Ten randomly selected strains of *A. pullulans* and 11 of *A. melanogenum* were used in a mouse experiment. Strains were grown on

TABLE 1 Strains examined, with source of isolation, growth and melanisation at 37°C, and strains used in larvae and murine inoculation experiments. P = *Aureobasidium pullulans*; P* = *Aureobasidium pullulans* clinical strain; M = *Aureobasidium melanogenum*; M* = *Aureobasidium melanogenum* clinical strain

					MEA (24°C)		MEA (37°C)		Lavae dead at 37°C	Mouse dead
Groups	Accession no.	ITS	Source	Origin	Diameter	Melanisation	Diameter	Melanisation		
A. pullulans white	CBS 114909	P	*Quercus robur*, green leaf	Netherlands	80	0	-	-	10	0
	CBS 117464	P	Orange juice	Netherlands	65	0	-	-	10	
	CBS 117466	P	Olive tree phylloplane	Italy	70	0	-	-		0
	CBS 121326	P	Wall in "sick building"	Greece	82	0	2	0	10	2
	CBS 116.14	P	Unknown	USA	80	0	-	-		
	CBS 590.75	P	*Tilia*, leaf	Netherlands	79	0	-	-		
	CBS 591.75	P	Decaying tuber	Netherlands	79	0	-	-		
	CBS 593.75	P	Asbestos concrete	Netherlands	81	0	-	-		
	CBS 703.76	P	*Quercus*, leaf	Russia	81	0	3	0	8	2
	CBS 566.78	P	*Sphaerotheca pannosa*	Ukraine	75	0	3	0	8	
	CBS 512.81	P	Barley leaf	France	80	0	-	-	10	1
	CBS 577.93	P*	Human, bronchoalveolar lavage	Sweden	81	0	4	0		
	CBS 351.96	P	Cold stored table grapes	Italy	78	0	5	0		
	CBS 100221	P	Driver seat	Netherlands	75	0	-	-	10	0
	CBS 100280	P	Salt pan	Slovenia	75	0	-	-	10	3
	CBS 100592	P	Mummified seal	Antarctica	65	0	-	-		
	CBS122385	P	Glace ice from sea water	Arctic	80	1	-	-	10	
	CBS 102662	P	*Malus*, fruit	Netherlands	70	1	-	-		
	CBS 249.65	P	Painted wood	Costa Rica	72	2	5	0	4	
	CBS 701.76	P	*Malus sylvestris*, fruit	Unknown	65	2	-	-		
	CBS 130469	P*	Cerebrospinal fluid	USA	76	2	-	-		
A. pullulans black	CBS 116758	P	Outdoor air sample	Germany	77	3	3	5	6	
	CBS 121328	P*	Human, onychomycosis	Greece	65	3	-	-	8	4
	CBS 298.56	P*	Human, lymph node	USA	82	3	2	5	6	0
	CBS 210.65	P	Wood	France	73	3	3	5	4	
	CBS 698.76	P	Fruit drink	Netherlands	80	3	-	5		
	CBS 700.76	P	Unknown	India	73	3	10	5		
	CBS 133.30	P	*Camellia sinensis*, leaf	Indonesia	78	4	5	1		
	CBS 117.39	P	Wood pulp	Sweden	83	4	11	5	10	
	CBS 585.75	P	*Olea europaea*, twig	Greece	72	4	-	-	0	
	CBS 115002	P	*Quercus robur*, green leaf	Netherlands	78	5	-	-		
	CBS 110.67	P	Cotton tissue	Netherlands	82	5	5	5	6	
	CBS 584.75^{NT}	P	*Vitis vinifera*, fruit	France	75	5	-	-	10	
	CBS 586.75	P	Flour	Netherlands	82	5	2	5		
	CBS 588.75	P	*Artocarpus integrifolia*, leaf	India	61	5	2	0	0	
	CBS 589.75	P	*Quercus*, leaf	Netherlands	80	5	2	0		
	CBS 592.75	P	Kitchen	Netherlands	75	5	-	-		
	CBS 699.76	P*	Human, thumb nail	Netherlands	78	5	5	5	0	0
	CBS 704.76	P	*Malus*, leaf	Netherlands	82	5	3	5		
	CBS 626.85	P*	Human, peritoneal dialysis fluid	Australia	82	5	10	3		
	CBS 259.93	P	Unknown	Unknown	75	5	-	-		
	CBS 796.97	P	Indoor environment	Netherlands	75	5	-	-		
	CBS 101160	P	Window frame	Sweden	80	5	3	0		
	CBS 109810	P	Wall surface	Ukraine	81	5	3	0		
	CBS 130542	P*	Toe nail	USA	79	5	4	3		

