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Cold adapted and closely related *mucoraceae* species colonise dry-aged beef (DAB)

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ABSTRACT

The dry ageing is a historically relevant method of meat preservation, now used as a way to produce the dry-aged beef (DAB) known for its pronounced flavour. Partially responsible for the taste of the DAB may be various microorganisms that grow on the surface of the meat. Historically, the fungal species colonising the DAB were described as members of the genera *Thamnidium* and *Mucor*. In this study we used both culture based approach as well as ITS2 rDNA metabarcoding analysis to investigate the fungal community of the DAB, with special emphasis on the mucoralean taxa. Isolated fungi were members of 6 different species from the family *Mucoraceae*, belonging to the genera *Mucor* and *Helicostylum*. Metabarcoding data provided supplementary information regarding the presence of other fungi including those from the *Thamnidium* genus. In both approaches used in this study isolates closely related to the *Mucor flavus* strain CBS 992.68 dominated.

1. Introduction

Meat has been a part of the human diet throughout the ages. Due to the short shelf life of raw meat, many different methods of its preservation (such as drying, smoking, and salting) have been invented (Dave and Ghaly, 2011). For a shorter term storage, these laborious processes were not necessary. In those cases, freshly portioned meat was hung in a dry and cold place to rest for several weeks in a process known as the dry-ageing. During this period, endogenous enzymes naturally present in meat, tenderise the product making it more palatable. Nowadays, this manufacturing method is employed in the production of the dry-aged beef (DAB), which is gaining popularity - especially in East and South-east Asia (Dashdorj et al., 2016).

Exposure of the outer pellicle of the DAB to the air during the ageing process allows many groups of microorganisms, both eukaryotic and prokaryotic, to colonise its surface. Different genera of bacteria, including *Pseudomonas* and *Lactobacillus*, are competing for dominance against yeasts and filamentous fungi (Ryu et al., 2020). Some of the most prevalent filamentous fungi present on the surface of the DAB are those belonging to the family *Mucoraceae*. Although in the late 20th century, some researchers perceived them as spoilage factors (Lowry and Gill, 1984), currently their presence is interpreted rather as a sign of the correct dry-ageing process (Mikami et al., 2021).

The presence of *Mucoromycota* representatives on the refrigerated meat was reported even more than 100 y ago (Brooks and Hansford, 1923; Monvoisin, 1918). At the time, the fungi found on DAB were often assigned to the genera *Mucor* and *Thamnidium* (Dave and Ghaly, 2011). In more recent studies other members of the family *Mucoraceae* were also reported such as *Helicostylum* (Mikami et al., 2021) and *Pilaira* (Oh et al., 2019). Although based on morphology these genera seem quite distinct, together with some *Mucor* genus members, they form monophyletic group inside broader *Mucor* genus (Walther et al., 2013) with a common trait being that they are able to grow in low temperatures but seem to struggle if the temperatures exceed 25 °C (Upadhyay, 1973; Lowry and Gill, 1984; Benny, 1992). One of the most commonly present on DAB seems to be *Mucor flavus* (Mikami et al., 2021; Oh et al., 2019; Ryu et al., 2020) which seems to be forming a species complex in a sense of being multiple cryptic species under one name.

Other psychrotolerant fungi may be present on the DAB surface. Majority of them are unicellular yeasts belonging to the *Ascomycota* such

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as *Debaryomyces* spp. (Ryu et al., 2018) or *Yarrowia* spp. (Patrignani et al., 2007). These fungi grow well in low temperatures, which makes them well adapted to the DAB storage conditions, but their growth is halted above 30 °C or even 25 °C. Due to this trait, and despite their strong proteolytic capabilities they are rarely human pathogens, and can be safely used in food fermentation (Huang et al., 2021). Filamentous ascomycetous fungi such as *Penicillium* spp. or *Aspergillus* spp. can be also observed on the DAB (Dave and Ghaly, 2011). In those genera there are species capable of toxin production, and due to this reason they are generally viewed as contaminants during the dry-ageing process (Koutsoumanis, 2023).

In contrast to *Ascomycetes*, growth of the members of the family *Mucoraceae* seem to promote correct dry ageing, positively contributing to the taste of the final product (Hanagasaki and Asato, 2018; Lee et al., 2019; Li et al., 2021; Mikami et al., 2021; Oh et al., 2019). Despite the seemingly ubiquitous presence of these fungi on the DAB reported in popular sources as "good moulds" the scientific literature reporting their occurrence on the DAB is less than extensive with a recent Scientific Opinion in the EFSA Journal (Koutsoumanis et al., 2023) only mentioning that those fungi "may have a positive influence on the ageing process" and mainly focusing on the bacteria influencing the quality of the DAB. To the author's knowledge no recent literature, that includes molecular data, describing the diversity of the mucoralean taxa on the DAB is available from Europe. The lack of scientific resources is an obstacle in standardisation of this process and creating appropriate legislative regulations. Creating safe ageing procedures would prevent the exposure of both people employed in the production of DAB as well as consumers to potential pathogens and toxins.

