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Functional diversity of *Bisifusarium domesticum* and the newly described *Nectriaceae* cheese-associated species

Océane Savary[a], Emmanuel Coton[a], Marie-Bernadette Maillard[b], Frédéric Gaucheron[c], Christophe Le Meur[a], Jens Frisvad[d], Anne Thierry[b], Jean-Luc Jany[a] and Monika Coton[a*]

[a]*Univ Brest, INRAE, Laboratoire Universitaire de Biodiversité et Écologie Microbienne, F-29280 Plouzané, France*

[b]*INRAE, Institut Agro, STLO, F-35000 Rennes, France*

[c]*CNIEL, Maison du Lait, 75009 Paris, France*

[d]*Department of Biotechnology and Biomedecine, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark*

Running title: Functional diversity of cheese-associated *Nectriaceae* spp.

[*]Corresponding author: Dr. Monika COTON, monika.coton@univ-brest.fr

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ABSTRACT

Bisifusarium domesticum is among the main molds used during cheese-making for its "*anticollanti*" property that prevents the sticky smear defect of some cheeses. Previously, numerous cheese rinds were sampled to create a working collection and not only did we isolate *B. domesticum* but we observed a completely unexpected diversity of "*Fusarium*-like" fungi belonging to the *Nectriaceae* family. Four novel cheese-associated species belonging to two genera were described: *Bisifusarium allantoides*, *Bisifusarium penicilloides*, *Longinectria lagenoides,* and *Longinectria verticilliformis*. In this study, we thus aimed at determining their potential functional impact during cheese-making by evaluating their lipolytic and proteolytic activities as well as their capacity to produce volatile (HS-Trap GC-MS) and non-volatile secondary metabolites (HPLC & LC-Q-TOF). While all isolates were proteolytic and lipolytic, higher activities were observed at 12°C for several *B. domesticum*, *B. penicilloides* and *L. lagenoides* isolates, which is in agreement with typical cheese ripening conditions. Using volatilomics, we identified multiple cheese-related compounds, especially ketones and alcohols. *B. domesticum* and *B. penicilloides* isolates showed higher aromatic potential although compounds of interest were also produced by *B. allantoides* and *L. lagenoides*. These species were also lipid producers. Finally, an untargeted extrolite analysis suggested a safety status of these strains as no known mycotoxins were produced and revealed the production of potential novel secondary metabolites. Biopreservation tests performed with *B. domesticum* suggested that it may be an interesting candidate for biopreservation applications in the cheese industry in the future.

Keywords: *Nectriaceae*, mould, proteolysis, lipolysis, volatilome, metabolites, bioprotection

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1. Introduction

With more than 1 500 varieties worldwide, exhibiting a multitude of flavors, odors, textures and colors (Fox et al., 2017), cheese is one of the most popular and consumed fermented foods. Cheese is also of particular interest for food microbiologists as it contains a highly diversified microbiota, ever evolving during fermentation but also between the cheese core and surface (Penland et al., 2021; Wolfe et al., 2014). Microbial ecology of cheese, in which many bacterial and fungal species intervene, has been the subject of a large number of studies (e.g. Dugat-Bony et al., 2016; Hermet et al., 2014; Marino et al., 2019; Penland et al., 2021; Wolfe et al., 2014; Yeluri Jonnala et al., 2018). In the case of fungi, not only can they positively impact cheese quality but some may act as cheese spoilers. The main spoilers belong to the *Penicillium* and *Aspergillis* genera (Pitt and Hocking, 2009) although others (e.g. *Mucor* spp., *Geotrichum candidum*) can also impact cheese organoleptic qualities. Regarding the fungal species which are frequently used by cheesemakers or isolated from cheese, one can cite the well-known domesticated species *Penicillium roqueforti* for blue-veined cheeses (e.g. Roquefort or Gorgonzola) (Coton et al., 2020) and *P. camemberti* in the case of bloomy rind cheeses (e.g. Camembert and Brie) (Dupont et al., 2017). Both species have been extensively studied in terms of genotypes and phenotypes (Caron et al., 2021; Dumas et al., 2020; Ropars et al., 2020, Gillot et al., 2017) and are of particular interest as intraspecific functional variability has been observed among strains belonging to the different genetic populations. This can also be linked to how they potentially influence the organoleptic qualities of the final product and thus used for future strain selection.

Among the key fungal metabolic activities in cheese, proteolysis and lipolysis are important as cheese aspect and texture can be modified but they also contribute to

flavor development by releasing metabolites or precursors (e.g. free amino acids, peptides and free fatty acids) involved in volatile compound production (McSweeney, 2017; Spinnler, 2017; Thierry et al., 2017). Another aspect is the ability of these fungi to produce extrolites (i.e. excreted secondary metabolites), or secondary metabolites, including mycotoxins, which can raise questions about strain safety status. Understandably so, these traits have already been studied in some cheese-related fungal species, especially *P. roqueforti* and *P. camemberti.* For *P. roqueforti*, various phenotypic traits (e.g. lipolysis, proteolysis, color, volatile compounds, extrolite production, growth on bread, cheese, salt tolerance) were shown to be linked to strains belonging to the described genetic populations (Caron et al., 2021; Dumas et al., 2020; Gillot et al. 2017). All of these traits can have a strong influence on blue-veined cheese organoleptic qualities (Caron et al., 2021; Martín and Coton, 2017). The same thing can be said for *P. camemberti*, a species presenting less genetic diversity, for which two varieties (*P. camemberti* var. ‘‘*camemberti*’’ and var. ‘‘*caseifulvum*’’) exhibiting contrasted phenotypic features in terms of color, growth, extrolite production, and competitive ability were identified (Ropars et al., 2020). Beyond the two mentioned emblematic *Penicillium* species, other mold species are also of interest during cheese-making but have not benefited from the same extensive research. This is the case for *Mucor lanceolatus* and *M*. *fuscus*, associated with Tomme de Savoie and Saint-Nectaire (Hermet et al., 2012), *Sporendonema casei,* associated with Cantal and Salers (Ropars et al., 2012), but also *Bisifusarium domesticum.* Interestingly, the latter species, which belongs to the *Nectriaceae* family, is used daily by cheesemakers as a commercially available ripening culture, especially for its ability to prevent surface stickiness (“*anticollanti*”) of some smear-ripened cheeses (e.g. Raclette, Tilsit) (Bachmann et al., 2003; 2005); but also for the typical moldy rind aspect it creates on

various other cheese surfaces (e.g. Saint-Nectaire, Reblochon). *B. domesticum* was originally identified as *Trichothecium domesticum*, then described as *Fusarium domesticum* by Bachman et al. (2003; 2005). Recently, we observed an unexpected diversity of *Nectriaceae* species from diverse cheeses and four novel cheese-associated species were described, namely *Bisifusarium allantoides and Bisifusarium penicilloides, Longinectria lagenoides,* and *Longinectria verticilliformis* (Savary et al., 2021). An in-depth physiological study was then performed on these species and highlighted distinct growth patterns suggesting they are well adapted to the cheese environment (Savary et al., 2022). However, to date there is still a major gap in knowledge to fully understand the role of these species in cheese.

In this context, the aim of this study was to evaluate the functional diversity of several isolates belonging to all five cheese-associated *Nectriaceae* spp. by investigating their lipolytic and proteolytic activities, volatilome from cultures on a cheese-mimicking medium and Raclette de Savoie cheese and their ability to produce secondary metabolites. We also evaluated their potential antimicrobial properties for use as bioprotective cultures in the future.

2. Materials and methods

2.1. Nectriaceae *isolate selection*

For *B. allantoides* (n=3)*, B. domesticum* (n=5), *B. penicilloides* (n=3), *L. lagenoides* (n=4) and *L. verticilliformis* (n=1), isolates were selected based on their availability in culture collections, except for *B. domesticum* isolates that were also selected based on Random Amplified Polymorphic DNA (RAPD) profiles (Savary et al., 2022) (Table 1). All the specimens studied originated from monosporal cultures.

2.2. Conidia production

For conidia production, each fungal isolate was pre-cultured for ten days at 25°C on Potato dextrose agar (PDA, Difco, Fisher Scientific), then two mL of Tween 80 (0.015 %, v/v) were added on the surface of each culture to collect a conidia suspension. Conidia were counted in Malassez cells and suspensions adjusted to 10^7 conidia/mL and conserved in 10 % (v/v) glycerol at -80°C until use.