(Continues)

TABLE 1 (Continued)

Groups	Accession no.	ITS	Source	Origin	MEA (24°C)		MEA (37°C)		Lavae dead at 37°C	Mouse dead
					Diameter	Melanisation	Diameter	Melanisation		
A. melanogenum white	CBS 130470	M*	Sputum	USA	79	0	-	-		
	CBS 771.97	M*	Polyethelene teraphthalate	Germany	73	0	5	0		
	CBS 130447	M*	Blood	USA	80	0	12	0		
	CBS 130479	M*	Blood	USA	80	0	9	0	8	
	CBS 130471	M*	Blood	USA	70	0	8	3		
	CBS 130482	M*	Sinus	USA	80	0	5	3		
	CBS 130477	M*	Bronchial washing	USA	81	0	10	5	10	
	CBS 130490	M*	Bone marrow aspirate	USA	78	1	8	1		
	CBS 130453	M*	Blood	USA	80	1	15	5		
	CBS 130541	M*	Blood	USA	71	1	15	5		
	CBS 130463	M*	Peritoneal fluid	USA	80	1	6	5		
	CBS 130456	M*	Peritoneal fluid	USA	65	2	3	0		
	CBS 130488	M*	Scalp	USA	80	2	4	1		
	CBS 130486	M*	Bronchial washing	USA	62	2	5	2		
	CBS 130459	M*	Urine	USA	75	2	3	3	8	
	CBS 110373	M	Soil	Thailand	65	2	10	5		
	CBS 122827	M	Polluted soil	Brazil	80	2	10	5		
	CBS 130458	M*	Forehead	USA	77	2	7	5	6	0
	CBS 130460	M*	Peritoneal fluid	USA	82	2	9	5	1	1
	CBS 130462	M*	Leg wound	USA	80	2	4	5		
	CBS 130480	M*	Kidney	USA	80	2	7	5	10	
	CBS 130483	M*	Paracentesis fluid	USA	73	2	8	5	4	
	CBS 130485	M*	Bone marrow aspirate	USA	80	2	10	5		1
A. melanogenum black	CBS 110374	M	Public fountain	Thailand	75	3	10	1	10	4
	CBS 130491	M*	Toe nail	USA	80	3	11	1		
	CBS 134079	M	Surface glacier ice	Norway	65	3	6	2		
	CBS 130454	M*	Sinus	USA	74	3	9	5	10	2
	CBS 130457	M*	Lung Biopsy	USA	82	3	7	5		
	CBS 130467	M*	Bronchial washing	USA	80	3	15	5	10	3
	CBS 130476	M*	Blood	USA	80	3	9	5	8	
	CBS 130481	M*	Scalp	USA	80	3	10	5		
	CBS 130489	M*	Bronchial washing	USA	76	3	10	5		
	CBS 621.80	M	Deteriorated army supplies	USA	83	4	5	3		
	CBS 130448	M*	Leg	USA	75	4	13	5	6	
	CBS 130455	M*	Bone marrow	USA	72	4	11	5	4	4
	CBS 130461	M*	Ethmoid sinus	USA	78	4	5	5	8	
	CBS 130465	M*	Blood	USA	78	4	6	5	10	0
	CBS 130466	M*	Sputum	USA	80	4	15	5	8	
	CBS 130475	M*	Lung	USA	81	4	13	5	4	3
	CBS 130484	M*	Blood	USA	82	4	11	5		
	CBS 130487	M*	Sputum	USA	80	5	5	0		
	CBS 130464	M*	Blood	USA	70	5	10	1		
	CBS 121327	M*	Ear canal of child	Greece	80	5	7	5		
	CBS 109800	M*	Peritoneal fluid	Greece	68	5	8	5		
	CBS 130450	M*	Blood	USA	83	5	13	5	6	2
	CBS 130472	M*	Sinus	USA	82	5	7	5	8	2
	CBS 130473	M*	Pelvis	USA	83	5	15	5	8	