With the growing popularity of the DAB in Europe we wanted to supplement currently available molecular data on fungal diversity, which is available mainly from East and Southeast Asia and not from Europe (Mikami et al., 2021; Oh et al., 2019; Ryu et al., 2020). The goal of this study was to characterise the fungal community of the DAB using in synergy ITS2 rDNA high-throughput amplicon sequencing (HTS) and culture based approach. Special emphasis was placed on the mucoralean taxa that are safe for humans and tend to promote the correct dry-ageing.

2. Materials and methods

2.1. DAB sample collection

Ten samples of a dried pellicle which develops on the outer surface during the dry-ageing of beef, were investigated. Samples of meat were the cuts from the rib area part of the longissimus dorsi which are often selected for the dry-ageing. Nine samples, that were aged for a minimum of 28 d, were collected from various butchers in Warsaw and one was obtained from a dry-ageing experiment performed in our laboratory (sample no 9). The dry-ageing performed in the laboratory was done in the same way as the generally recommended good practice for the dry ageing with the temperature of 1–4 °C and a relative humidity of 80–90 % for 28 d (Dashdorj et al., 2016). This experiment was done in order to observe the fungal diversity developing during the ageing done in a safe and strictly controlled environment and was used as the control for other samples.

2.2. High-throughput ITS2 amplicon sequencing

From each DAB sample 10 g of meat was collected in several subsamples (5–10) and homogenised in 90 ml of saline solution (0.9 %). One millilitre of homogenate was collected and centrifuged (10000×*g* for 5 min) as recommended by Ryu et al. (2020). The pellet was collected and resuspended in 675 μl of CTAB solution for DNA extraction (EURx, Gdańsk, Poland). Then, 25 μl of proteinase K (20 mg/ml; EURx, Gdańsk, Poland) was added and the solution was incubated in 55 °C for 1 h. The temperature was raised to 65 °C for 15 min to inactivate any remaining enzymes. Afterwards, 700 μl of chloroform:isoamyl alcohol (24:1) was added to dissolve nonpolar impurities such as fatty acids. The resulting solution was mixed gently by inverting tubes for 10 min and centrifuged for 5 min at 3000×*g*. The top layer was transferred into new tubes to which 5 μl of RNAse (Blirt S.A., Gdańsk, Poland) was added. Then, the samples were gently mixed and incubated in 37 °C for 20 min. After the incubation, 700 μl of chloroform:isoamyl alcohol (24:1) was added again, and the tubes were gently mixed and centrifuged for 10 min at 3000×*g*. The top layer was transferred into 15 ml tubes. Then, 300 μl of 5 M NaCl solution and 1.8 ml of 96 % ethanol cooled to −80 °C were added. Samples were subsequently cooled for 1 h in −20 °C to facilitate DNA precipitation. Afterwards, samples were centrifuged and the supernatant was discarded. DNA was washed with 1 ml of 70 % ethanol. To avoid DNA dissolving during the washing, the work was done on ice and the alcohol solution was cooled to −20 °C. DNA samples were centrifuged in 4 °C for 7 min at 3000×*g* and the supernatant was discarded. Washing was repeated. Samples were left to dry overnight and were dissolved in a DNAse free water. The DNA quantity and quality were assessed spectrophotometrically with NanoPhotometer NP80 (Implen GmbH, Munich, Germany). This DNA extraction method using generally available reagents provided a substantial amount of the DNA of a quality suitable for Illumina sequencing with an added benefit of being a relatively low cost DNA extraction.

ITS2 (Schoch et al., 2012) marker was selected to be used in this study as it is suitable sequence for identifying fungi within the *Mucoromycotina* and *Saccharomycotina* (Mbareche et al., 2020) from which members where observed in other recent studies on the DAB microbiome (Mikami et al., 2021; Oh et al., 2019; Ryu et al., 2020). For ten samples ITS2 rDNA sequence was amplified according to good practice for the purposes of amplicon HTS. For amplification *Q5 Hot Start High-Fidelity 2x Master Mix* and ITS3f (5′-GC ATC GAT GAA CGC AGC-3′), ITS4r (5′-TCC TCC GCT TAT TGA TAT GC-3′) primers (Tedersoo et al., 2015; White et al., 1990) were used. The amplicons were sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) by the Genomed company (Warsaw, Poland) using a v3 MiSeq chemistry kit in the paired-end mode (read lengths 2 × 300 bp). Raw sequencing data were deposited in the National Center for Biotechnology Information's (NCBI) Sequence Reads Archive (SRA) under the accession number: PRJNA858677.

2.3. Bioinformatic processing of the ITS2 amplicon sequencing data

To analyse the obtained amplicon data we used both ASV and OTU approaches since each of these methods has its own advantages and disadvantages (Kauserud 2023). The quality of the ITS2 sequencing reads was controlled using FastQC (Andrews et al., 2012).

