LWT - Food Science and Technology 243 (2026) 119179

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

The effect of fungal proteolytic enzymes on myofibrillar proteins in dry-aged beef

Grzegorz Ostrowski [a,*], Danuta Jaworska [b], Natalia Kasałka-Czarna [c], Magdalena Montowska [c], Anna Muszewska [d], Magdalena Płecha [d], Wiesław Przybylski [b], Krzysztof Sawicki [e], Łukasz Słowik [a], Julia Pawłowska [a]

[a] University of Warsaw, Biological and Chemical Research Centre, Faculty of Biology, Institute of Evolutionary Biology, Żwirki i Wigury 101, 02-089, Warsaw, Poland
[b] Warsaw University of Life Sciences (WULS), Institute of Human Nutrition Sciences, Nowoursynowska 166, 02-787, Warsaw, Poland
[c] Poznań University of Life Sciences, Faculty of Food Science and Nutrition, Wojska Polskiego 28, 60-637, Poznań, Poland
[d] Polish Academy of Sciences, Institute of Biochemistry and Biophysics, Pawińskiego 5a, 02-106, Warsaw, Poland
[e] CHRIS, Dry-Ager, Szwedzka 23/75, 30-324, Kraków, Poland

ARTICLE INFO

Keywords:
Aspartic peptidase
Beef
Tenderisation
Transcriptome
Mucor

ABSTRACT

The role of fungi in traditional food fermentation is not fully understood. The aim of this study was to assess the enzymatic potential of a fungal biostarter isolated from dry-aged beef. We have applied a multi-omics approach, i.e. combined the analysis of genomic and transcriptomic data with the observation of protein degradation patterns in the fungus-inoculated meat. Thanks to this, it was possible to pinpoint the enzymes produced by the fungus responsible for the changes occurring during dry-ageing. By comparing samples of meat inoculated with the fungal biostarter and those without, we were able to establish the effect of fungal enzymes on meat protein composition. The presence of the fungus on meat increased the relative amount of selected low-molecular-weight proteins (i.e.113.9 kDa, 103.5 kDa, and 18.9 kDa). We believe this change may be caused by fungal endopeptidase activity. Additionally, results of transcriptome analysis indicate that several aspartic endopeptidases were expressed by the fungus. The identified enzymes have a similar structure to the aspartic peptidases used as microbial rennets. Overall, the changes caused by the fungus during dry-ageing seem to be linked to the activity of released aspartic endopeptidases.

1. Introduction

Contemporary omics technologies enable easier characterisation of the microbial diversity, further elucidating the role of fungi and bacteria in the production of traditionally fermented foods. Combining next generation sequencing technologies with traditional biochemical methods provides unique insight into the dynamism of microbial communities and their influence on the quality of the final product. Though the role of the fungi used in production of: doenjang-meju (Jung et al., 2014), dongchimi, (Lee et al., 2025), and Baixi sufu (Wan et al., 2020) has already been investigated, the focus was particularly placed on *Aspergillus oryzae,* commonly used as a biostarter for fermentation of various soy products (Han et al., 2024; Zhao et al., 2015). However, other fungi employed in food fermentation, especially members of *Mucorales* order, lack proper descriptions of their enzymatic capabilities (He et al., 2019; Vellozo-Echevarría et al., 2024).

One of the fungi-engaging processes that is especially poorly understood is the dry-ageing of meat. General safety recommendations suggest following practices that minimise microbial growth on meat (Koutsoumanis et al., 2023), however, similarly to artisanal cheese manufacturing, inducing "good" microbial growth may potentially improve the final product's quality. This practice is widely used in sausage making, thus providing a reliable way to ensure that only non-pathogenic strains colonise the product (Hüfner & Hertel, 2008; Sunesen & Stahnke, 2003). In the case of dry-aged beef, psychrotolerant Mucoraceae together with ascomycetous yeasts have been noted as the dominant eukaryotic taxa (Capouya et al., 2020; Coton et al., 2024; Rodrigues et al., 2025). Although the presence of these microorganisms is currently considered a sign of a correct ageing process (Hanagasaki & Asato, 2018; Mikami et al., 2021; Oh et al., 2019; Ryu et al., 2020), the correlation between microbial growth and the quality of the final product is still limited (Cheng et al., 2023; Savini et al., 2024).

* Corresponding author.
E-mail address: g.ostrowski@uw.edu.pl (G. Ostrowski).

https://doi.org/10.1016/j.lwt.2026.119179
Received 30 September 2025; Received in revised form 27 January 2026; Accepted 18 February 2026
Available online 19 February 2026

The microorganisms developing on the beef's surface can tenderise the meat and improve its quality through production of proteolytic enzymes. Simultaneously, some volatile organic compounds released during dry-ageing process, such as alcohols and aldehydes, are responsible for the characteristic fruity, nutty and buttery aromas (Hanagasaki & Asato, 2018, 2023; Jaworska et al., 2025; Liu et al., 2024; Przybylski et al., 2023). On the other hand, excessive proteolysis of the muscle tissue can compromise the integrity of the meat, resulting in undesirable texture and can lead to protein oxidation and aggregation, reducing the nutritional value of the product (Savas et al., 2024; Utrera et al., 2011). Conversely, some of the volatile organic compounds produced by the microorganisms can be responsible for putrid or rancid aromas negatively influencing the perception of the final product (Mikami et al., 2022; Ribeiro et al., 2023).