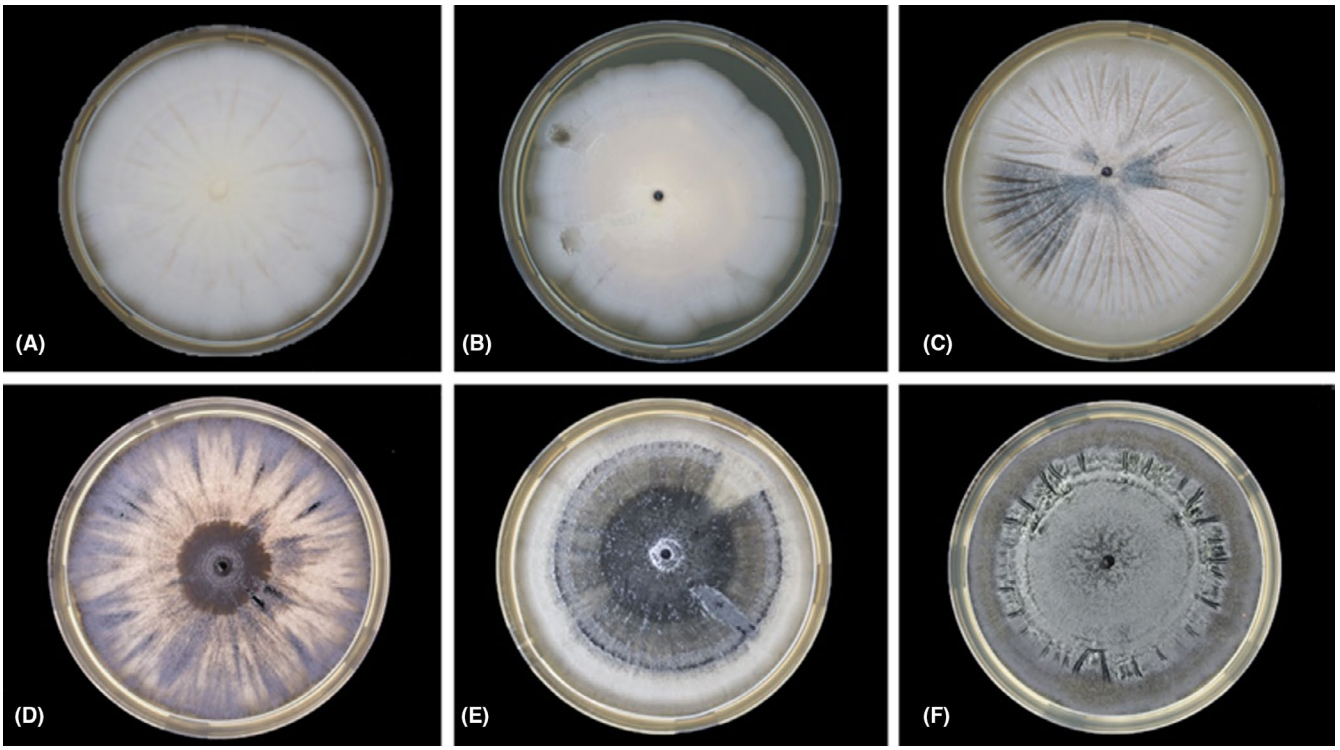

FIGURE 1 Degrees of melanisation in macrocultures on MEA at 24°C. (A) Colony without darkening (0); (B) Melanisation ≤ 20% (1); (C) Melanisation 21%-40% (2); (D) Melanisation 41%-60% (3); (E) Melanisation 61%-80% (4); (F) Melanisation 81%-100% (5)