For ASV approach raw Illumina MiSeq paired reads were processed using tools and pipelines wrapped by QIIME2 version 2022.2 (Bolyen et al., 2019). The DADA2 implemented as QIIME2 plugin (Callahan et al., 2016) was used for filtering to reduce the number of chimeric sequences. Both reverse and forward sequences were trimmed to 220 bp which gave the highest percentage of input non chimeric sequences. Reads with phred quality score less than 20 (Q20 %) were discarded. QIIME2 was also used to create ASVs table and later for taxonomic assignments based on a naive Bayes classifier trained on the UNITE database: sh_qiime_release_29.11.2022 (Nilsson et al., 2019). Obtained ASVs were also analysed using BLASTn (Altschul et al., 1990) search against the NCBI databases ITS_RefSeq_Fungi (2022-10-28) and nt_euk (2023-07-19) to verify accuracy of the identification done using bayesian methods.

For the OTU approach PIPITS (Gweon et al., 2015) version 3.0 pipeline was used for filtering sequences and to create OTUs with 97 % sequence identity. QIIME2 was then used for taxonomic assignments using the same classifier as in the ASV approach to give comparable results.

The phyloseq package (version 1.42.0) (McMurdie and Holmes,

2013)for R (R Core Team, 2022) was used for further data analysis (rarefaction curves, diversity bar plots). For graphical plotting the ggplot2 R version 3.3.5 package was used (Wickham, 2016) with R software version 4.2.1 (R Core Team, 2022).

2.4. Obtaining of fungal cultures

Subsamples of the pellicle used for HTS were placed in the moist chambers (Benny, 2008; Skirgiełło et al., 1979), incubated at room temperature (ca. 18 °C) and checked daily for mycelial development and sporulation. This method was selected to induce growth and sporulation for *Mucoraceae* fungi previously reported as main components of the microbial community of DAB (Mikami et al., 2021; Oh et al., 2019; Ryu et al., 2020). Spores were collected using glass capillary and placed on 4 % potato dextrose agar (BTL, Łódź, Poland) plates. Fungal cultures were incubated at room temp. For up to 14 d. The specimens were then inspected under the microscope (Nikon Eclipse E200) and preliminary identified. Isolates assigned to the family *Mucoraceae* were selected for further studies. For preservation purposes mycelium was collected from each pure culture and placed under 10 % glycerol solution for long term storage at −80 °C (Kitamoto et al., 2002). Dried cultures were deposited as voucher herbarium specimens in Herbarium of the University of Warsaw (Table 1.).

Apart from the fungi isolated from the DAB additional 7 *Mucoraceae* strains from the University of Warsaw collection as well as 2 strains from the Westerdijk Fungal Biodiversity Institute (Utrecht, Netherlands) culture collection were also included as the references for growth experiments.

2.5. DNA extraction from cultures, amplification and sequencing

A small amount of mycelium (1 cm^2) was collected from each fungal culture (Table 1.) Then, the DNA was extracted from each sample using DNA extraction kit EM13 (Blirt S.A., Gdańsk, Poland) according to the manufacturer's instructions. The internal transcribed spacer (ITS) and putative cyclopropane fatty-acyl-phospholipid synthase (CFS) were selected as markers for molecular identification purposes. ITS is widely considered as a universal fungal barcode (Schoch et al., 2012) while CFS was recommended to recognise closely related *Mucor* species (Wagner et al., 2020). The following primers pairs were used: ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) for ITS amplification (White et al., 1990) and CFS-f1 (5′-TTY TCY CGI TTY GCT CCT CGT-3′) and CFS-r1 (5′-ACC ARA TRA ART CYT CRT ATT GCC A-3′) for CFS amplification (Wagner et al., 2020). PCR mix consisted of: 10 µl of *2x TaqNova-RED PCR Master Mix* (Blirt S.A., Gdańsk, Poland), 4 µl of water, 1.5 µl of each of forward and reverse primers in 10 µM concentration, and 3 µl of template DNA for a final reaction volume of 20 µl. For both PCR reactions Wagner et al. (2020) setup was used: Initial denaturation 95 °C for 5 min, 30 cycles of denaturation (95 °C for 30 s), annealing (ITS 52 °C (White et al. (1990), CFS 54 °C (Wagner et al., 2020) for 30 s), and extension (72 °C for 45 s), final extension 72 °C for 7 min. Presence of the expected product was checked on 1 % agarose gel with *Midori Green* (Nippon Genetics Europe, Düren, Germany) PCR products were cleaned using *DNA purification kit EM26* (Blirt S.A., Gdańsk, Poland) and bidirectionally sequenced using *BigDye™ Terminator v3.1 Cycle Sequencing Kit* (Thermofisher Scientific, Waltham, MA, USA). The product was cleaned using *Zetadex-50 Fine* (EMP BIOTECH GMBH, Berlin, Germany) and sent to the external company Genomed (Warsaw, Poland) for further processing and sequencing. The obtained sequences were deposited in the GenBank database under the accession numbers listed in Table 1.