Since fungi belonging to the Mucoraceae family occur naturally on beef (Ryu et al., 2020) they may potentially be employed to promote positive changes during the dry-ageing process. The studies of Hanagasaki and Asato (2018) and Przybylski et al. (2023) reported that *Mucor flavus* could be used as a biostarter for dry-ageing of beef. It is a non-pathogenic, fast-growing, easy-to-culture fungal species well adapted to near-freezing temperatures (Danilova et al., 2024) allowing to employ both the organism itself and its enzymes in food production (Morin-Sardin et al., 2017; Qasim et al., 2022; Yegin et al., 2011). Studies on the enzymatic activity of Mucoraceae fungi usually focus on optimisation of the industrial scale production and potential applications (Dzurendova et al., 2021; Morin-Sardin et al., 2017) with some of the enzymes even being successfully expressed in yeast to further optimise their production (Gama Salgado et al., 2013; Sun et al., 2018; Yegin & Fernandez-Lahore, 2013). However, studies exploring how these fungi produce enzymes in more natural conditions, especially the strains incapable of growing in elevated temperatures, are mostly limited; available descriptions of mesophilic and psychrotrophic *Mucor* species mainly focus on the strains relevant to cheese making (Lebreton et al., 2019; Ozturkoglu-Budak et al., 2016; Qasim et al., 2022; Zhang et al., 2018). Though recent studies on how psychrophilic Mucoraceae fungi can be employed in dry-ageing of beef showed the increased tenderness of the product as a perceived advantage, this effect on tenderness can be overshadowed by naturally occurring proteolytic enzymes (Hanagasaki & Asato, 2018; Jaworska et al., 2025; Mikami et al., 2021; Przybylski et al., 2023). Gaining understanding of fungal proteolytic activity and its links to improving the tenderness of dry-aged beef may further optimise the production process.

As such, in this study we try to characterise the enzymatic capabilities of the *Mucor* isolate KKP 2092p and its influence on the meat proteolysis and the status of muscular protein affected by the released enzymes. To achieve this, fungal transcriptome and protein degradation patterns observed in meat inoculated with the fungal biostarter were subjected to synergistic analysis aimed at identifying fungal enzymes relevant to the dry-ageing of beef (Fig. 1).

2. Materials and methods

2.1. Dry-ageing experiment

2.1.1. Meat samples

Meat samples for the dry-ageing experiment were taken from the R class (taking into account the muscularity) and class 2 (considering the fatness) according to the EUROP system of post-slaughter cattle classification. The animals were slaughtered in accordance with the European Union Council Regulations (EC) No. 1099/2009. From each animal, a pair of samples was taken from the left and right *Longissimus dorsi* muscle. From each pair, one control and one test sample were randomly selected. The meat originated from five individuals crossbred from Holstein-Friesian cows with bulls of meat breeds. The average weight of the samples was 3.5 ± 0.5 kg. Control samples and samples inoculated with the biostarter were dry-aged in a specialised fridge (DryAger, Bad Saulgau, Germany) at 80-90% relative humidity and 1.5 °C for 28 days.

2.1.2. Preparation of fungal biostarter

The fungal biostarter (isolate KKP 2092p) used in this study is a strain originally obtained from dry-aged beef retrieved from a local Warsaw butcher in 2020 (Ostrowski et al., 2023). The fungal isolate KKP 2092p for use in dry-ageing of beef is protected under Polish patent law as part of the patent nr: P.443 722. The biostarter was prepared by cultivating the fungus in solid-state fermentation on cracked rice at room temperature, which was then lyophilised and ground into fine powder. The powder was rehydrated in water (125 g/l) for 24 h at room temperature prior to application onto meat and then spread evenly onto the meat's surface with a silicone brush. To achieve reliable fungal growth, each kilogram of meat was inoculated with 20 ml mycelium suspension (10^6 cfu/ml). The control samples were inoculated with cooked and lyophilised rice prepared in a similar manner.

2.1.3. Evaluation of the meat quality traits, protein degradation, and polymerisation

The following physicochemical parameters were assessed after aging: pH, colour parameters, peak shear force, chemical composition of muscle, the content of malondialdehyde, muscular protein proteolysis, and polymerisation (SDS-PAGE). The pH was measured three times using an automatic pH meter 330i (WTW®, Weilheim, Germany) with specialised SenTix® SP Number 103645 electrodes. Colour parameters (CIE Lab*) were recorded five times using a CR-310 Konica Minolta® Chroma Meter (Osaka, Japan) on the meat's cross-section. After grilling (PK2745E, 3000 W, 60 Hz, Potis GmbH, Goettingen, Germany) for 3 min at 250 °C (medium rare) and cooling the samples to room temperature five longitudinal square cross-section cores (1 cm × 1 cm) were obtained and peak shear force was measured using a Warner-Bratzler attachment fitted with a flat knife at 50 mm/min until the sample was fully cut (Silva et al., 2017). The protein content was determined based on the method described in ISO 166634 (ISO 2009). An extraction method was employed to determine the free fat content of the meat, following the procedure outlined in Polish Committee for Standardization (2013). The

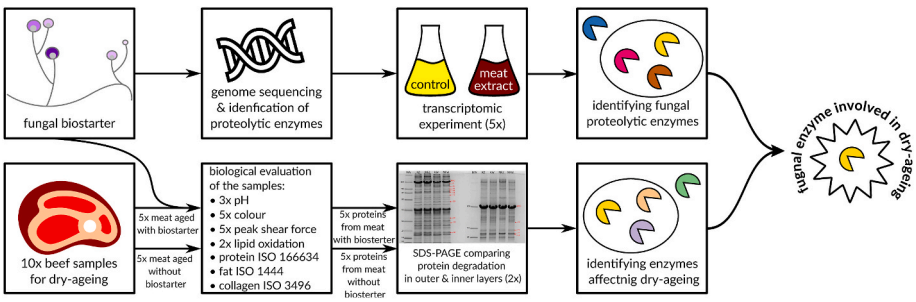

Fig. 1. Schematic overview of the study design, methodology used, and the associated hypotheses.

total collagen content of the samples was determined by the hydrolysis of the samples with sulfuric acid and the measurement of absorbance, as described in ISO-3496 (ISO 1994). The level of lipid oxidation in aged meat was evaluated in duplicate using the 2-tiobarbituric acid (TBA) methodology, based on the content of malondialdehyde (Shahidi, 1990, pp. 1008–1014).