Sabouraud Glucose agar (SGA, Oxoid) for 2-3 days at 24°C using suspensions in water as inocula. When colonies were still unpigmented, the surface of colonies was washed gently with phosphate-buffered saline (PBS) and conidia were collected in falcon tubes, taking care that no hyphae were included. Freshly harvested conidia were checked microscopically to avoid swelling or germination. Harvested conidia were washed three times in sterile PBS. Conidia were counted and adjusted to designated concentration of 10^8 conidia/mL using counting chambers. Aliquots of 100 µL were used, with inocula of 10^7 conidia/mice. Ten mice were used per strain. Ten control mice were injected with PBS only, and 10 untouched mice served as control. Infected mice were examined at least three times daily. Female Kunming mice (20-25 g body weight, 4-5 week, Guizhou Medical University; n = 230) were inoculated via injection into lateral tail vein. The study was conducted under the guidelines and approval of the Research Ethics Committee of Guizhou Medical University (SCXKqian2018-0001). Animals were housed under standard conditions (SYXKqian2018-0001), with food and water supplied ad libitum. Survival (in days post-infection) was recorded for each mouse in each group. Mice were monitored at least twice daily, and dead mice were dissected immediately. Brain, liver, spleen and kidneys from each mouse were harvested and weighed. After 15 days of infection, all mice were sacrificed for anatomical examination. Organs excised from all infected mice were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin-eosin (H&E) according to Kumar et al.[20]

2.5 | Galleria infection model

The experiment was conducted at the Parasitology Laboratory of the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy) using 40 *Aureobasidium* strains from CBS (Utrecht, The Netherlands), including 20 *A. pullulans* and 20 *A. melanogenum* (Table 1). Inocula of 10^8 yeast cells/mL were prepared in PBS. Only 25°C was applied, since not all strains of *A. pullulans* were able to grow at 37°C. In addition, at this temperature, there is significant mortality of controls in the Galleria infection model. *Galleria mellonella* larvae were obtained from the animal house of the IZSVE and were stored at 18°C before the experiment. For all experiments, inocula were prepared with 1-week-old cultures grown on SGA plates and rubbing the surface with a glass spreader to release conidia. Conidia were released from hyphae by filtering through sterile miracloth, centrifuged and resuspended in 5 mL sterile phosphate-buffered saline, pH 7.4 (PBS). Conidia were counted using a Bürker chamber, and inocula were prepared by adjusting conidia to 1×10^8 cells/ mL in sterile PBS. Larvae of similar size were randomly selected for each experiment (10 larvae/strain), and those showing discoloration were discarded. Each larva was injected with 10 µL of inoculating solution just below the second to last left proleg. For each strain, ten larvae received inocula diluted in PBS, 10 control larvae were injected with PBS only and 10 untouched larvae served as controls. Injected larvae were maintained in Petri dishes at 25°C under standard aerobic conditions, and survival was recorded twice daily for 10 days. Larvae

TABLE 2 Diagnostic *Aureobasidium pullulans* and *Aureobasidium melanogenum* SNPs in the rDNA Internal Transcribed Spacer region

	Position					
Species	**373**	**375**	**416**	**418**	**469**	**470**
A. pullulans	C	T	A	T	A	C
A. melanogenum	T	C	T	A	C	T

were considered dead when they displayed no movement in response to gentle prodding with a pipette tip. To confirm vitality of *Aureobasidium* strains, 10 µL suspensions of each strain were cultured on SGA and incubated at 25°C and the number of colony-forming units (CFU) per plate was counted. Four dead larvae (two inoculated with *A. pullulans* and two with *A. melanogenum*) were formalin-fixed and used for histopathology after H&E staining.