2.6. Phylogenetic analysis

The obtained reads were assembled using the CAP3 algorithm implemented in UGENE v.37.0 (Okonechnikov et al., 2012). Resulting consensus sequences were compared with the NCBI nucleotide database using BLASTn (Altschul et al., 1990) algorithm for preliminary taxonomic placement. Additional 30 reference sequences were retrieved from GenBank to prepare a phylogenetic tree (Table 2.). Sequences coding for ITS and CFS were aligned separately using the MAFFT program with localpair setting recommended for alignment of less than 200 sequences (Katoh and Standley, 2013). Subsequently, the alignments were trimmed in the trimAl program using automated1 algorithm

Table 1
Strains and isolates investigated during this study with GenBank accession numbers for deposited sequences.

Species	Herbarium culture number	Source	Dry-aged beef sample no.	ITS accession number	CFS accession number
Thamnidium elegans	WA 71819	UW culture collection	–	OP060156	OP122170
Thamnidium elegans	WA 74551	UW culture collection	–	OP060157	OP122171
Thamnidium elegans	WA 74552	UW culture collection	–	OP060158	OP122172
Thamnidium elegans	WA 71820	UW culture collection	–	OP060159	OP122173
Thamnidium elegans	CBS 341.55	CBS culture collection	–		OP122174
Mucor flavus	WA 71817	dry aged beef	1	OP060160	OP122175
Thamnidium elegans	WA 18081	UW culture collection	–	OP060161	OP122176
Helicostylum elegans	WA 71818	dry aged beef	1	OP060162	OP122177
Helicostylum elegans	WA 71816	dry aged beef	2	OP060163	OP122178
Mucor circinelloides	WA 12397	UW culture collection	–	OP060164	OP122179
Mucor circinelloides	WA 71815	dry aged beef	2	OP060165	OP122180
Thamnidium elegans	WA 71813	UW culture collection	–	OP060167	OP122182
Mucor griseocyanus	CBS 366.70	CBS culture collection	–		OP122183
Mucor circinelloides	WA 74555	dry aged beef	3	OP060197	OP122214
Mucor aligarensis	WA 74556	dry aged beef	3	OP060198	OP122215
Mucor aligarensis	WA 110631	dry aged beef	4	OP060199	
Mucor aligarensis	WA 110630	dry aged beef	4	OP060200	
Mucor aligarensis	WA 110629	dry aged beef	5	OP060201	
Mucor aligarensis	WA 110628	dry aged beef	5	OP060202	
Mucor aligarensis	WA 110624	dry aged beef	5	OP060203	
Mucor mucedo	WA 110625	dry aged beef	5	OP060204	OP122217
Mucor mucedo	WA 110626	dry aged beef	5	OP060205	OP122218
Mucor flavus	WA 134633	dry aged beef	6	OP060211	OP122224
Mucor flavus	WA 134634	dry aged beef	6	OP060212	OP122225
Mucor flavus	WA 146188	dry aged beef	7	OP060213	OP122226
Mucor saturninus	WA 146189	dry aged beef	6	OP060214	OP122227
Mucor flavus	WA 146190	dry aged beef	8	OP060215	OP122228
Mucor flavus	WA 146191	dry aged beef	10	OP060216	OP122229

Table 2
GenBank sequences used to calculate the phylogenetic tree with accession numbers.

Species	Culture collection	Status	Source	ITS	CFS
Helicostylum pulchrum	CBS 259.68		Air-dried raw sausage, Germany	JN206052	MT533678
Helicostylum pulchrum	CBS 107.23		n. a.	JN206053	
Helicostylum pulchrum	CBS 258.59	T of *H. venustellum*	Isopod, UK, England	JN206054	
Mucor aligarensis	CBS 993.70	T	Soil, India	JN206056	MT533735
Mucor aligarensis	CBS 244.58		Human; ear, UK	JN206057	
Mucor circinelloides	CBS 195.68	NT	Air, Netherlands	JN205961	MF495008
Mucor circinelloides	CBS 196.68		*Triticum aestivum*, Turkey	JN205968	MF495000
Mucor flavus	CBS 230.35	T of *M. attenuatus*	Dung of roe Germany	JN206061	MT533673
Mucor flavus	CBS 234.35	NT	Germany	JN206051	MT533674
Mucor flavus	CBS 126.70	T of *M. mephitis*	Dung of mouse, USA	JN206049	MT533641
Mucor flavus	CBS 197.71	T of *M. meridionalis*	Dung of wood mouse Ukraine	JN206066	MT533662
Mucor flavus	CBS 893.73	T of *M. sciurinus*	Forest soil, Russia	JN206062	MT533731
Mucor flavus	CBS 992.68		coastal gravel flat covered by *Bryum argenteum*, Antarctica	JN206068	
Mucor griseocyanus	CBS 116.08	T	soil, Norway	JN206003	MF494967
Mucor griseocyanus	CBS 366.70		canned strawberries, Netherlands	JN206001	MF494971
Mucor griseocyanus	CBS 223.56		Netherlands	JN206000	MF494970
Mucor minutus	CBS 586.67		India	JN206048	MT533722
Mucor mucedo	CBS 640.67	NT	nose effluent of cow, Netherlands	JN206085	MT533724
Mucor mucedo	CBS 228.29	T of *M. murorum*	Russia	JN206088	
Mucor rongii	CICC 41 725	T	Moist walls, China	MK903014	MT815279
Mucor rongii	CICC 41 726		Mouldy tofu, China	MK903012	MT815280
Mucor saturninus	CBS 974.68	NT	Soil, Netherlands	JN206072	MT533734
Mucor strictus	CBS 576.66	NT	soil at lake shore, Austria	JN206037	MT533719
Mucor piriformis	CBS 169.25	NT	decaying pear	JN206028	MT533655
Mucor plasmaticus	CBS 177.46		dung of rabbit, UK	JN206076	
Pilaira caucasica	CBS 523.68	T	mouse dung, Armenia	JN206299	MT533708
Pilaira anomala	CBS 131.23		dung of rabbit, UK	JN206097	
Thamnidium elegans	CBS 411.52		Dung of bat, Poland	JN206058	
Thamnidium elegans	CBS 641.69		USA	JN206059	
Thamnidium elegans	CBS 341.55		USA	JN206060	MT533690