2.3. Lipolytic and proteolytic activities investigation

Lipolytic and proteolytic activities were determined based on methods described by Ozturkoglu-Budak et al. (2016) and Dumas et al. (2020). Briefly, 10 mL tributyrin agar (5 g/L peptone, 3 g/L yeast extract, 15 g/L agar, 10 mL/L tributyrin - ACROS, Organics -) and skim milk agar (100 g/L skim milk - Difco, Fisher Scientific -, 20 g/L agar and 10 g/L yeast extract) were prepared in glass tubes. Skim milk agar was prepared by first autoclaving the skim milk separately at 104°C for ten min, while the other components were autoclaved at 121°C for 15 min. The two parts were then homogenized for one min using an Ultraturrax (IKA, Heidelberg, Germany) and distributed into sterile glass tubes (10 mL/tube).

All sixteen isolates were studied as well as *P. roqueforti* FM164 (MNHN collection strain), as a positive control. Spores suspensions were adjusted to 10^5 conidia/mL in Tween 80 (0.015 % v/v) and 25 µL were inoculated on the medium surface (or Tween 80 - 0.015% v/v - for negative control) and incubated at 12°C and 25°C to represent typically cheese ripening and optimal growth conditions, respectively. Three replicates were performed for each condition. Lipolytic and proteolytic activities were then monitored every two to three days, during a minimum of 24 days and up to 40 days,

by measuring the clear zone that formed just under the fungal culture. Surface growth was not determined although visual inspection was done to ensure replicates were the same. Statistical analyses for testing differences in lipolysis and proteolysis activities between isolates, culture temperatures and their interactions were performed using the R software (version 4.2.2, https://www.r-project.org/). Given that assumptions of normality and homoscedasticity of residuals, as tested using Shapiro-Wilk and Bartlett tests, were rejected, an aligned Rank transform procedure for non-parametric factorial analyses was applied on the dataset prior to ANOVA and contrast post-hoc tests using the ARTool package (Wobbrock et al., 2011; Elkin et al., 2021).

2.4. Volatile profiling by headspace gas chromatography-mass spectrometry.

For volatile profiling, all isolates (n=16) were spread (100 µL of 10^5 conidia/mL suspensions or 0.015 % (v/v) Tween 80 as a negative control) onto cheese agar (pH 4.7 ± 0.01 adjusted with lactic acid) prepared as described by Morin-Sardin et al. (2016) with the following modifications: no HCl addition and 3 mL lactic acid instead of 5.6 mL. Additionally, for the *B. domesticum* isolates, calibrated conidia suspensions (100 µL of 10^5 conidia/mL suspensions) were also spread onto industrial unripened Raclette de Savoie cheese (~pH 5.4) slices prepared using the same procedure described by Savary et al. (2022) in triplicates and incubated at 12°C for 25 days. After incubation, 2.5 ± 0.02 g samples were transferred into 22 mL Perkin Elmer vials sealed with polytetrafluoroethylene/silicone septa and stored at −80 °C until analysis. Volatile compounds were extracted by headspace (HS) trap coupled to gas chromatography-mass spectrometry (GC-MS) using a Perkin Elmer Turbomatrix HS-40 trap automatic headspace sampler with trap enrichment and a Clarus 680 gas chromatograph coupled to a Clarus 600 T quadrupole mass spectrometer (PerkinElmer, France), as

previously described (Pogačić et al., 2015), with modifications of the chromatographic conditions according to Harlé et al. (2020). Samples were injected in a random order. Volatile compounds were identified by comparing their retention index (RI) and mass spectra from the NIST 2008 Mass Spectral Library (Scientific Instrument Services, Ringoes, NJ, United States), and with those reported in the literature but also, when available, with standards (Sigma Aldrich, France) analyzed in the same conditions.

Data were analyzed using R software (R Core Team, 2019). A principal component analysis (PCA) was first performed to identify the mains trends and ensure that no aberrant samples were observed (one out of three replicates of the *B. domesticum* MNHN-RF-05627 isolate on Raclette de Savoie was removed due to a different macroscopic aspect). To highlight volatile differences between isolates, analysis of variance (ANOVA) and mean comparison were performed on $\log_{10}$-transformed data , to identify compounds that significantly increased or decreased in abundance compared to the negative control. Then, data were centered and scaled by compound and hierarchically clustered by Ward's minimum variance method, and Euclidean distance metric with the *hclust* R function before being plotted by the *heatmap.2* function of R *ggplots* package.

2.5. Metabolite profiling by liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-Q-TOF)

All studied isolates were inoculated in three points using 5 µL of 10^6 conidia/mL suspensions onto Yeast Extract Sucrose Agar (YES, pH 5.2) and skim milk agar plates (two replicates), and incubated for fourteen days at 25°C. Then, five six mm plugs were removed from the agar colonies for extraction with ethyl acetate / isopropanol (3:1)

containing 1 % (v/v) formic acid using ultra-sonication (Elmasonic S30H, Elma Schmidbauer, Germany) for 60 min. Extracts were then resuspended in 300 µL methanol and sonicated for 20 min before being filtered (0.45 µm, PTFE) into amber vials and conserved at -20°C until analysis. Each extract was analyzed by injecting five µL into a HPLC 1260 coupled to a 6530 Accurate-Mass quadrupole-time-of flight mass spectrometry Q-TOF (Agilent Technologies, Santa Clara, CA) equipped with a dual electrospray ionization source (Agilent Technologies, Santa Clara, CA). Molecules were separated using a ZORBAX Extend-C18 column (2.1 x 50 mm and 1.8 µm) equipped with a guard column and analytes were ionized in positive electrospray ionization (ESI+) mode in a scan range of 50 to 1700 m/z and 2 scan/s. The column was maintained at 35°C with a flow rate set to 0.2 mL/min. Mobile phases were as follows: LC-MS grade water containing 0.1% formic acid (solvent A) and methanol containing 0.1 % formic acid (solvent B) (Carlo Erba Reagents, France). Solvent B was maintained at 10 % for the first 4 min followed by an increase from 10 to 100 % for 15 min before maintaining solvent B at 100% for 7 min. Samples were maintained at 10°C in a well plate autosampler until injection and the total run time was 22 min followed by 5 min post-time to wash and re-equilibrate the column before the next injection. The mass spectrometer conditions were as follows: capillary voltage 4000 V, source temperature 325 °C, nebulizer pressure 50 psig, drying gas 12 L/min.

The secondary metabolites were identified using the Mass Hunter analytical software applying the METLIN-AMRT-PCD database to obtain presumptive identifications (based on best hits) and generate putative formulas. Then, the Mass Profiler software was applied to compare the profiles between the inoculated samples and the negative control, the compounds only found in inoculated samples were retained and so considered as produced by the isolate.

Additionally, fatty acid profiling was also performed for the type strains of *B. allantoides*, *B. domesticum*, *B. penicilloides*, *L. lagenoides* and *L. verticilliformis*. To do so, additional extracts (three replicates) were prepared as described above, after 14 days growth on skim milk and YES agar media at 25°C. These extracts were then analyzed by the LIPIDOCEAN platform (Brest University, France) as described by Sardenne et al. (2021). Statistical analyses for testing differences in total fatty acid production between species, culture temperatures and their interactions were performed using the R software (version 4.2.2, https://www.r-project.org/). Given that assumptions of normality and homoscedasticity of residuals, as tested using Sahpiro-Wilk and Bartlett tests, were not rejected, a two-way ANOVA was performed.

2.6. Evaluation of B. domesticum *bioprotective potential*

The biopreservation potential was first evaluated for three *B. domesticum* isolates (MNHN-RF-05627, UBOCC-A-120001, UBOCC-A-120004) against the growth of *P. biforme* (Ei5, Ei18, UBOCC-A-112059) and *P. roqueforti* (FM164, LCP00146, LCP04180) isolates. To do so, 100 µL of a *B. domesticum* spore suspension, adjusted to 10^6 conidia/mL, were spread onto the surface of PDA or cheese agar plates and incubated at 25°C. After one or four days incubation, the target isolates were inoculated using a 2 µL spore suspension adjusted to 10^5 conidia/mL directly onto the *B. domesticum* cultures (or uninoculated medium for controls) in three points, and in triplicate. Radial growth of each target isolate was then monitored for ten days. Given that assumptions of normality and homoscedasticity of residuals, as tested using Shapiro-Wilk and Bartlett tests, were rejected, statistical analyses for testing differences in radial growth between the different bioprotective assays (*i.e.* a

Penicillium sp. strain grown on a given cultured medium inoculated with a given *B. domecticum* strain during a given culture time) was achieved using Kruskall-Wallis tests followed by Dunn tests as post-hoc tests with the R software (version 4.2.2, https://www.r-project.org/). Tests to evaluate the impact of the culture medium, culture time and *B. domesticum* isolates as well as their interactions were performed using Rank transform procedures for non-parametric factorial analyses prior to ANOVA using the ARTool package (Wobbrock et al., 2011).