2.6 | Statistics

Data processing and analysis was done using SPSS v19.0 statistical package. A paired samples t test was conducted to compare the growth of *A. pullulans* and *A. melanogenum* at two temperatures and on two media. Different mortality rates among larvae inoculated with the fungi vs PBS and controls were screened using the Fisher exact test. The mean number of dead larvae in each group was compared with each other also using analysis of variance (ANOVA), software SPSS for windows and GRAPHPAD. Survival of mice was compared using log-rank test and Gehan-Breslow-Wilcoxon test, using GRAPHPAD PRISM v7 for Windows. *P*-values ≤ 0.05 were considered to be significant.

3 | RESULTS

Complete/partial rDNA ITS and LSU and partial translation elongation factor 1-α (*TEF1*) were used as molecular markers for final identification of 92 strains. Neotype strains CBS 584.75 of *A. pullulans* and CBS 105.22 of *A. melanogenum* were used as controls in the alignments. Diagnostic SNPs in the ITS region were used for separation of the two groups (Table 2). Based on these sequences, 45 strains were identified as *A. pullulans* and 47 as *A. melanogenum*.

Expansion growth was measured for both species after 15 days of incubation (Table 1). On average, growth of *Aureobasidium* strains differed significantly between 24°C (76.68 ± 5.46 cm, n = 92) and 37°C (5.42 ± 4.60 cm, n = 92) on MEA ($P < 0.01$) as well as on OA. Growth responses were similar on MEA (Table 1) and OA (data not shown). At 24°C on MEA and OA, no significant difference was observed between *A. pullulans* (76.38 ± 5.54 cm, n = 45) and *A. melanogenum* (76.98 ± 5.42 cm, n = 47) ($P > 0.05$). However, at 37°C on both media, *A. melanogenum* grew faster (8.60 ± 3.61 cm, n = 47) than *A. pullulans* (2.11 ± 2.86 cm, n = 45) ($P < 0.01$).

No significant difference between the two species was observed at 24°C in the structure of hyphae, conidia, endoconidia, yeast cells or chlamydospores (Figure S1). Chlamydospores and swollen cells may show a microscopically visible, irregular mantle of EPS. In both species, colony colours varied from off-white, pink, creamish to dark brown and occasionally black in *A. pullulans*, and off-white, creamish to black for *A. melanogenum*. *Aureobasidium pullulans* showed poor growth at 37°C (22 out of 45 strains did not grow), while *A. melanogenum* grew well at 37°C. Melanisation was scored at 24°C and 37°C on MEA and OA according to a 5-grade scale (Table 1). No significant difference was found in melanisation of *A. pullulans* (M = 1.9 ± 1.9) and *A. melanogenum* (1.8 ± 1.55) at 24 °C ($P > 0.05$). A significant difference in melanisation of *A. melanogenum* was noted at 24°C (M = 2.60 ± 1.61, n = 47) compared to 37°C (3.64 ± 1.96, n = 47) on MEA ($P < 0.01$) as well as on OA. The strains of *A. pullulans* (1.26 ± 2.11, n = 45, the 22 strains that did not grow were recorded as 0) that showed some growth at 37°C were less melanised than those of *A. melanogenum* (3.58 ± 1.98, n = 47) at this temperature ($P < 0.01$) on both media.

Comparative virulence of *Aureobasidium* strains was tested in a murine model. Fifteen-day survival rates of all mice are listed in Figure 2. While all of the animals infected with *A. pullulans* and *A. melanogenum* died between days 1 and 15 compared to the controls that remained alive, analysis by log-rank test and Gehan-Breslow-Wilcoxon tests showed overall differences in survival were significant ($P < 0.05$). Figure 2 shows that survival rates of the *A. melanogenum* group with melanisation rates 3–5 were significantly lower compared with other groups. No significant differences were found between remaining groups, that is, *A. melanogenum* with M = 0-2 and *A. pullulans* with M = 0-2 and 3-5 ($P > 0.05$). The anatomical results