To evaluate the changes in protein degradation and to check for products of protein aggregation and visible polymerisation, SDS-PAGE was run in technical duplicates, comparing separately the outer and inner layers (in the geometric centre, i.e. about 4-5 cm deep) of meat in each sample. To do this, meat samples of 0.1 g were separately homogenised in 1 mL of sodium-phosphate buffer (10 mM, 2% SDS, 25 mM NEM, pH 7.0) with Ultra-Turrax T25 (IKA Labortechnik, Staufen, Germany) for 2 × 20 s at 9500 rpm, and for 15 s at 13 500 rpm. These samples were centrifuged at 14 000 rpm for 10 min at 10 °C, and the obtained supernatant was stored at −80 °C for further analysis. The protein concentration was determined using a 2-D Quant kit (GE Healthcare Bio-Sciences, Fairfield, CT, USA). Protein (15 μg) aliquots were diluted 1:2 in 2 × SDS-PAGE loading buffer (4% w/v SDS, 125 mM Tris-HCl, 20% v/v glycerol, 0.004% w/v bromophenol blue, pH 6.8) and subsequently heated for 5 min at 95 °C. The samples were separated in 15% or 6% separation gel prepared in a Hoefer SE 250 system (GE Healthcare Bio-Sciences). A reference broad-range molecular weight standard (Bio-Rad Laboratories, Inc., CA, USA) was applied. Gels were run at a constant current of 20 mA per gel, then stained with Coomassie brilliant blue, scanned (Gel Doc XR + System, Bio-Rad Laboratories, Inc., CA, USA), and processed using Image Lab 6.0.1 software (Bio-Rad Laboratories).

2.1.4. Statistical analysis of physicochemical parameters

From each SDS-PAGE run, the surface of the individual bands was estimated. Then, it was assumed that the total area of all bands separated on the gel is 100%. Band percentage values were imported into Statistica 13.1 (StatSoft Inc., Tulsa, OK, USA) software. The results were analysed with one-way ANOVA. Groups of mean values with statistically significant differences were identified using Tukey's test, at $P < 0.05$. For meat quality traits (pH, colour parameters, chemical composition of muscle, and peak shear force), normality of the distribution was assessed using the Shapiro–Wilk test, and then the differences between groups were identified using t-Student's test. The results were expressed as mean ± standard error.

2.2. Genomic data sequencing, assembly, and annotation

For genomic sequencing, the biostarter strain was cultured on petri dishes with potato dextrose agar 4% (BTL, Łódź, Poland). Genomic DNA of the KKP 2092p isolate was obtained from the fungus following a previously established protocol which utilises CTAB for nucleic acid extraction, chloroform:isoamyl alcohol (24:1) for preliminary purification, and ethanol for final washing (Ostrowski et al., 2023). The reads were obtained using Illumina Nova-Seq paired-end 150 bp sequencing. The obtained sequencing data quality was assessed using FastQC. De novo genome assembly was conducted using SPAdes (version 3.11.1) (Prjibelski et al., 2020) with default parameters and using a –careful flag to enhance assembly accuracy. Assembly quality was assessed with BUSCO 5.8.2 (Manni et al., 2021). Gene prediction and functional annotation were performed using Funannotate (version 1.8.17) (Palmer & Stajich, 2020) and the integrated Augustus package using a model trained on *Rhizopus oryzae* due to its high genetic similarity to the analysed specimen. Functional annotation was done through Eggnog-Mapper v2.1.12 using eggNOG 5.0 database (Cantalapiedra et al., 2021; Huerta-Cepas et al., 2019).

2.3. Transcriptomic experiment

2.3.1. Culture conditions

For transcriptomic experiments, solid-state cultures were first established on the petri dishes with potato dextrose agar (BTL, Łódź, Poland). After incubation for a week at room temperature with light exposure, spores were harvested using a sterile saline (0.9%) solution by gently scraping mycelium with a glass spreader. Harvested spores were counted and used to inoculate (5×10^5/L) complete liquid medium consisting of: $NaNO_3$ 2,00 g/L; K_2HPO_4 1,00 g/L; KCl 0,50 g/L; $MgSO_4 * 7H_20$ 0,50 g/L; $FeSO_4 * 7H_20$ 0,01 g/L; glucose 25,00 g/L; peptone 2,00 g; yeast extract 2,00 g. The obtained pre-cultures (6 × 100 ml) were incubated in half-litre flasks in darkness at 20 °C and 200 RPM until a sufficient amount of mycelium could be harvested for the physiological experiments (38h). After preincubation the mycelium was gently harvested, washed with sterile saline (0.9% NaCl) solution and equally split into 10 flasks (250 ml) each containing 50 ml of medium: 5 with only minimum medium consisting of: $NaNO_3$ 2,00 g/L; K_2HPO_4 1,00 g/L; KCl 0,50 g/L; $MgSO_4 * 7H_20$ 0,50 g/L; $FeSO_4 * 7H_20$ 0,01 g/L; glucose 5,00 g/L and 5 flasks with the same medium which was additionally enriched in 5 g/L of beef extract (BIOCORP, Warsaw, Poland). After 5 h of further incubation (darkness, 20 °C, 200 RPM), the mycelium was harvested, briefly dried with a paper towel, and immediately placed in liquid nitrogen. The obtained mycelium was stored in −80 °C awaiting RNA extraction.