Secondly, the *B. domesticum* isolate exhibiting the highest activity was selected to further investigate its antifungal ability with a cell-free extract. To do so, fermentations were carried out in 250 mL Erlenmeyer flasks with baffles containing either 100 mL of skim milk (SM) broth or yeast extract sucrose (YES, pH 5.2) broth. Each flask (two replicates) was inoculated with 40 µL suspension adjusted to 10^6 conidia/mL (or Tween 80 for the negative control) and incubated at 25°C for 10 days with agitation (180 rpm). After growth, cultures were first centrifuged at 7000 g for ten min to remove fungal mycelium, then prefiltered under vacuum using 2 µm glass fiber prefilters (Merck, Milipore, Germany) before filtration using 0.45 µm nitrocellulose filters (Merck, Milipore, Germany). Cell-free supernatants (CFS) were collected and stored at 4°C overnight before their inclusion without pH adjustment (25% v/v) in PDA media (60 mm Petri dishes). Finally, 2 µL inoculum of a spore suspension adjusted to 10^5 conidia/mL of both *P. biforme* and *P. roqueforti* isolates were used and radial growth was measured over two predetermined perpendicular directions after five days incubation at 25°C.

3. Results

3.1. Lipolytic and proteolytic activity

For proteolytic and lipolytic activities, all strains showed clearing zones below the surface mycelium at both temperatures and for both activities (Figure S1). For lipolytic activity on tributyrin agar after 24 days at 12°C and 25°C (Figure 1A), clearing zone measurements varied from 3.0 to 10.3 mm and 4.0 to 13.0 mm, respectively. Statistical tests indicated that temperature ($P=1.05x10^{-12}$) isolate ($P=1.86x10^{-4}$) and their interaction ($P=3.92x10^{-5}$) impact proteolysis activity revealing significant differences among the different lipolysis assays (Figure 1A). In comparison to *P. roqueforti,* used as a control, all the tested *Nectriaceae* strains showed smaller clearing zones at 25°C and only three *Nectriaceae* strains (UBOCC-A-120036, UBOCC-A-120004 and ESE 00140) did not show significantly smaller clearing zones at 12°C than *P. roqueforti*. Interestingly, although growth was clearly slower at the lowest temperature, clearing zones observed at 12°C were significantly larger than those at 25°C for only two isolates pertaining to the species *B. domesticum* (MNHN-RF-05627, 6.0 ± 0.0 vs. 4.0 ± 0.0 mm at 12°C and 25°C, respectively and UBOCC-A-120001, 6.7 ± 0.6 vs. 5.0 ± 0.0 mm) (Figure 1A). Conversely, a significantly higher activity was observed at 25°C for one out of five *B. domesticum* isolates (UBOCC-A-120004, 3.0 ± 0.0 vs. 8.3 ± 0.6 mm at 12°C and 25°C, respectively), one out of three *B. allantoides* isolates (UBOCC-A-120035, 6.3 ± 1.5 vs. 9.3 ± 0.3 mm), one out of four *L. lagenoides* isolates (UBOCC-A-120039, 5.0 ± 1.7 vs. 8.3 ± 2.1 mm) and *L. verticilliformis* UBOCC-A-120043 (3.0 ± 0.0 vs. 6.8 ± 0.8 mm). The remaining isolates showed similar results at 12°C and 25°C (Figure 1A).

For proteolytic activities, casein degradation in skim milk agar according to temperature was even more contrasted between isolates (Figure S1, 1B). As for lipolysis, statistical tests indicated that temperature (P=6.07.10-7) isolate (P<2.22.10-

16) and their interaction (P<2.22.10-16) impact proteolysis activity revealing significant differences among the different lipolysis assays (Figure 1B). Indeed, despite reduced growths, clearing zones were significantly larger at 12°C than 25°C for several isolates belonging to *B. domesticum* (MNHN-RF-05627, 19.0 ± 0.0 vs 13.7 ± 0.6 mm, UBOCC-A-120002, 15.3 ± 0.6 vs. 10.3 ± 0.6 mm and UBOCC-A-120003 18.3 ± 0.6 vs. 13.0 ± 1.0 mm), *B. penicilloides* (VTT-D-041022, 17.7 ± 2.3 vs. 10.0 ± 1.0 mm, UBOCC-A-120021, 15.7 ± 0.6 vs. 6.8 ± 0.8 mm and UBOCC-A-120034, 16.3 ± 1.2 vs. 6.7 ± 1.2 mm) and *L. lagenoides* (ESE00140, 13.2 ± 0.3 vs. 10.0 ± 0.0 mm) (Figure 1B). Conversely, significantly larger clearing zones were observed at 25°C for *B. domesticum* UBOCC-A-120001 (17.3 ± 1.2 vs. 21.0 ± 0.0 mm at 12°C and 25°C respectively) and *L. verticilliformis* UBOCC-A-120043 (5.3 ± 0.6 vs. 11.3 ± 0.6 mm)(Figure 1B). Moreover, at 25°C, low proteolytic activity was observed for *B. domesticum* UBOCC-A-120004 (5.7 ± 0.6 mm) while no clearing zone was observed at 12°C after 25 days incubation and only 3.3 ± 0.6 mm after 40 days while this value reached 28.7 ± 0.6 mm for *B. domesticum* MNHN-RF-05627 after 40 days (data not shown). Noteworthy, clearing zones obtained for *P. roqueforti* on skim milk agar were much smaller (4.3 ± 0.6 mm after 24 days at 25°C) than the other tested strains despite intense mycelium growth on the medium surface. Moreover, regarding the clearing zone observed at 12°C, the lowest activity was noted for *L. verticilliformis* (5.3 ± 0.6 mm) while for some *B. allantoides*, *B. domesticum, B. penicilloides* and *L. lagenoides* isolates, the clearing zone reached up to 17.0, 19.0, 17.7 and 15.3 mm, respectively.

3.2. Volatile profiling

A total of 68 volatile compounds were detected and identified by headspace GC-MS in all cheese agar (for all isolates) and Raclette de Savoie samples (only for *B.*

domesticum isolates), including twenty-two ketones, twelve alcohols, nine aldehydes, seven esters, seven fatty acids, four carboxylic acids, three sulfur compounds, two phenols, one pyrazine and one styrene (Table S1). An ANOVA performed on all samples, including the negative controls, showed that among these compounds, 49 and 26 were significantly impacted by fungal growth (p value ≤ 0.01) on cheese agar and Raclette de Savoie, respectively (Table S1). ANOVA results identified which compounds were significantly more abundant (e.g. heptan-2-ol, decan-2-one, nonan-2-one on cheese agar samples) or less abundant (e.g. ethanol, benzaldehyde, 1-hydroxypropan-2-one) for the different fungal isolates when compared to the negative controls (non inoculated medium or cheese) and so considered as produced or not by the isolate (Table S1).