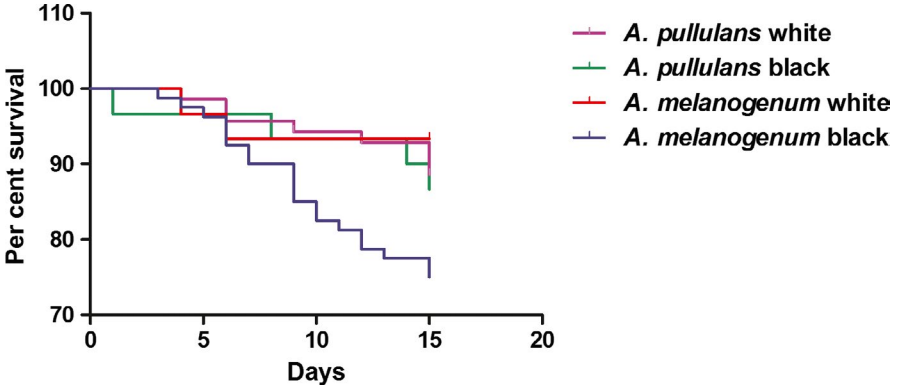

FIGURE 2 Survival of Kunming mice following i.v. infection with *Aureobasidium pullulans* and *Aureobasidium melanogenum* monitored during 15 days. Survival curves compare four groups: *A. pullulans* light (0-2); *A. pullulans* dark (3-5); *A. melanogenum* light (0-2); and *A. melanogenum* dark (3-5) at 24°C

TABLE 3 Results of *Galleria* infections. P = *Aureobasidium pullulans*; M = *Aureobasidium melanogenum*

25°C	n	Dead	Alive	% mortality	Comparisons	*P*
A. pullulans	400	212	188	53.00	P vs M	$P < 0.01$
A. melanogenum	400	129	271	32.25	P vs Control	$P < 0.01$
Control	80	7	73	8.75	P vs PBS	$P < 0.01$
PBS only	90	8	82	8.89	M vs Control	$P < 0.01$
					M vs PBS	$P < 0.01$

showed that the heart, liver, spleen, lung, kidney and intestinal tract of *A. melanogenum* and *A. pullulans* infected mice had different degrees of pathological changes, among which the intestinal tract and liver were the most obvious. The liver of mice infected by *A. melanogenum* was black with white patches, and the gastrointestinal tract was strongly inflated. *Aureobasidium pullulans* infected mice showed small white spots on the liver, and the gastrointestinal tract was lightly inflated. Histopathology showed inflammatory lesions in the heart, liver, spleen, lung, kidney and intestinal tissues of *A. melanogenum* and *A. pullulans* infected mice after routine HE staining; lesions in intestines and liver were the most obvious. The intestinal mucosa of the *A. melanogenum* group showed significant epithelial shedding, and the muscular layer had become thin. Hepatocytes showed partial necrosis, and histopathology in both species showed infiltration of inflammatory cells such as neutrophils and lymphocytes. *Aureobasidium pullulans* infected mice had mild destruction of the intestinal mucosa and thinning of the muscular layer.

In a *Galleria* experiment, using 40 *Aureobasidium* strains, rates of mortality of larvae compared between *A. pullulans* and *A. melanogenum*, relative to controls of PBS and untouched larvae, are given in Table 1. Inoculated larvae with fungal cells invariably showed significantly higher mortalities than controls of untouched larvae and PBS alone ($P < 0.01$). At 25°C, the rate of mortality of *Galleria* larvae inoculated with *A. pullulans* was significantly higher than those inoculated with *A. melanogenum* (53.00% vs 32.25%; $P < 0.01$; Table 3). Average survival per species is shown in Figure 3. Histopathology in *Galleria* larvae showed identical results, both species producing melanised, chlamydospore-like cells in tissue (Figure 4).