2.3.2. RNA isolation and processing

After thorough homogenisation with steel balls, 1 ml of TRIzol solution was added to each tube containing a frozen mycelium sample and the tube was gently mixed. Samples were incubated at room temperature for 15 min, followed by adding 200 μL of chloroform:isoamyl alcohol (24:1) to remove any nonpolar impurities, and the samples were then gently mixed and incubated for 5 min at room temperature. Samples were centrifuged (15 min, 1.2×10^4 g, 4 °C) and the top layer was transferred into a new tube. If needed, washing with chloroform:isoamyl alcohol (24:1) solution was repeated. 500 μL of isopropanol was added to the transferred top layer to precipitate the nucleic acids. Samples were incubated at room temperature for 10 min and then the supernatant was removed. Precipitated nucleic acids were washed twice with 70% ethanol solution, air dried, and resuspended in nuclease-free water. For purification and DNA removal from the sample, GeneJET RNA Purification Kit (Thermofisher Scientific, Waltham, MA, USA) was used. Further processing of the RNA was performed at Genomics Core Facility of the University of Warsaw and included assessing the quality of the RNA using High Sensitivity D1000 ScreenTape (Agilent Technologies, Inc., Waldbronn, Germany), enrichment of the mRNA fraction using oligo-dT primers, construction of the cDNA libraries, qualitative and quantitative analysis of the obtained cDNA, and sequencing of the product using paired-end 2x100 cycles technique in NovaSeq 6000 system (Illumina, San Diego, CA, USA).

2.3.3. Transcriptome bioinformatic analysis

Raw reads quality was first evaluated using FastQC and summarised with MultiQC (Ewels et al., 2016). Low-quality reads and the initial 10 nucleotides were trimmed using fastp to improve the overall quality of the data (Chen et al., 2018). An indexed genome of KKP 2092p isolate was produced with HISAT2 (Kim et al., 2019). The reads were then aligned to the indexed genome with HISAT2 (Kim et al., 2019) and sorted with Samtools (Danecek et al., 2021) to prepare the data for downstream analysis. Quantification of gene expression was performed using HTSeq (Anders et al., 2015). The count matrix output was used for the analysis of differentially expressed genes. To identify differential gene expression between experimental conditions, the DESeq2 (Love et al., 2014) package in R was used. The analysis involved specifying the experimental design within the DESeq2 model (Love et al., 2014), with correction for factors influencing gene expression. The results were

trimmed for statistically significant differences against a criterion of a threshold-adjusted p-value of 0.05. Then log_2FC (the logarithmic ratio of expression levels difference between the two conditions) was calculated. Apart from analysing each gene separately, another analysis was performed on the genes grouped based on their orthology. To investigate which enzymes could be potentially responsible for the observed extracellular proteolytic activity, a list of PFAM enzyme families was made using the MEROPS database. Based on this list, the orthologues present in the genome were then identified using eggNOG 5.0 database (Huerta-Cepas et al., 2019) and further characterised using InterPro database (Blum et al., 2025).

All of the obtained genomic data were deposited at the National Center for Biotechnology (NCBI) as part of BioProject under the number: PRJNA1250995. Genomic data including: contigs, a file containing all of the annotated features, RNA count matrix, and DESeq2 analysis results, are also provided as supplementary data to this article.

3. Results and discussion

3.1. Meat aging and fungal influence

Application of the fungal biostarter KKP 2092p had no influence on colour, pH, chemical composition of muscle, or malondialdehyde content in the beef steaks. The pH and colour parameters, as well as the chemical composition of the observed meat were comparable with the results obtained by Sha et al. (2017) and Oh et al. (2019) in similar studies where no biostarter was used. The pH of the meat is especially important because it influences the colour, microbiota and innate enzyme activity (Ribeiro et al., 2025). The values provided in Table 1 are in optimal range for the activity of endogenous proteases, especially μ- and m-calpains and cathepsins which contribute to the degradation of myofibrillar and connective proteins improving meat tenderness (Ribeiro et al., 2025). Naturally occurring surface microbiota including *Thamnidium, Mucor*, *Penicillium species*, and yeasts such as *Debaryomyces* and *Yarrowia* can also influence meat maturation (Ryu et al., 2020).

Similarly to results obtained by Przybylski et al. (2023) and Hanagasaki and Asato (2018) a small decrease in shear force could be observed but the effect of the fungus was not significant, given inherent heterogeneity of meat as a substrate (Table 1) and relatively low number of investigated samples. However, in the study of Przybylski et al. (2023) despite the lack of significant differences in the measured peak shear force using the Warner-Bratzler method, the beef aged with the *Mucor* isolate was significantly more tender according to consumers who participated in hedonic evaluations.

In the columns indicated as biostarter- and biostarter+ are means of the relevant measurements; SEM (standard error of the mean) provides overall measure of the dispersion of individual sample means for all of the investigated beef samples; p-value <0.05 would indicate that the biostarter- and biostarter + samples were significantly different from each other regarding the investigated parameter.