CBS: Westerdijk Institute, Culture collection of fungi and yeasts, Utrecht, the Netherlands CICC: China Center of Industrial Culture Collection, Beijing, China URM: Federal University of Pernambuco, Recife, Brazil T ex-type strain, NT neotype strain.

(Capella-Gutiérrez et al., 2009). CFS and ITS alignments were then concatenated. The phylogenetic tree was calculated using the maximum likelihood approach in RAxML software (Kozlov et al., 2019) on partitioned dataset using GTR + FO + G4m substitution model. The tree robustness was assessed by bootstrap analyses with 1000 replicates. The isolates were assigned to species based on their position on the phylogenetic tree.

2.7. Maximal growth temperature experiment

To check which of the isolates were indeed incapable of growth in the temperatures exceeding 25 °C and to confirm whether this trait may be linked to the taxonomic position of the isolates an experiment was performed. Plates with 4 % potato dextrose agar (BTL, Łódź, Poland) were inoculated in the centre with 10 μl of spore suspension of the analysed isolates and placed in 20 °C. The temperature was increased daily in 5 °C increments until it reached 40 °C. Observed growth was marked on the plates each time the temperature was increased.

3. Results

3.1. Metabarcoding of fungal diversity on DAB

From the Illumina MiSeq sequencing, 1 241 612 paired-end reads were obtained. Raw data was deposited in GenBank under accession number: PRJNA858677. In the ASV approach after quality evaluation, 968 856 sequences remained and were grouped into 111 ASVs. In the OTU approach, after filtering, 1 063 770 sequences were clustered with 97 % sequence identity forming 107 OTUs. Rarefaction curves can be found in Supplementary Materials S1.

Although samples differed by overall composition, the dominant ASVs and OTUs were similar among samples on the genus level in both approaches (Supplementary Materials S2 and S3). The representatives of genera *Candida, Debaromyces Yarrowia* and *Mucor* were the most common (Fig. 1). *Mucor* genus was detected in all of the samples, however in samples 2 and 3, its relative abundance was low. Based on the results obtained from BLASTn (Altschul et al., 1990) search of the NCBI nt_euk 2023-07-19 database dominating ASVs' centroid sequences had the highest similarity to sequences of the strain CBS 992.68 (GB acc. No. JN206067) representing *Mucor flavus* species complex (Walther et al., 2013), while the ASVs with the highest relative abundance from ascomycetous yeasts was the most similar as this represented by *Candida zeylanoides* (GB acc. No. MH459420) Blast values for ASVs are listed in Supplementary Materials S2.

3.2. Culture based mucoralean diversity on DAB

Nineteen isolates of fungi belonging to the family *Mucoraceae* were isolated from DAB pellicle samples. Based on the obtained sequences they were assigned to 6 different species (Fig. 2.): *Helicostylum pulchrum, Mucor circinelloides*, *Mucor mucedo*, *Mucor saturninus*, *Mucor aligarensis*, and *M. flavus*. Sequences assigned to the last species, group in a single clade inside the *M. flavus* species complex (Walther et al., 2013), and based on the BLASTn (Altschul et al., 1990) search in the nt_euk 2023-07-19 NCBI database have the highest similarity to the sequence from the *M. flavus* CBS 992.68 strain which, however, does not form monophyletic group with *M. flavus* neotype strain (Fig. 2.).

3.3. Maximal growth temperature experiment

All the fungi isolated from the DAB except the one assigned to the *M. circinelloides* species were indeed incapable of growth in temperatures exceeding 25 °C (Fig. 2.).