4 | DISCUSSION

Aureobasidium is a genus of black-yeast-like fungi comprising numerous species with plant-associated or extremotolerant life styles, for example, colonising bare rock or hypersaline habitats.[21] The fungi are ubiquitously found on lice-inhabited, sugar-rich phyllosphere.[4] Also, environments low in nutrients such as moist glass or metal are colonised. Their efficient production of extracellular polysaccharides (pullulan, aubasidan) may enhance survival under conditions of dryness and solar irradiation. Further survival structures are the chlamydospores that, if present, are thick-walled and heavily melanised.[11] In addition to thermotolerance with clinical occurrence, osmotolerance/osmophily was also identified as a major factor correlated with opportunism.[7]

Aureobasidium is among the most common fungi in moist compartments of indoor and domesticated environments. Human infection is extremely rare, and invariably of opportunistic nature, that is, mild superficial on otherwise healthy individuals, or systemic only upon severe impairment of the hosts' immunity or general condition. Opportunism in this genus is likely to be enhanced by undirected survival factors such as thermotolerance, thick, melanised cell walls and protective EPS. Also, the ubiquitous nature and risk

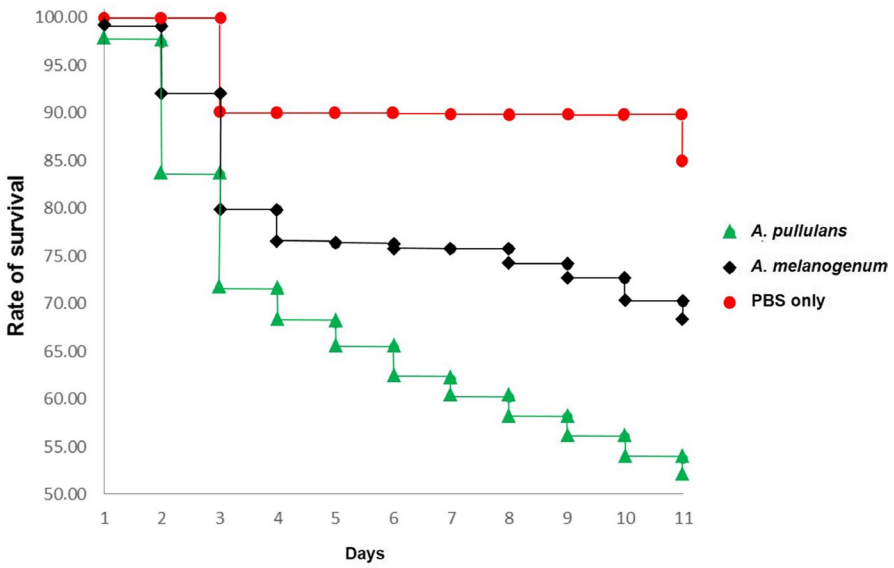

FIGURE 3 Survival of *Galleria mellonella* larvae following inoculation of *Aureobasidium pullulans* and *Aureobasidium melanogenum* during 10 days. Untouched control curve was identical to the PBS curve

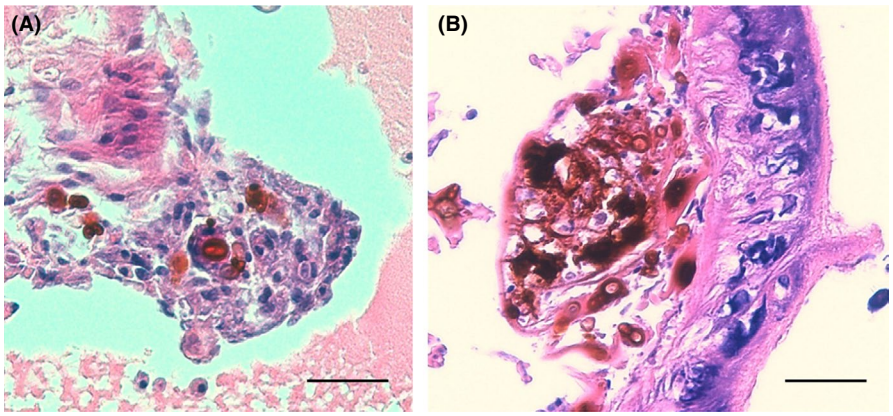

FIGURE 4 *Aureobasidium pullulans*, CBS 100280, histology in dead larva 3 days after inoculation, H&E (A); *Aureobasidium melanogenum*, CBS 110374, histology in dead larva 2 days after inoculation at 24°C, H&E (B). (scale bar = 50 μm)