The analysis of muscle proteins' degradation using the SDS-PAGE method showed that significant differences can be observed between meat samples aged with the biostarter (Table 2). When it comes to the effect of fungus application, the SDS-PAGE showed relatively higher levels of selected short-chain proteins (Table 2). Their elevated presence can be caused by a partial proteolysis of longer proteins - such as myosin heavy chain protein (MHC) - cut specifically by the enzymes released by the fungus. Bhat et al. (2018) showed that the amount of 110 kDa muscle protein increases during ageing time, and together with the 90 kDa protein can be an indicator of proteolysis of the MHC. The proteins' group of 27-32 kDa appears during postmortem muscle degradation and is also correlated with proteolysis and tenderisation of beef during ageing (Huff-Lonergan et al., 2010; Kemp et al., 2010). These low-molecular-weight proteins appear together with the degradation of troponin-T which is another indicator of postmortem proteolysis of muscle proteins and beef tenderness (Gagaoua et al., 2021; Huff-Lonergan et al., 2010; Kemp et al., 2010). Levels of those low-molecular-weight proteins were overall slightly higher in the samples aged without the biostarter possibly due to enzymatic activity of the fungus. However, a band of around 18.9 kDa was much more prominent in the inoculated samples. It is similarly sized as the bands that increased in intensity with aging of beef in the study of Liu, Yu, et al. (2025). There were small but significant differences in proteolysis related to the sampling site (inner/outer part), likely caused by moisture loss in the outer layer and easier access of the enzymes secreted by the fungus growing on the meat's surface. Another study investigating the influence of microorganisms on proteolysis both on the surface and inside showed that free amino acid concentration was two times lower on the surface of the dry-aged beef (Bischof et al., 2023). This may be caused by slower proteolysis in the outer layer due to high moisture loss.

As shown by the aforementioned results, protein degradation in the control group is consistent with previous results and is a result of the proteolysis of endogenous proteolytic enzymes present in muscle tissue such as: cathepsins, calpain system and caspases (Huff-Lonergan et al., 2010; Kemp et al., 2010). However, the proteolysis of myofibrillar proteins in the group with dry-aged beef (with the application of the *Mucor* biostarter) was additionally supported by exogenous enzymes produced by the fungus. According to Mikami et al. (2021) dry aging of beef with moulds produced different levels of flavour compounds and according to Kim et al. (2018) and Oh et al. (2019) could affect muscle protein degradation and sensory quality. Trigueros et al. (1995) showed, that the proteolytic capability of the *Penicilium*, *Mucor* and *Aspergillus oryzae* strains for degradation of myofibrillar and sarcoplasmic proteins in dry sausages differed by even up to 30-fold and were highly dependent on media, pH and temperature. Casaburi et al. (2007) demonstrated a significant impact of starter cultures on the degradation of myofibrillar proteins such as myosin heavy chain (MHC), actin and alpha-actinin during the ripening of traditional fermented sausages. The proteolytic activity of the *Mucor* isolate and its ability to degrade proteins was proven in soybean fermentation at a low temperature by Cheng et al. (2009). These proteolytic properties of *M. flavus*, manifested by an increase in free amino acids in dry aged beef, were also clearly confirmed in the studies of Hanagasaki and Asato (2018, 2023). In the case of the *Mucor* isolate KKP 2092p, the degradation of myofibrillar proteins of the muscle tissue may even occur at very low temperatures providing a safe way to increase beef tenderness during the dry-ageing.

No high molecular mass protein aggregates and no visible polymerisation were observed in the product both in the control and the samples inoculated with the fungus. These changes can be induced by oxidation of meat during the dry-ageing process (Jongberg et al., 2017; Zhang et al., 2013). Their lack in the investigated samples points to correct dry-ageing conditions and shows that the enzymatic activity of the fungus had no negative effect on the meat quality.

Table 1
Impact of the fungus on the biochemical parameters of the meat [n = 10].

Traits	Biostarter-	Biostarter+	SEM	p-value
pH	5.70	5.74	0.02	0.57
L*	35.28	34.85	1.14	0.89
a*	20.09	20.60	0.43	0.65
b*	18.28	18.01	0.26	0.70
Protein content (%)	22.05	21.70	1.64	0.94
Fat content (%)	17.85	16.60	5.83	0.95
Collagen content (%)	1.45	1.48	0.09	0.92
The content of malondialdehyde (mg/kg)	0.17	0.25	0.03	0.26
Peak shear force after grilling (N)	83.18	76.66	16.63	0.23

3.2. Genome characteristics

Sequencing of *Mucor* KKP 2092p using Illumina NovaSeq short-read

Table 2
SDS-PAGE experiment results showing the influence of the biostarter on changes in the selected protein bands.

Molecular weight (kDa)	Control outer part	Biostarter outer part	Control inner part	Biostarter inner part
170.6	1.11b ± 0.14	0.99ab ± 0.09	0.83ab ± 0.09	0.58a ± 0.16
135.8	0.63ab ± 0.10	0.31a ± 0.03	1.56b ± 0.46	0.26a ± 0.05
113.9	0.87ab ± 0.19	1.15b ± 0.13	0.53a ± 0.07	1.15b ± 0.13
103.5	2.26a ± 0.12	3.89b ± 0.31	2.09a ± 0.19	3.64b ± 0.27
99.6	3.66b ± 0.18	1.97a ± 0.20	2.08a ± 0.15	2.40a ± 0.24
81.7	0.79b ± 0.07	0.57a ± 0.04	0.47a ± 0.05	0.61ab ± 0.05
60.2	1.87b ± 0.07	0.97a ± 0.05	2.43c ± 0.24	1.30a ± 0.11
34.0	1.31b ± 0.12	0.39a ± 0.05	1.63b ± 0.20	0.55a ± 0.11
28.9	1.55b ± 0.08	0.67a ± 0.05	1.78b ± 0.16	1.05a ± 0.12
18.9	4.45ab ± 0.10	6.19c ± 0.34	3.73a ± 0.36	5.32bc ± 0.42

Means with different letters (a/b/c) in the same row show a significant difference ($P \leq 0.05$). ab marks results different from c. bc marks results different from a. The colours signify these differences as well; purple a, green b, orange c, and intermediate colours showing either ab or bc. Following ± signs are the values of standard error for the measured bands.

technology resulted in 11 714 952 paired-end reads, all of them uncontaminated and of very good quality (average PHRED score per base above 35 for all bases); therefore, no additional cleaning steps were performed and all reads were retained. After discarding contigs shorter than 1000 nucleotides, we obtained 2606 contigs with a total length of 64.19 Mbp.