4. Discussion

In both HTS-based and traditional approaches the observed fungal species seemed to form a stable community. It was composed mainly of

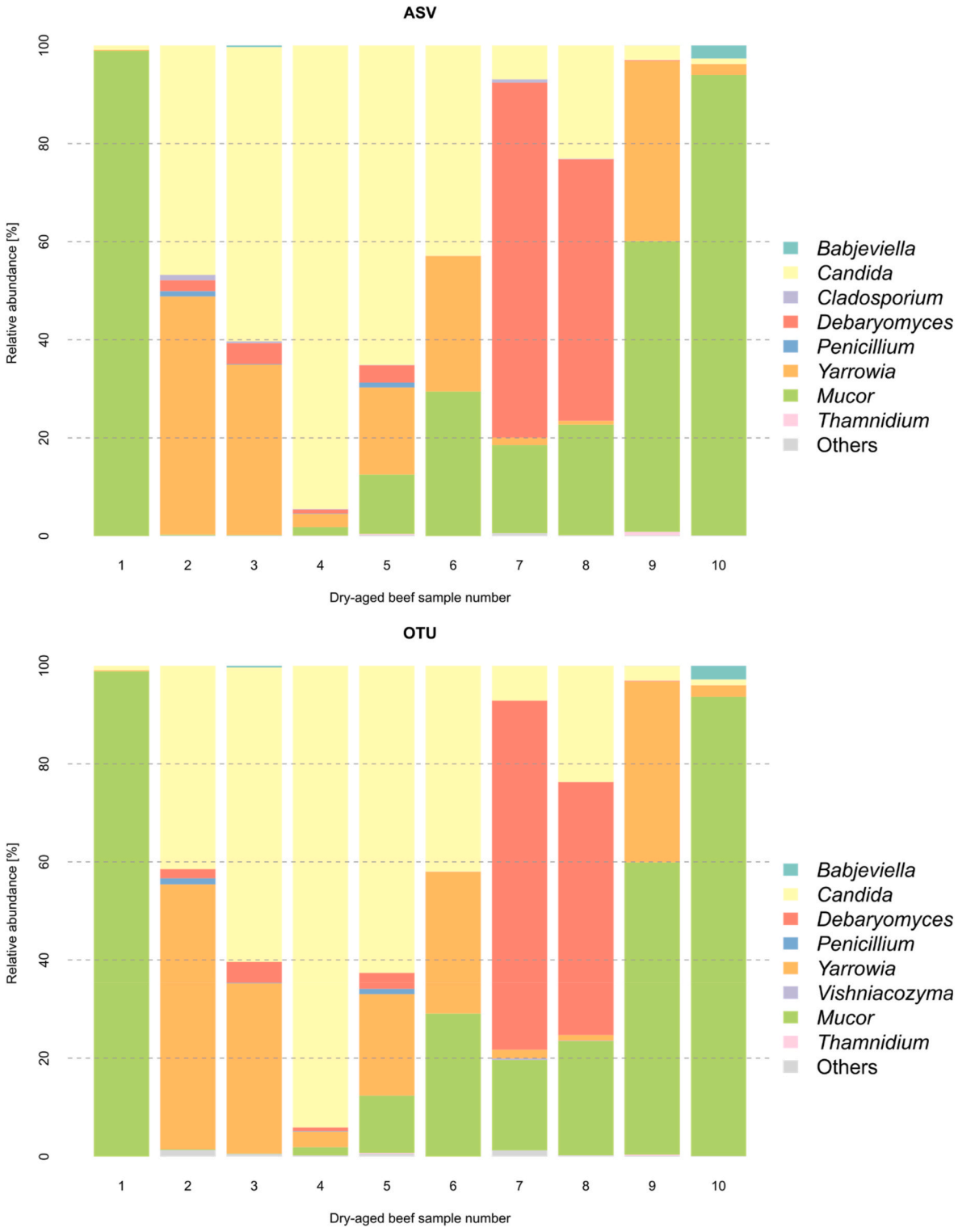

Fig. 1. Relative abundance of ASVs and OTUs at a genus level in the analysed samples. Taxonomic assignment was done using a naive Bayes classifier trained on the UNITE database: sh_qiime_release_29.11.2022 (Nilsson et al., 2019). For each method eight genera with the highest relative abundance among the investigated samples are shown. Other genera were merged into the "Others" category.

different ascomycetous yeasts and *Mucoraceae* representatives. The representatives of the first group belonged to genera *Yarrowia, Candida* and *Debaryomyces* (Fig. 1.). All detected taxa are known to possess high proteolytic and lipolytic activity (Patrignani et al., 2007) and were reported in other recent studies as colonisers of dry aged meat products (Groenewald et al., 2014; Mikami et al., 2021; Ryu et al., 2020).

These finds align with other recent studies on the diversity of the DAB. Almost the same taxa as those reported in Asian studies (Mikami et al., 2021; Oh et al., 2019; Ryu et al., 2020) seem to also colonise the DAB in Europe.