PCA and heatmap analyses indicated that replicates of a given isolate were well grouped together, except for a few cases such as *B. domesticum* UBOCC-A-120001 or UBOCC-A-120003 replicates on cheese agar or Raclette cheese (Figures 2 and 3, Figure S2). It was globally also the case for isolates belonging to the same species, although intraspecific variability was observed. First, at the species level, *B. domesticum* and *B. penicilloides* produced more volatile compounds with respectively thirteen to nineteen (except four for UBOCC-A-120001) and ten to eighteen volatile compounds, while only six to seven were observed for *B. allantoides,* and nine to thirteen for *L. lagenoides* (Figure 4). Conversely, *L. verticilliformis* produced only one compound, namely oct-1-en-3-ol. Volatile compounds produced by the different isolates corresponded mainly to alcohols and ketones for all species, while a few esters and phenols were also produced by *B. domesticum* and *B. penicilloides* and/or *B. allantoides,* and aldehydes by *L. lagenoides*. Moreover, while only oct-1-en-3-ol was produced by the five species, three compounds were produced by all four other

species, namely 3-methylbutan-1-ol, 3-methylpentan-2-one, and heptan-4-one (Figure 4). Other compounds were common to two or three species. As for *B. domesticum*, *B. penicilloides* and *L. lagenoides,* seven compounds were systematically produced, (e.g. heptan-2-ol, nonan-2-one and undecan-2-one), and without significant differences in abundances (Figure 4). Six other compounds were produced only by *B. domesticum* and *B. penicilloides* isolates, including heptyl acetate, heptan-2-one and pentan-2-one (Figure 4). Finally undecan-2-ol was only produced by *Bisifusarium* spp., 4-methylphenol by *B. allantoides* and *B. domesticum,* and octan-3-one by *B. allantoides* and *L. lagenoides.* Conversely, other compounds were specific to one species such as propan-2-one for *B. penicilloides,* (E)-dec-7-en-2-one for *B. domesticum* and non-3-en-1-ol, (E)-non-2-enal, (E)-oct-2-enal and oct-1-en-3-one for *L. lagenoides* (Figure 4).

At the intraspecific level, variations in volatile profiles according to the considered isolate were also observed (Figure S3). This was particularly the case among *B. domesticum* isolates, as UBOCC-A-120001 produced less compounds (n=5), namely 3-methylpentan-2-one, heptan-4-one, oct-1-en-3-ol, 4-methylphenol (although they were also produced by other *B. domesticum* isolates but with no quantitatively significant differences) and 3-methylbutan-1-ol, specific to this isolate. Noteworthy, the latter compound was also produced by *B. allantoides* UBOCC-A-120035 and UBOCC-A-120037, *B. penicilloides* VTT-D-041022, and *L. lagenoides* ESE00140 with no differences in abundance between these isolates. Still, for *B. domesticum*, it could be noted that with the exception of the UBOCC-A-120001 isolate, ten compounds (e.g. heptan-2-ol, nonan-2-ol, nonan-2-one, undecan-2-ol and undecan-2-one) were produced by all *B. domesticum* isolates (Figure S3). Moreover, MNHN-RF-05627 also exhibited a different volatilome from a quantitative point of view, as several compounds (e.g. nonan-2-ol, heptyl acetate, undecan-2-ol and (Z)-oct-3-en-2-ol) were produced at

significantly higher concentrations when compared to the other *B. domesticum* isolates.

Concerning *B. allantoides*, a lower number of volatile compounds were produced by the three strains (e.g. heptan-4-one, oct-1-en-3-ol, octan-3-one, 3-methylpentan-2-one and 4-methylphenol) while undecan-2-ol and 3-methylbutanol were not produced by UBOCC-A-120035 and UBOCC-A-120036, respectively (Figure S3). Beyond these qualitative differences, no significant differences in abundances were observed between the three *B. allantoides* isolates. Concerning *B. penicilloides*, nine compounds were common to the three isolates (e.g. undecan-2-ol, nonan-2-ol, heptan-2-one) (Figure S3), while six compounds (e.g. decan-2-one, propan-2-one, undecan-2-one) were produced by UBOCC-A-120034 and VTT-D-041022 but not by the third isolate (i.e. UBOCC-A-120021) (Figure S3). Fewer compounds and no production of specific compounds was observed for the latter isolate. Overall, no significant abundance differences were noted among isolates, except for heptan-2-ol, propan-2-one and 3-methylpentan-2 for which higher abundances were observed for VTT-D-041022. For *L. lagenoides*, five compounds were common to the four strains (i.e. 3-methylpentan-2-one, oct-1-en-3-ol, (E)-non-2-enal, (E)-oct-2-enal and (Z)-oct-3-en-2-ol), (Figure S3) while two compounds were produced by three out of four isolates (i.e. nonan-2-one and oct-1-en-3-one not produced by UBOCC-A-120039 and UBOCC-A-1200038, respectively). Others were produced by two out of four isolates or specific to a given isolate (Figure S3). However, among these productions, no significant quantitative differences were noted between *L. lagenoides* isolates.

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Regarding volatile profiling on Raclette de Savoie, which were only performed for the five selected *B. domesticum* isolates, ten to fifteen compounds were produced, including seven compounds (e.g. 3-methylpentan-2-one, furan-2-carbaldehyde, oct-1-en-3-ol and octan-3-one) common to all isolates (Figure S4). Otherwise, only a few qualitative differences could be noted. Indeed, non-8-en-2-one and nonan-2-one were produced by all isolates except UBOCC-A-120001, while undecan-2-one and propan-2-one were not produced by UBOCC-A-120003 and UBOCC-A-120004, respectively. Some compounds were also specific to a given isolate (i.e. (E)-oct-2-enal for UBOCC-A-120004 or heptan-2-ol, nonan-2-ol, heptan-2-one for MNHN-RF-05627) (Figure S4). Among the common compounds produced, only a few quantitative differences were observed. For instance, while oct-1-en-3-ol and 4-methylphenol were produced by all isolates, the former was detected at a lower abundance for MNHN-RF-05627 and the latter at a higher abundance for UBOCC-A-120002 and UBOCC-A-120004. Conversely, for other compounds such as undecan-2-one, nonan-2-one and non-8-en-2-one, no significant differences were noted.

When comparing *B. domesticum* volatilomes on cheese agar and Raclette de Savoie, it could be noted that even if several compounds were produced on both media by some isolates (e.g. oct-1-en-3-ol, heptan-4-one undecan-2-one, nonan-2-one, non-8-en-2-one), others were only produced on Raclette de Savoie cheese (e.g. furan-2-carbaldehyde, octan-3-one, propan-2-one) (Figure S5). Noteworthy, for UBOCC-A-120001, the *B. domesticum* isolate which produced only four compounds on cheese agar, a distinct volatilome with greater production of volatile compounds was observed on Raclette de Savoie (Figure S5). MNHN-RF-5627 was the only isolate to produce heptan-2-ol, nonan-2-ol and heptan-2-one on Raclette de Savoie while on cheese agar

several isolates produced them. Finally, among the compounds produced on both media, only few quantitative differences were noted, which could be linked either to a matrix effect or to isolate activity, or both. Indeed, the same compounds could be found with different abundances on both media due to better production on one but this may also be due to a better extraction.

3.3. Secondary metabolite production

Secondary metabolite profiles were assessed for the five species by LC-MS/Q-TOF. Compounds (putative identifications) that were identified in more than 50 % of all replicates (which included at least 2 replicates per triplicate for a given isolate) for a given species were considered as part of the core metabolome and results are presented in Figure 5 and Figure S6. For skim milk agar cultures, alpha linolenic acid and enigmol (putative identifications) were produced by all species and isolates while 63, 40, 72, 82 and 140 compounds were produced by *B. allantoides*, *B. domesticum*, *B. penicilloides*, *L. lagenoides* and *L. verticilliformis,* respectively (Figure 5A). Among them, several compounds were unique to one species (25, 14, 32, 36 and 87 compounds, respectively) and the database proposed several putative identifications although confirmations are needed. As for YES agar cultures, linoleic acid, enigmol, phytosphingosine and phytosphingosine-1-P (external database identifications) were produced by all isolates regardless of the species, while 34, 22, 55, 30 and 65 compounds were produced by *B. allantoides*, *B. domesticum*, *B. penicilloides*, *L. lagenoides* and *L. verticilliformis,* respectively with 10, 11, 26, 13 and 48 compounds produced only by one of these respective species (Figure 5B). When comparing both media used for secondary metabolite profiling, more compounds were produced on skim milk agar compared to YES agar for the five species (Figure S6). However, it

could be noted, for some species, that several compounds (on average 11%) would be produced on both media. Fatty acid profiling was also carried out for each neotype strain per species from YES and skim milk agar cultures compared to the medium without culture. The obtained profiles (Figure 6) did not reveal significant differences in production neither between both media ($P>0.05$) nor between *Nectriaceae* species ($P>0.05$). For all five species, the main fatty acids were palmitic (9 to 30 µg/mg), stearic (4 to 12 µg/mg), oleic (4 to 35 µg/mg), linoleic (10 to 22 µg/mg) and linolenic (0.4 to 3.9 µg/mg) acids.