The obtained genome was almost twice as big as the genome of *Mucor flavus* (Table 3) that was recently sequenced and assembled by Hosono et al. (2024). When the *Mucor* KKP 2092p genome assembly quality was checked with BUSCO v. 3.0.2 (Simão et al., 2015) (database: fungi_odb12), the majority of the relevant genes were observed in duplicates (Table 3). Once the identity of these duplicated orthologous sequences was assessed, one copy per pair was indeed identical with its equivalent obtained from *Mucor flavus* K1Ta, while the second one was indistinguishable from counterparts obtained from *Helicostylum pulchrum* KT1b. The contigs obtained for KKP 2092p were sorted using Kraken2 with two genomes (K1Ta, KT1b) serving as custom databases. Based on those sorted contigs, two almost complete genomes were assembled: that of *Mucor flavus* and *Helicostylum pulchrum*.

Such similar tendencies of duplicating genetic material were observed in clinically relevant species belonging to *Mucorales* (Nguyen et al., 2020). Some of the *Rhizopus* strains whose genome was sequenced also seem to have undergone recent whole genome duplications (Gryganskyi et al., 2018), but to the authors' knowledge, no such events have been reported for members of the *Mucor* genus. Following the whole genome duplication, some of the copies may have already been lost explaining the observed single-copy genes (Table 3) and why some of the expressed genes of KKP 2092p seem to have missing domains. Carrying multiple copies of the genes may potentially help in adapting to various environmental conditions, but systematic analysis of this phenomenon and its environmental relevance is lacking (Vellozo-Echevarría et al., 2024).

3.3. Transcriptome characteristics

3.3.1. Gene overexpression in samples supplemented with meat extract

Overall, there were 385 upregulated and 567 downregulated genes (LFC < -1 or >1, p-value <0.05) in the cultures supplemented with meat extract compared to the control ones. Several genes (e.g. malate synthase ENOG503NX6Q) present in duplicates were expressed differently in the control and the test samples. When the overall expression for the whole orthologous group encompassing a copy from the *Mucor* and another from *Helicostylum* was compared, no significant changes in their expression were observed. However, in the majority of pairs of duplicated genes, only one copy showed substantial expression. Many of the genes with significant differences in expression, including 4 with the highest expression differentiation (Fig. 2a) had an unspecified function.

The characterised upregulated genes in cultures supplemented with meat extract were primarily connected to mitochondrial biogenesis and gluconeogenesis/glycolysis. One of these genes was chalcone isomerase

Table 3
Genome assembly comparison between the Isolate KKP 2092p, and the strains KT1a and KT1b sequenced by Hosono et al. (2024).

Species strain		Isolate KKP 2092p	Contigs matched to *M. flavus* KT1a	*M. flavus* KT1a	Contigs matched to *H. pulchrum* KT1b	*H. pulchrum* KT1b
Number of contigs		3053	508	69	1719	91
Contig N50		59 747	167 227	857 634	31 466	946 389
The longest contig		761 426	761 426	3 272 716	147 997	2 241 229
Total (bp)		64 496 461	32 347 132	33 728 218	31 283 200	34 232 018
GC contents (%)		34.38	34.64	34.60	34.13	34.19
Coverage		54.75	-	37.0	-	33.3
BUSCO	Complete and single copy	135	909	914	852	920
	Complete and duplicated	962	171	176	137	174
	Fragmented	13	19	14	64	13
	Missing	12	23	18	69	15