As for hyphal fungi, since this study focused on *Mucoraceae* members, methods that exploited their fast growth in optimal conditions

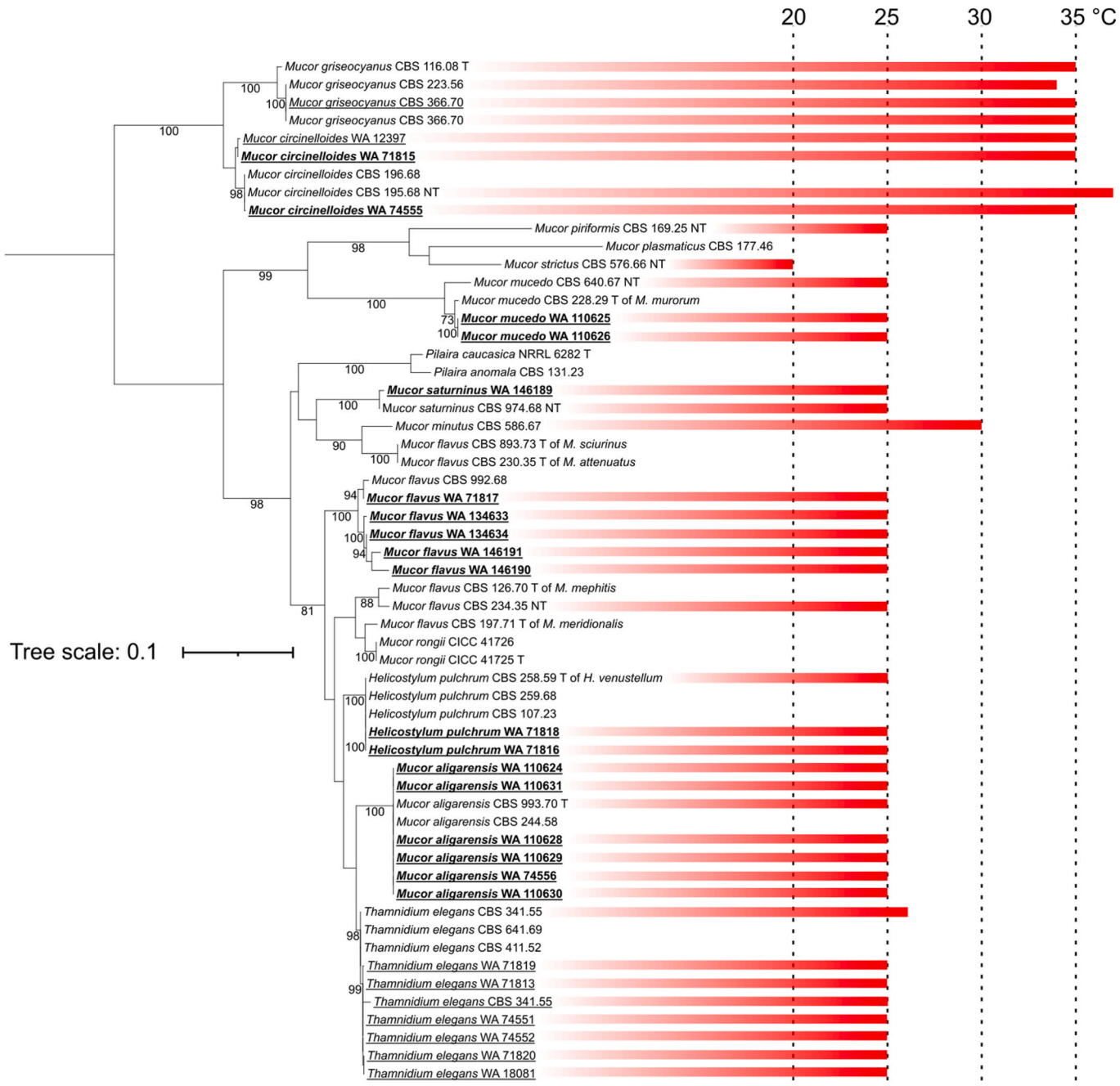

Fig. 2. RAxML phylogram of the isolated *Mucoraceae* based on the concatenated ITS and CFS sequences supplemented with molecular data from type specimens. Sequences from *Mucor circinelloides* and *M. griseocyanus* were used as an outgroup (Walther et al., 2013). Bootstrap values above 70 are shown. In bold are the isolates from the DAB. Maximum recorded growth temperatures are provided for each of the analysed strains and isolates. Underscored are the strains whose maximum growth temperature was measured in this study. For other strains maximal growth temperatures, preferably for the type specimens, are shown from other studies (Benny, 1992; Schipper 1975; Upadhyay, 1973; Wagner et al., 2020). Sequences that are not obtained during this study and were used in creation of this figure are listed in Table 2.

were used for their isolation from the DAB. Elevating humidity and providing slightly higher temperature (circa 18 °C) induced fast growth of the aerial hyphae and abundant sporulation. These conditions allowed to avoid contamination by hyphal ascomycetous fungi such as members of the genera *Penicillium* and *Aspergillus* since their growth is generally slower than those of the *Mucoromycota* members. While *Penicillium* and *Aspergillus* fungi can potentially grow under refrigerated conditions this difference in physiology may also partially explain why they were almost absent from the metabarcoding data. Colonisation and subsequent growth seem to happen mostly when meat is still moist on the surface. As the meat dries during the dry ageing and the pellicle develops, only the already established communities seem to survive. Here fast growth of the mucoralean fungi may give them an advantage in this primary colonisation but confirming this hypothesis would require more experiments. Another reason for relatively weak presence of *Penicillium* and *Aspergillus* representatives on the DAB may be the fact that the meat harbouring those fungi is generally discarded during the dry-ageing as many members of these genera produce secondary metabolites that are harmful to humans (Dave and Ghaly, 2011). During this study they were also perceived rather as contaminants than innate parts of the DAB mycobiota.