3.4. Bioprotective activities of B. domesticum *cultures*

The potential bioprotective activities of one and four days *B. domesticum* cultures were tested against two fungal species that commonly contaminate dairy products, namely *P. biforme* and *P. roqueforti*.

Overall, a decrease in radial growth of isolates of both *Penicillium* species was observed in the presence of *B. domesticum* cultures, regardless of culture time and medium, when compared to the control condition. Indeed, statistical tests indicated a significant lower growth for the majority of the bioprotective assays (47 of 72) compared to the control (Figure 7) especially for four-day *B. domesticum* inoculations (30 of 36) and in CA medium. *Bisifusarium domesticum* culture time and culture medium impacted the bioprotective effect as confirmed by statistical tests (data not shown). For *P. biforme*, with one day *B. domesticum* inoculations, radial growth of all three isolates was decreased by 61–100 % and 13–100 % on PDA and cheese agar media, respectively, depending on the considered *B. domesticum* isolate. *B. domesticum* UBOCC-A-120004 had less impact on *P. biforme* growth than the other *B. domesticum* isolates and reduced the radial growth by 63–75 % and 13–90 % on PDA and cheese

agar, respectively, while *B. domesticum* MNHN-RF-05627 or UBOCC-A-120001 reduced growth by 83–100 % and 73–99 % on PDA and by 73–99 % and 83–100 % on cheese agar, respectively.

For *P. roqueforti*, in the presence of one day old *B. domesticum* cultures, radial growth of all three isolates was decreased by 20–94 % and 48–93 % on PDA and cheese agar, respectively. Important differences were observed between *B. domesticum* isolates, especially *B. domesticum* UBOCC-A-120001 that decreased *P. roqueforti* radial growth by only 20–32 % and 53–83 % on PDA and cheese agar, respectively, while MNHN-RF-05627 decreased growth by 68–94 % and 85–93 % on PDA and cheese agar, respectively. However, for the former, this decrease was higher with four days old cultures, in particular on cheese agar medium (i.e. 82–97 % on cheese agar vs. 36–54 % on PDA) while for MNHN-RF-05627 and UBOCC-A-120004, growth was totally inhibited.

Regarding the tests performed with PDA medium supplemented with *B. domesticum* MNHN-RF-05627 YES or SM CFS), differences in antifungal activities were observed depending on the considered medium and fungal target (Figures S7A and B, S8, S9). First, for *P. biforme* isolates, no effect of the *B. domesticum* supernatant prepared from YES cultures was observed while those prepared from SM inhibited growth for *P. biforme* Ei18 and decreased growth for UBOCC-A-112059. For *P. roqueforti* isolates, YES-based supernatants did not inhibit growth. However, an impact on all three *P. roqueforti* strains growth was observed for PDA supplemented with *B. domesticum* SM CFS. Their radial growth was reduced by at least 50 % and no conidia were produced (absence of blue-green color, only white mycelium was observed) compared to the control (Figure S9).

4. Discussion

The involvement of bacteria and fungi via the action of several metabolic pathways, including lipolysis and proteolysis, during cheese-making has been well established (Ardö et al., 2017; McSweeney, 2017; Spinnler, 2017; Thierry et al., 2017). For fungi, while these aspects have been largely studied for some of the main cheese species (e.g. *P. roqueforti*) (Caron et al., 2021; Dumas et al., 2020; Gillot et al., 2017), for others, further knowledge is still needed. This is particularly the case for technological cheese species *B. domesticum* as well as the four recently described *B. allantoides*, *B. penicilloides, L. lagenoides,* and *L. verticilliformis* species (Savary et al., 2021). In fact, no study has yet been published to decipher their potential roles during cheese-making or determine whether they are simply contaminants. In this context, we focused on studying these species in relation to their relevant functional traits for cheese-making, namely proteolytic and lipolytic activities as well as fatty acid, volatile and extrolite production profiles as well as biopreservation potential.

First, lipolytic and proteolytic activities were determined as these are key reactions occurring during cheese production and strongly participate to the final cheese characteristics (e.g. aspect, texture but also by releasing aroma compounds and precursors) (Ardö et al., 2017; Martín and Coton, 2017; McSweeney, 2017; Thierry et al., 2017). All isolates were confirmed to be proteolytic and lipolytic, however, within each species, isolate-dependent activities were observed. For lipolytic activity, lower intraspecific variability among isolates was observed although a temperature effect was seen. Interestingly, at 12°C, despite reduced growth, higher activity was noted only for two isolates *(*MNHN-RF-05627 and UBOCC-A-120001) pertaining to the species *B. domesticum*. This is of particular interest as cheese ripening is performed

at similar temperatures, suggesting these isolates are well adapted to these conditions. However, compared to the *P. roqueforti* control included in this study, the tested *Nectriaceae* species exhibited lower lipolytic activity so the relevance of this activity during cheese-making is of interest to study further. The same observations were made for proteolytic activities for some isolates as they were clearly more proteolytic at 12°C than 25°C, despite slower growth. Some intraspecific variability was also observed as one *B. domesticum* isolate had low proteolytic activity at 25°C and no clearing zone was observed at 12°C after 25 days. Interestingly, this isolate (UBOCC-A-120004) was also shown to have a distinct genetic profile (based on RAPD), be less tolerant to salt as well as low temperatures (5°C) in a previous study (Savary et al, 2022). In comparison to *P. roqueforti,* used as a control as well known for both its intense lipolytic and proteolytic activities (Caron et al., 2021; Dumas et al., 2020; Gillot et al., 2017), lower proteolytic activities were observed at 12°C while at 25°C, a similar activity was observed with several isolates.

Volatilomes of *Nectriaceae* isolates were then determined after growth on a cheese-mimicking medium, but also on Raclette de Savoie cheese for five *B. domesticum* isolates to determine the potential impact of this technological cheese species on a cheese matrix. Among the fungal cultures, distinct volatile profiles were distinguished, mainly grouping isolates of the same species together. Based on cheese agar results, *B. domesticum* and *B. penicilloides* were qualitatively differentiated from the other species as they produced more volatile compounds. Conversely, *L. verticilliformis* produced only one compound in the tested conditions. The former compound corresponded to oct-1-en-3-ol, produced by all isolates from the five species, except *B. penicilloides* UBOCC-A-120021, and is well known to have a fresh mushroom, earthy or oily odor (Frisvad, 2014; Spinnler, 2017). This compound is frequently

produced by many fungal species (Elmassry et al., 2020) and in relation to cheese, *P. camemberti* is a well known producer and this compound is derived from the degradation of unsaturated fatty acids (Thierry et al., 2017). Oct-1-en-3-ol is also known to contribute to the characteristic Camembert aroma (Ganesan and Weimer, 2017; Molimard and Spinnler, 1996) thus the studied *Nectriaceae* isolates may also impact overall cheese aroma. Some specific compounds with interesting odor descriptors for cheese-making were also produced by the studied other species. Regarding the *Bisifusarium* species, only undecan-2-ol (odor descriptor: fresh, waxy) was solely produced by the three *Bisifusarium* species (except UBOCC-A-120035 and UBOCC-A-120001 isolates) with a significantly higher production by *B. domesticum* MNHN-RF-05627 compared to *B. domesticum* UBOCC-A-120004, *B. penicilloides* UBOCC-A-120021 and *B. allantoides* UBOCC-A-120036. Moreover, *B. penicilloides* and *B. domesticum* produced six specific compounds with fruity or sweet descriptors including heptan-2-one, non-8-en-2-one, and pentan-2-one. They also produced heptan-2-ol and nonan-2-ol, two alcohols that are also known to participate to the well-known Camembert aroma (Thierry et al., 2017). Among volatile compounds, methyl-ketones (e.g. nonan-2-one - fresh, sweet -, undecan-2-one - fruity, creamy, fatty -) were the most abundant and well associated with *B. domesticum, B. penicilloides* and *L. lagenoides*. These compounds result from fatty acid beta-oxidation, which is particularly described in blue-veined cheese but also in Parmigiano Reggiano, Camembert de Normandie or Cheddar cheese, and associated with *P. camemberti*, *P. roqueforti* or *Geotrichum candidum* metabolism (Gillot et al., 2017; Spinnler, 2017; Thierry et al., 2017). Regarding the other main volatile compounds of interest, heptan-4-one is known to have a “cheese” odor and was produced by all *B. allantoides, B. domesticum* and *B. penicilloides* isolates. Propan-2-one, a compound commonly

associated with cheese as Queso Blanco (Ganesan and Weimer, 2017), was only produced by *B. penicilloides* UBOCC-A-120034 and VTT-D-041022 on cheese agar and by *B. domesticum* on Raclette de Savoie (except UBOCC-A-120004). Furthermore, only a few aldehydes were produced by these species, conversely to alcohols that were the second most abundant family of compounds produced by the studied *Nectriaceae*. This result might be explained by aldehyde reduction into alcohols during ripening (Thierry et al., 2017).