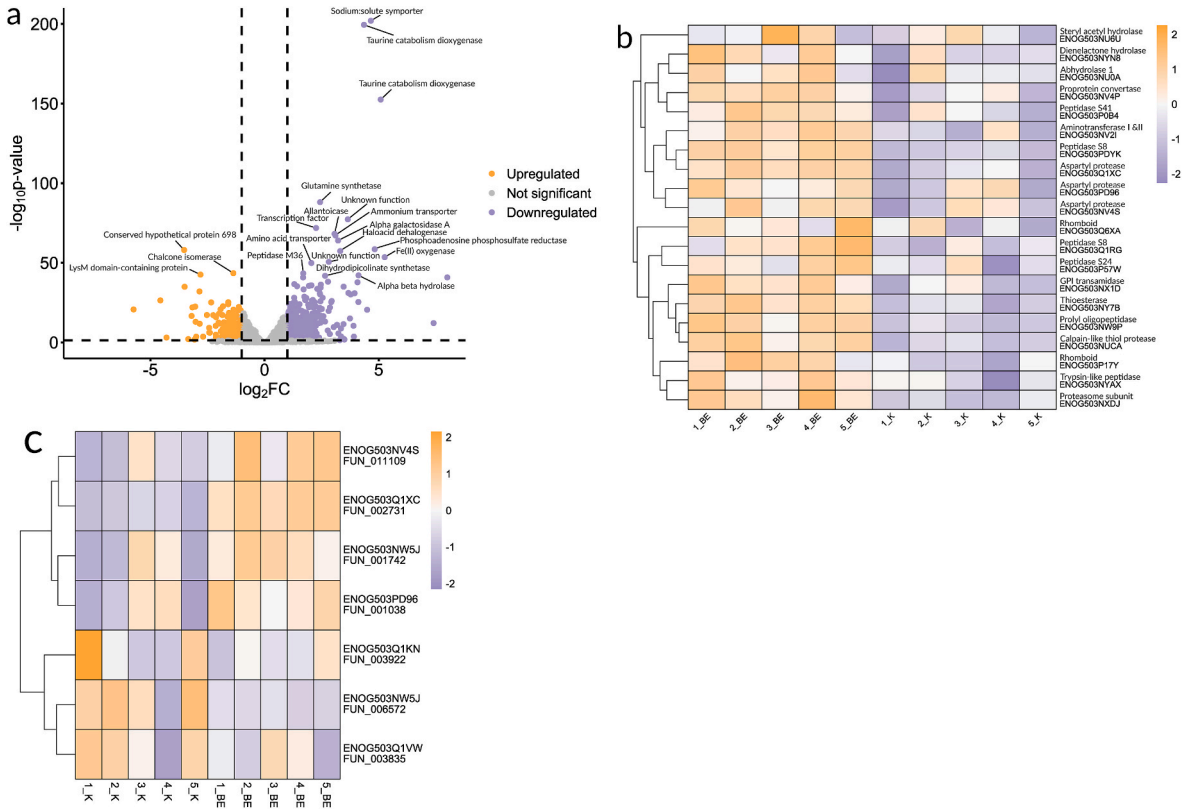

Fig. 2. Observed differential gene expression. BE designates the samples supplemented with beef extract, and K the control samples. (a) shows the overall biggest differences in expression patterns between the control and the investigated samples. Top 20 results were selected based on the p-value. On the left (orange) are the orthologous groups that were upregulated in the samples supplemented with beef extract; on the right (purple) the downregulated ones; (b) shows the top 20 (based on p-value) upregulated hydrolyses that are reported as potentially used in extracellular digestion of protein by fungi; (c) shows differential expression of aspartic peptidase genes.

(Fig. 2a) (ENOG503Q16X; log_2FC −1.37; p-val 3.11E-44), a gene involved in mitochondrial functions and haem binding (Schmitz et al., 2023). Cytochrome oxidase assembly protein (ENOG503NWB3; log_2FC −1.74; p-val 3.45E-05) was another example of an upregulated gene participating in the assembly of the mitochondrial respiratory chain. To supply mitochondria, the genes involved in gluconeogenesis/glycolysis were also overexpressed in the samples supplemented with meat extract. For instance, several copies of glyceraldehyde 3-phosphate dehydrogenase (ENOG503NUCI) were overexpressed (log_2FC −1.28; p-val 3.36E-06) in the samples supplemented with beef extract, but since the genes involved in gluconeogenesis/glycolysis and mitochondrial biogenesis are parts of basic cell metabolism, the relative changes in their expression were not high. However, elevated levels of transcripts related to mitochondrial activity indicate an overall faster metabolism in the extract-supplemented samples. This theory is further supported by the observed overexpression of genes involved in the import of oligopeptides (ENOG503NXS7; log_2FC −3.50; p-val 1.11E-35) (ENOG503-NUA6; log2FC −1.72; p-val 1.12E-04) that could be substrates for gluconeogenesis.

Another transport protein that seems to be related to the usage of meat extract as an energy source is the hypothetical protein 698 (ENOG503PBKY; log_2FC −3.52; p-val 4.71E-56). It consists of several helices typical of membrane transporters grouped in the YeiH-like family (IPR004630). This protein is similar to putative prokaryotic sulphate exporters classified in the transporter database as TCDB 2.A.98. In bacteria, their expression can be triggered by utilising taurine, an amino sulfonic acid commonly found in red meat (Denger et al., 2006). Metabolism of amino acids containing sulphur, such as cysteine and methionine, may cause an excess of sulphur compounds that necessitates their removal from the cell to maintain cell homeostasis (Stipanuk, 2020). Sulphate permease (ENOG503NW17) also showed slightly elevated expression (log_2FC −0.584; p-val 4.85E-07) in the samples supplemented with meat extract, further highlighting the importance of managing sulphur levels when utilising amino acid-rich substrates as the main energy source.

Chalcone isomerase and hypothetical protein 698 are among the top most overexpressed genes when the fungus is exposed to meat extract (Fig. 2a). Their elevated expression seems to be connected to utilising meat extract as an energy source and interacting with the surrounding environment, but connecting them to biological processes in eukaryotes is difficult and their exact functions remain insufficiently described.

3.3.2. *Proteases expression in samples supplemented with meat extract*

Overall, not many peptidases were upregulated in the samples supplemented with meat extract and those with elevated expression were primarily involved in protein turnover, protein maturation, had a regulatory function (e.g. tripeptidyl peptidase II from S8A family, peptidase M49, prolyl oligopeptidase S9A) (Muszewska et al., 2017), or were not yet fully characterised (S8A peptidase with unknown function). Due to that, the search for candidate enzymes that could be responsible for the observed proteolysis was extended to other promising peptidases showing high expression in the samples supplemented with meat extract. Among them were numerous cysteine peptidases, serine peptidases, and metalloproteases (Fig. 2b). However, many of these enzymes are either known for performing intracellular functions or are uncharacterised. Some of these enzymes could also influence the dry-ageing process, but to understand their exact impact, further studies would be needed.