to be traumatically inoculated or inhaled increase the frequency of human infection. The relatively small number of described cases indicates that infective abilities and virulence probably are low.[11]

Clinical strains have been collected over the decades both at CBS and UTHSC; in the former also environmental strains were maintained. Strains were traditionally enlisted into the collections under the umbrella name *A. pullulans*, as the separation of *A. melanogenum* is only of recent date.[18] After retrospective identification of the two taxa, it was noticed that the two species had nearly equal frequency in the CBS-UTHSC data set (*A. pullulans* / *A. melanogenum* = 45/47). In this set of strains, significantly more clinical isolates appeared to belong to *A. melanogenum* (42/47), while *A. pullulans* (35/45) was more often derived from environmental sources such as leaves, soil and fruits. Both species showed a worldwide distribution (Table 1). The two species show low MICs of amphotericin B, itraconazole and posaconazole. However, they were resistant to fluconazole and had high MICs of voriconazole, isavuconazole, caspofungin and micafungin.[22]

This led us to verification of possible association of the generally hypothesised virulence factors thermotolerance and melanisation with virulence in animal models. Comparing growth performance of the two species, *A. melanogenum* showed better growth at 37°C on all media tested. The species also showed more intense melanisation at 37°C. The same was noted by Gostinčar et al[18] based on a smaller data set. While strains of the two species on average are similar at 24°C, *A. melanogenum* increases melanisation at elevated temperature, while this response remains absent in *A. pullulans*. Nearly, all strains of *A. melanogenum* were able to grow at 37°C, compared to only 50% of the strains of *A. pullulans* (Table 1).

In general, growth at body temperature is a prerequistic for fungal pathogens in order to survive inside the mammal body.[7] *Aureobasidium melanogenum* showed significantly better survival at 37°C than *A. pullulans*, which is expected to enhance its infectivity. Possibly, this was due to the response with increased melanisation at elevated temperature, which was not observed in *A. pullulans*. In the murine model, the observed difference in virulence was not linked to *A. melanogenum* vs *A. pullulans*, but to the *A. melanogenum* black group vs the rest. It may be concluded that *A. melanogenum* black group can survive at 37°C, as well as melanin is produced, which shows a higher pathogenicity. Only when these two factors combine, that is, in the black group of *A. melanogenum*, significant pathogenicity is noted in the murine model. With only a single virulence factor, as in the black group of *A. pullulans* or the white group of *A. melanogenum*, virulence is low. Provided that optimal conditions are available, melanin can be regarded as a virulence factor. Note that, in contrast, black yeasts in *Exophiala* were observed to decrease melanisation in response to hydrogen peroxide stress; this seemingly inappropriate use of melanin by the fungus led to the conclusion that this type of stress was unlikely to occur in the fungus' natural habitat.[23]

In the *Galleria* infection model at 25°C, mortalities due to *A. pullulans* were higher than in larvae injected with *A. melanogenum*. These results are thus in conflict with the murine model results. At 25°C, there is no difference in growth and melanisation between the two species, and thus the observed difference in mortality may be coincidental. The *Galleria* infection model is judged not to be useful for *Aurobasidium*. Compared to the *Galleria* infection model, the mouse is closer to the human host and has the same body temperature. The pronounced virulence of the melanised fraction of *A. melanogenum* provides a logical explanation of the observed higher frequency on clinical strains in the *A. pullulans/A. melanogenum* data set. We conclude that the murine model accurately reflects the infectious potential of the two species.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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