As already stated, we chose two experimental conditions that mimicked cheese-making conditions to determine volatile compound production. Cheese agar medium provided a standardized and controlled condition to determine a given strains' volatilome without any biotic interactions classically encountered in the complex cheese ecosystem, while unripened Raclette de Savoie cheese was used as it is voluntarily inoculated with *B. domesticum* at the industrial scale for its *anticollanti* properties was thus closer to technological conditions. Some specific volatile profiles were clearly observed between isolates and according to medium. For example, butan-2-one was not produced on cheese agar by *B. domesticum* strains MNHN-RF-05627, UBOCC-A-120001 and UBOCC-A-120002 while clearly identified from Raclette de Savoie cheese samples. In addition, *B. domesticum* UBOCC-A-120001 showed qualitatively, the lowest production of volatile compounds on cheese agar compared to the other *B. domesticum* isolates while on Raclette de savoie cheese, the latter exhibited a similar profile with the other isolates (the other isolate profiles were only slightly impacted by medium). Based on these results, *B. domesticum* MNHN-RF-05627 and *B. penicilloides* VTT-D-041022 cultures on cheese agar had the highest aromatic potential as more volatile compounds were produced and/or with higher abundances, which could clearly be of interest for future screening of strains for use

during cheese-making. However, the volatilome in a given condition, only provides a partial view of the isolate aromatic potential. Indeed, as stated before, the volatilome could be linked to the methodology applied and the medium used. Moreover, the aromatic potential by a given strain should be confirmed by sensorial analyses of cheese samples which were not compatible with our conditions as this would require a large number of samples for orthonasal sensory evaluation and a dedicated expert panel.

Regarding secondary metabolite production, a recent study by Savary et al. (2021) already showed that no known mycotoxins were produced by *B. allantoides*, *B. penicilloides*, *L. lagenoides* and *L. verticilliformis* in the tested conditions, this is also the case for *B. domesticum* (data not shown, also based on *in silico* genome searches), thus in agreement with the Bachmann et al. (2005) study. Moreover, in the latter study, no formation of cytotoxic metabolites and metabolites with antibiotic activity against *Geobacillus stearothermophilus* were observed, which reinforces the potential safety status of this species (based on the European Qualified Presumption of Safety or the US Generally Recognized As Safe statuses). Based on a global metabolite profiling analysis performed by LC-MS/Q-TOF, on fourteen days YES and skim milk agar cultures, several common compounds were produced by the five species with, in general, twice as many compounds produced on skim milk agar medium vs. YES agar. In general, *B. domesticum* produced about half as many metabolites, regardless of medium, than the four other species and *L. verticilliformis* produced the highest number of compounds. Using the external METLIN-AMRT-PCD database, several compounds were identified based on their *m/z* values. Common extrolite productions among all isolates were noted on skim milk and/or YES agar for some compounds such as linolenic and linoleic acids, fatty acids already known to be produced by other fungal

species such as *Mucor* spp., *G. candidum* or *Yarrowia lipolytica* (Chan et al., 2018; Cordova and Alper, 2018; Luo et al., 2018; Mohamed et al., 2020), but also a sphingoid base analog named “enigmol” (based on the database identification), not associated with other fungal species but studied for anticancer properties (Symolon et al., 2011), and sphingolipids, phytosphingosine and phytosphingosine-1-P, already described for *Y. lipolytica* (Han et al., 2021). According to the species, some fatty acids and sphingolipids (*i.e.* ceramides and sphingosines), frequently produced by several fungal species and already described to have antimicrobial properties (Schorsch et al., 2013), were putatively identified in our study although further confirmation with corresponding standards would be necessary. Finally, these results also highlighted the production of several compounds with high molecular weights (potentially mid-cyclic lipopeptides or complex fatty acids, based on Savary et al., 2021) that could not be precisely identified here. Genome sequencing of these *Nectriaceae* strains would be of clear interest to perform a genome-wide search for secondary metabolite biosynthetic gene clusters, including those that may be cryptic or silent pathways in our experimental conditions. In parallel, fatty acid profiles were also determined from 25°C skim milk and YES cultures for the five neotypes species. For all strains, several fatty acids were produced at different levels and included palmitic, stearic, oleic and linoleic acids, which have all been described among those classically produced by fungal species (Kosa et al., 2018). Thus, while these fatty acid profiles could be expected, the studied species can be considered as good lipid producers as their production rates are comparable to other species such as *Mucor* spp., *Mortierella* spp. or *Rhizopus* spp. (Kosa et al., 2018). Furthermore, these results were well correlated with the previous investigations and also confirmed the lipolytic activities observed on the same medium. Finally, the highest fatty acid production profile was noted for *B. allantoides* on YES medium.

Finally, to assess whether the produced metabolites have potential antifungal activity, bioprotective activities were investigated for three *B. domesticum* isolates. Interestingly, we observed that one day old *B*. *domesticum* cultures induced preventive protection as growth of all *P. biforme* and *P. roqueforti* isolates was significantly decreased. This is particularly of interest considering that *B. domesticum* has slow growth, so this effect is most likely linked to metabolite production rather than competition for nutrients in the medium. Interestingly, this antifungal property appeared more important on cheese agar which is the closest to actual cheese conditions. More precisely, different activities were observed depending on the tested *B. domesticum* isolate. Higher bioprotection potential was clearly seen for MNHN-RF-05627 while it was lower for UBOCC-A-120001. We then performed preliminary tests to investigate whether cell-free *B. domesticum* extracts were also active, using the isolate that showed the highest activity in cheese agar. The *B*. *domesticum* supernatant produced from SM cultures clearly displayed antifungal activities against the common dairy industry spoiler, *P. roqueforti.* This was not observed for the supernatant obtained from YES culture, indicating that the medium impacted the antifungal activity. Not only did we observe reduced growth but also stress as the mycelium remained white. Moreover, in this study, and as already seen for volatile and extrolite compounds, differences were observed between the two studied media and also between the tested *P. biforme* and *P. roqueforti* isolates. These results clearly highlighted the importance of using several media to investigate the functional properties of a given species as described for the OSMAC approach (one strain many compounds) (Wei et al., 2010). Together with the observed fatty acid, volatile, lipolytic or proteolytic profiles, these potential antifungal properties could be of great interest for technological strain selection for industrial applications in the future. The metabolites involved in this activity should also

be further studied. It would clearly be of interest to extend these tests using several *B. domesticum* isolates against other fungal spoilers but also to extend this to the other four newly described cheese-associated *Nectriaceae* species.

5. Conclusions

The obtained results provide new insights about the functional properties of *B. domesticum* and the newly described cheese-associated *Nectriaceae* species,i.e. *B. allantoides*, *B. penicilloides*, *L. lagenoides* and *L. verticilliformis*. The observed functional traits are of clear interest for future *B. domesticum* strain selection by cheesemakers to improve overall cheese quality and safety. Our findings also highlighted the potential interest to further investigate the newly described cheese-associated *Nectriaceae* species, in particular *B. penicilloides*, during cheese-making as they could be used as novel adjunct or ripening cultures in the dairy industry. Moreover, secondary metabolite profiling reinforced the atoxigenic character of the tested *B. domesticum* and *Nectriaceae* spp. isolates as no known mycotoxins were produced. Yet, further investigation of the potentially novel secondary metabolites will be of interest in the future and also to determine their function. The potential bioprotective antifungal activities of *B. domesticum* cultures and their cell-free fractions clearly highlighted promising properties for the dairy industry although further studies will be needed.

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Authors' contributions

EC, J-LJ, FG and MC obtained the funding, supervised the study, edited and proofread the manuscript. EC, J-LJ, MC and OS designed the experiments. OS performed the experimental work, analysed the data and drafted the manuscript. AT and M-BM performed GC-MS analyses and AT and MC provided assistance for statistical and correlation analyses. OS and MC performed all extrolite profiling experiments by LC-Q-TOF and data analyses. JF provided extrolite profiling methodology and performed extrolite identifications. All authors contributed and proofread the article.