One group of peptidases that matched the protein degradation patterns observed in SDS-PAGE and was highly expressed in the

investigated samples were aspartic endopeptidases: saccharopepsin (ENOG503NW5J), and several other (ENOG503Q1KN, ENOG503Q1XC, ENOG503NV4S, ENOG503PD96, ENOG503Q1VW) (Fig. 2c). All of these genes belonging to different orthologous groups contain the A1 peptidase domain that is usually present in enzymes with extracellular destination; however, some of these enzymes may be related to intracellular processes such as protein turnover (Silva et al., 2023) and function as chaperones (Hulko et al., 2007). Several of the highly expressed enzymes contained both A1 peptidase domain and various ribosomal domains, indicating their intracellular function. On the other hand, there are numerous examples of extracellular aspartic peptidases synthesised by members of the *Mucor* genus, which are known as mucorpepsins or rhizopuspepsins (Herman et al., 2024). They can be used in cheese manufacturing as an alternative to animal rennet (Yegin et al., 2011). In particular, aspartic peptidases extracted from *Rhizomucor miehei* seem to be widely used for this purpose. Importantly, the *Mucor flavus* orthologues of ENOG503NV4S and ENOG503PD96 shared the FDTGSSD motif characteristic of microbial rennets (Fig. 3) (Yegin & Dekker, 2013). Especially ENOG503PD96 (FUN_001038) shows a high similarity to the microbial rennet extracted from closely related *Mucor mucedo* (Yegin et al., 2012) and other aspartic peptidases (Fig. 3). The observed pH of the meat (5.70-5.74) aligns with the pH in which the microbial aspartic proteases used in cheese making are usually active (Yegin & Dekker, 2013). One such enzyme, aspartyl protease from *Rhizomucor miehei*, had already been successfully employed in meat tenderisation. In the slightly acidic environment of meat, *Rhizomucor miehei* A1 peptidase outperformed commercially available papain, a peptidase commonly used for meat tenderisation (Sun et al., 2018). Endogenous enzymes naturally occurring in meat, such as Cathepsin D and E, classified as aspartic endopeptidases with a mechanism of activity similar to fungal aspartic peptidase, may also be responsible for the degradation of proteins such as titin, myosin, nebulin, actin, and tropomyosin, improving the meat's texture (Adamczak et al., 2011). Therefore, it is likely that aspartic endopeptidases synthesised by the fungus could be partially responsible for the proteolysis of beef proteins during the dry-ageing.

These findings suggest that aspartic endopeptidases produced by the fungus are one of the enzymes responsible for the differences in observed protein degradation patterns between the test and the control samples. The effect of low temperatures (~1.5 °C) present during dry-ageing on the efficiency of the proteases secreted by the KKP 2092p isolate remains to be studied.

4. Conclusions

Multi-omics approach, combining the comparison between protein degradation patterns and transcriptomic data, helped to better understand the influence of the *Mucor flavus* biostarter on the dry-ageing of meat and aided in preliminary identification of the enzymes relevant to this process. The presence of the fungus altered the profile of short-chain proteins present in meat, i.e. 113.9 kDa, 103.5 kDa, and 18.9 kDa levels increased. This difference can be attributed to the activity of endopeptidases which cleave the longer proteins into smaller products. Some of the genes coding for aspartic endopeptidases were highly expressed when the fungus was exposed to meat extract. Rennet-like enzymes seem to be good candidates for explaining observed proteolytic changes. Their activity is limited to specific cleavage sites in long-chain proteins, which can potentially improve meat's tenderness without compromising its integrity. The properties of these peptidases could be useful in low-temperature enzyme-assisted ageing of meat. The fungus itself also has several metabolic adaptations to protein-rich substrates, is non-pathogenic and capable of surviving in freezing temperatures, making it ideal not only for dry-ageing of meat but also for other similar biotechnological applications.

CRediT authorship contribution statement

Grzegorz Ostrowski: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Danuta Jaworska:** Writing – review & editing, Investigation, Formal analysis. **Natalia Kasałka-Czarna:** Writing – review & editing, Validation, Investigation. **Magdalena Montowska:** Writing – review & editing, Validation, Formal analysis. **Anna Muszewska:** Writing – review & editing, Writing – original draft, Validation, Software, Data curation, Conceptualization. **Magdalena Płecha:** Writing – review & editing, Investigation, Data curation. **Wiesław Przybylski:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition. **Krzysztof Sawicki:** Project administration, Funding acquisition. **Łukasz Słowik:** Writing – review & editing, Visualization, Software. **Julia Pawłowska:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial

Fig. 3. *Mucor* isolate KKP 2092p aspartic peptidase A1 compared to known rennet-like peptidases from Mucorales (Gama Salgado et al., 2013; Sun et al., 2018; Yamazaki et al., 1999; Yegin & Fernandez-Lahore, 2013). In orange are the sequences of the signal peptides, in light violet are the sequences corresponding to eukaryotic aspartyl proteases (PFAM PF00026), with saturated violet sections being pepsin (A1) aspartic protease family signatures (PRINTS PR00792) as assigned using InterPro database (Bateman et al., 2025). In blue are the motifs characteristic for aspartyl proteinases used as microbial rennets (Yegin & Dekker, 2013).

interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was partially supported by The National Centre for Research and Development: as part of the NCBR Tango-IV-C/0005/2019 project: "Biostarters development for dry aged beef production". We also acknowledge the help of Kamil Krakowski with genomic data analysis and Małgorzata Orłowska with her outlining key nuances of complex karyogamous states.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2026.119179.

Data availability

Data will be made available on request.

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