Of the *Mucoraceae* representatives, which were isolated using

plating, isolates phylogenetically belonged to the *M. flavus* group as defined by Walther et al. (2013) where present in the most of the investigated samples. Additionally, in HTS the ASV with the highest relative abundance was related to CBS 992.68 strain belonging to the *M. flavus* species complex (Walther et al., 2013). In other studies in which the mycobiota of the DAB was investigated, fungal isolates closely related to this lineage were also found (Hanagasaki and Asato 2018; Mikami et al., 2021). *Helicostylum* sp. which was also reported recently as DAB coloniser (Ryu et al., 2020) was likewise identified in this study. These data suggest the existence of a specific mucoralean community adapted to the cold and dry environment characteristic for DAB surface. DNA obtained from all of the strains mentioned in Table 1., including the *Helicostylum* sp. isolated in this study, gave good products in a PCR using the same primers for ITS2 sequence as those used in HTS.

While fungi belonging to the *Thamnidium* genus were not isolated directly from the DAB surface, they have been detected using metabarcoding methods. They are capable of growth in low temperatures and were previously described as the main agents colonising the DAB (Dashdorj et al., 2016). They were not, however, detected in other recent studies investigating fungal diversity of DAB (Mikami et al., 2021; Oh et al., 2019; Ryu et al., 2020) and seem to be less prevalent than some of the literature may suggest (Dashdorj et al., 2016). This phenomenon could be explained by the superficial similarity of *Thamnidium* and *Helicostylum* species. In the past, both taxa belonged to *Thamnidiaceae* family, which included *Mucoromycota* representatives that on small branchlets are forming sporangiola, structures similar to sporangia but often without internal columella and a limited number of spores (Upadhyay 1973). The first described species that gave name to the whole family was *Thamnidium elegans* Link (1809). Later, new fungi were discovered, with different shaped sporangiola bearing hyphae, and those were separated into new genera, like *Helicostylum* Corda (1842). For a long time there was no scientific consensus, whether these morphological differences were important enough to classify those fungi as separate genera and several taxonomic reclassifications were proposed (Upadhyay, 1973). Another explanation could be that "*Thamnidium*" could have been used as a misnomer for other morphologically similar members of the family *Thamnidiaceae*, in the same way as "*Mucor*" is still sometimes used to describe other members of the order *Mucorales*.

Another problem was the assignment of the isolates whose ITS sequence was the most similar to the *M. flavus* strain CBS 992.68. The neotype of species *M. flavus* is a strain CBS 234.35, which does not cluster together with strain CBS 992.68, also assigned to this taxon. In the past, several separate taxa were described in this group, but they were synonymised with *M. flavus* by Schipper (1975). In our phylogenetic reconstructions *M. flavus sensu* Schipper (1975) is not monophyletic. This topology is consistent with previous results of Walther et al. (2013), who named this group *M. flavus* complex and refrained from further taxonomic changes awaiting more detailed studies. Additionally, "*M. flavus* Bainier, 1903" is not a legitimate name as this name was previously assigned to a different species (namely *Thelactis flava* H. Mart (1817) MB#142860), and thus it does not follow the principle of priority. Interestingly, representatives of the *M. flavus* complex turned out to be particularly common among the isolates from DAB. All of them (including ones from study of Hanagasaki and Asato (2018) and Mikami et al. (2021)) cluster together with strain CBS 992.68 but none of former ex-type strains (from before Schipper's revision) is falling into this group.

Thermotolerance seems to be one of the distinguishing features among *Mucor* genus members. Representatives of the *M. circinelloides* group (Walther et al., 2013) are ubiquitous and can be found in various environments (including the DAB). Due to being capable of growing in temperatures up to 39 °C (Wagner et al., 2020) (Fig. 2.) they are also pathogens of warm-blooded animals including humans causing deadly mucormycosis (Wagner et al., 2020). In contrast, members of the *M. flavus* group (including *Pilaira* genus) and its sister clade namely *M. mucedo* group as defined by Walther et al. (2013) seem to be better adapted to lower temperatures (Fig. 2.). A possibility could be a creation of a separate genus encompassing those two cryotolerant clades. These taxonomic revisions could simplify distinguishing the fungi regarded as safe for the dry-ageing.

5. Conclusions

The isolates belonging to the *M. flavus* complex were found as a dominant fungal species. The fungi representing genera *Helicostylum* and *Mucor* were also isolated from the DAB. The metabarcoding data indicated that members of the *Thamnidium* genus may be growing in this environment too. Majority of these taxa belong to a monophyletic group inside the *Mucor* genus with a common trait being their adaptation to low temperatures and a lack of growth in temperatures exceeding 25 °C. Due to this characteristic their presence on the DAB may be indicative of the safe and correct dry-ageing process.

Declaration of competing interest

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2023.09.005.

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