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Functional diversity of the cheese-associated *Nectriaceae* species, *B. allantoides*, *B. domesticum*, *B. penicilloides*, *L. lagenoides* and *L. verticilliformis*.

Figure captions

Figure 1: Lipolytic (A) and proteolytic (B) activities of the studied cheese-associated *Nectriaceae* species. The clear zone was measured on tributyrin agar after 24 days culture at 12°C and 25°C for lipolysis while halo size was measured on skim milk agar after 24 days culture at 12°C and 25°C for proteolysis. The following species and isolates are represented with a mean value of three replicates (error bars corresponding to standard deviation): *B. allantoides* (n=3 isolates), *B. domesticum* (n=5), *B. penicilloides* (n=3), *L. lagenoides* (n=4), *L. verticilliformis* (n=1) and *P. roqueforti* (n=1), as a positive control. No clear zone was observed on the negative control (data not shown). The lowercase letters above the bars indicate statistical significance; mean values with different letters are significantly different ($P < 0.05$).

Figure 2: Normalized heat-map representation of the studied cheese-associated *Nectriaceae* species volatilomes after 25 days culture on cheese agar at 12°C. Hierarchical clustering was done using Ward's linkage and Euclidean distances. Sample names are on the bottom and volatile compound names are on the right. The green to red color range corresponds to low to high compound abundances. The samples were noted with the abbreviations described below with the information "_CAR1", "_CAR2", "_CAR3" corresponding to replicate 1, 2, 3 on cheese agar, respectively. BA_A, BA_B and BA_C correspond to *B. allantoides* UBOCC-A120035, UBOCC-A-120036 and UBOCC-A-1200037. BD_A, BD_B, BD_C, BD_D and BD_E correspond to *B. domesticum* MNHN-RF-05627, UBOCC-A-120001, UBOCC-A-120002, UBOCC-A-120003 and UBOCC-A-120004. BP_A, BP_B and BP_C correspond to *B. penicilloides* UBOCC-A-120021, UBOCC-A-120034 and VTT-D-041022. LL_A, LL_B, LL_C and LL_D correspond to *L. lagenoides* ESE000140,

UBOCC-A-120038, UBOCC-A-120039 and UBOCC-A-120041. LV_A corresponds to *L. verticilliformis* UBOCC-A-120043 while the negative control was noted NEG.

Figure 3: Normalized heat-map representation of *B. domesticum* isolate volatilome after 25 days culture on cheese agar or Raclette de Savoie cheese at 12°C. Hierarchical clustering was done using Ward's linkage and Euclidean distances. Sample names are on the bottom and volatile compound names are on the right. The green to red color range corresponds to low to high compound abundances. The samples were noted with the abbreviations described below with the information "_CAR1", "_CAR2", "_CAR3" and "_RCT1", "_RCT2", "_RCT3". corresponding to replicate 1, 2, 3 on cheese agar and 1, 2, 3 on Raclette de Savoie, respectively. BD_A, BD_B, BD_C, BD_D and BD_E correspond to *B. domesticum* MNHN-RF-05627, UBOCC-A-120001, UBOCC-A-120002, UBOCC-A-120003 and UBOCC-A-120004, while the negative control was noted NEG.

Figure 4: Venn diagrams representing interspecies volatilome comparison on cheese agar (25 days culture at 12°C). For each species (*B. allantoides*, *B. domesticum*, *B. penicilloides*, *L. lagenoides* and *L. verticilliformis)*, the volatile compounds produced by at least one isolate are represented. Numbers correspond to the compounds specific to one species or shared by several species highlighted by the circles. The diagrams were obtained using the "*jvenn"* online tool (Bardou et al., 2014).

Figure 5: Venn diagrams representing interspecies extrolite profiles comparison on skim milk agar and YES agar (14 days culture at 25°C). Numbers correspond to the compounds specific to one species or shared by several species highlighted by the circles. The diagrams were obtained using the "*jvenn*" online tool (Bardou et al., 2014).

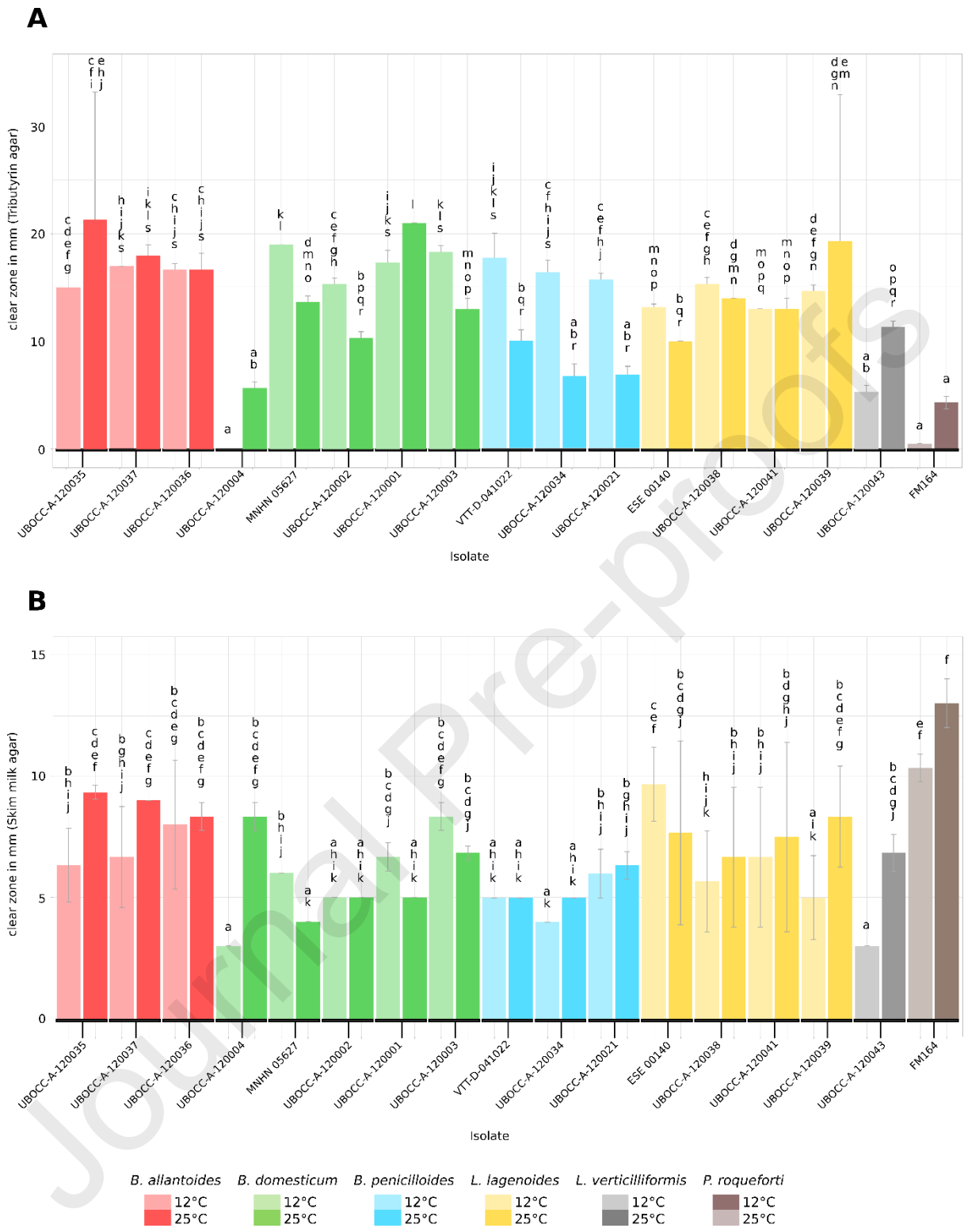
Figure 6: Fatty acid profiles of *B. allantoides*, *B. domesticum*, *B. penicilloides*, *L. lagenoides* and *L. verticilliformis* on YES and Skim Milk (SM) agar media. The analyses were performed from fourteen growth cultures (25°C) and with three replicates of each neotype strain. The average value with the standard deviation is reported. Fatty acid production is represented as per microgram of fatty acid quantified in the sample. A two-way ANOVA was conducted and did not reveal any differences between temperature nor medium.

Figure 7: *B. domesticum* biopreservation tests against *P. biforme* (A) and *P. roqueforti* (B) isolates, on PDA and cheese agar. Radial growth of the target isolates are represented after ten days growth on either one or four days *B. domesticum* lawn culture at 25°C. The lowercase letters above the bars indicate statistical significance; mean values with different letters are significantly different ($P < 0.05$). Pink asterisks above the bars indicate

biopreservation assays for which the mean value was significantly different ($P < 0.05$) from the mean value of the corresponding control (the same *Penicillium* isolate grown in medium not inoculated with an isolatre of *B. domesticum*).

Figure 1A, B

Journal Pre-proofs



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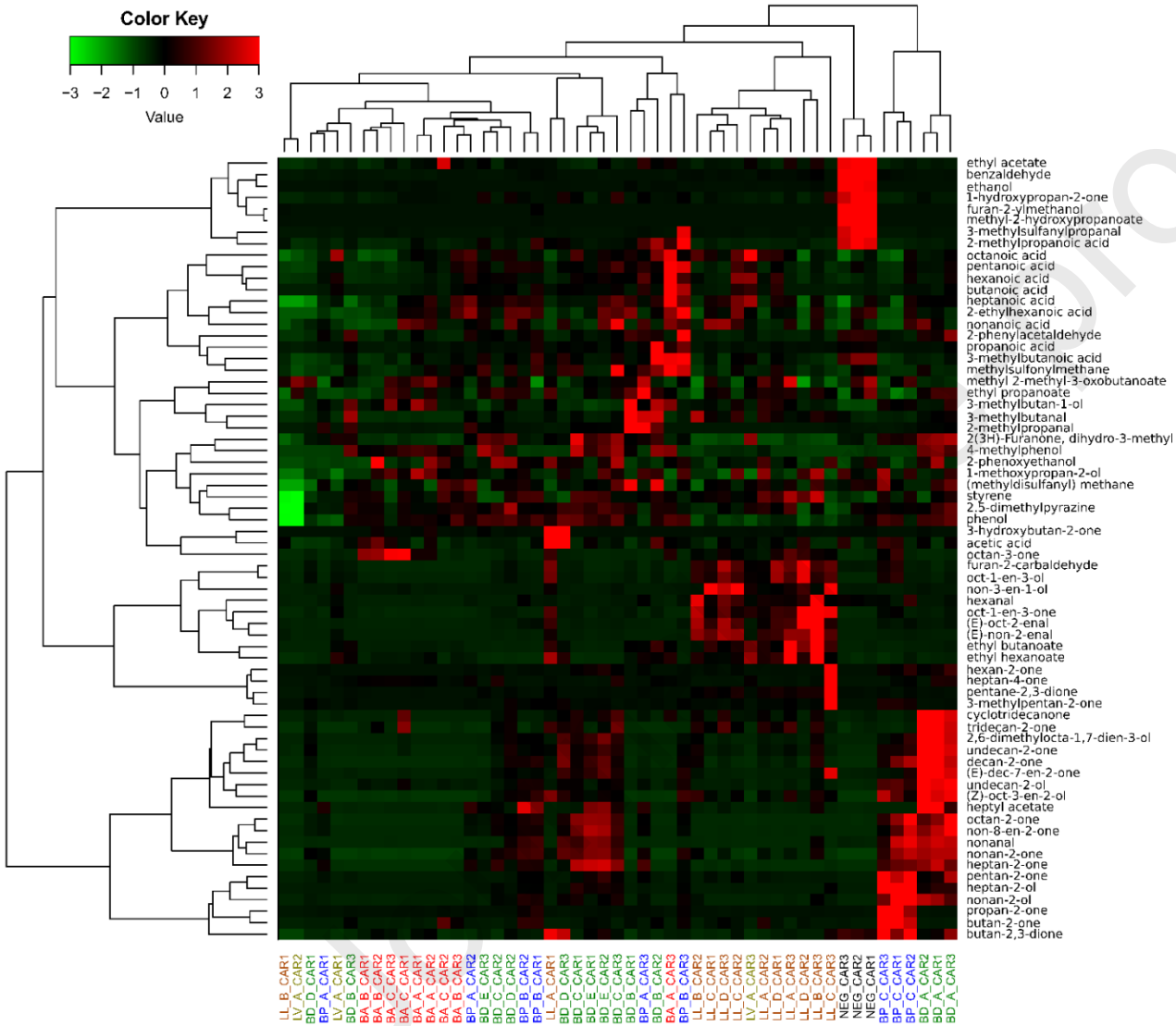

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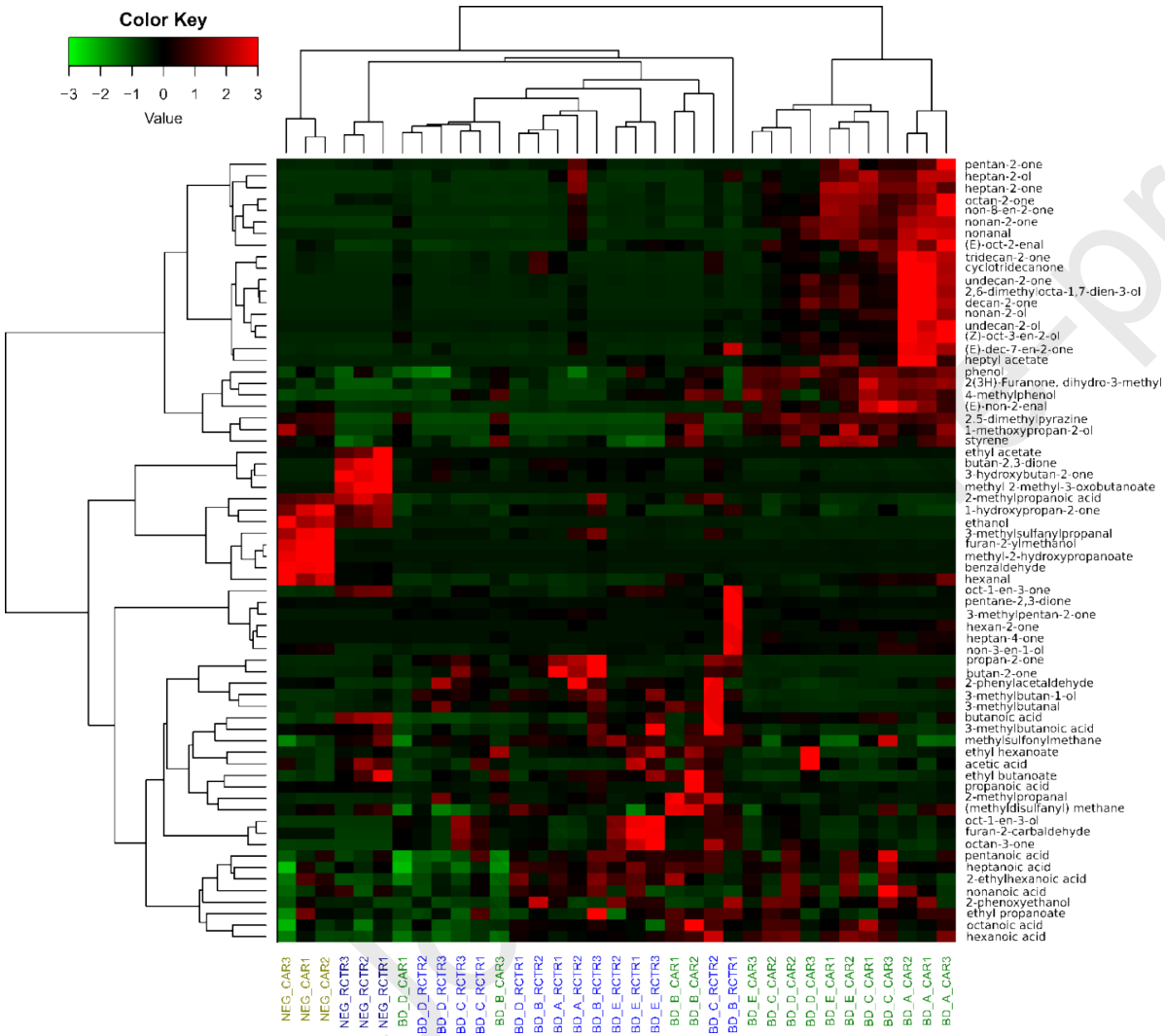

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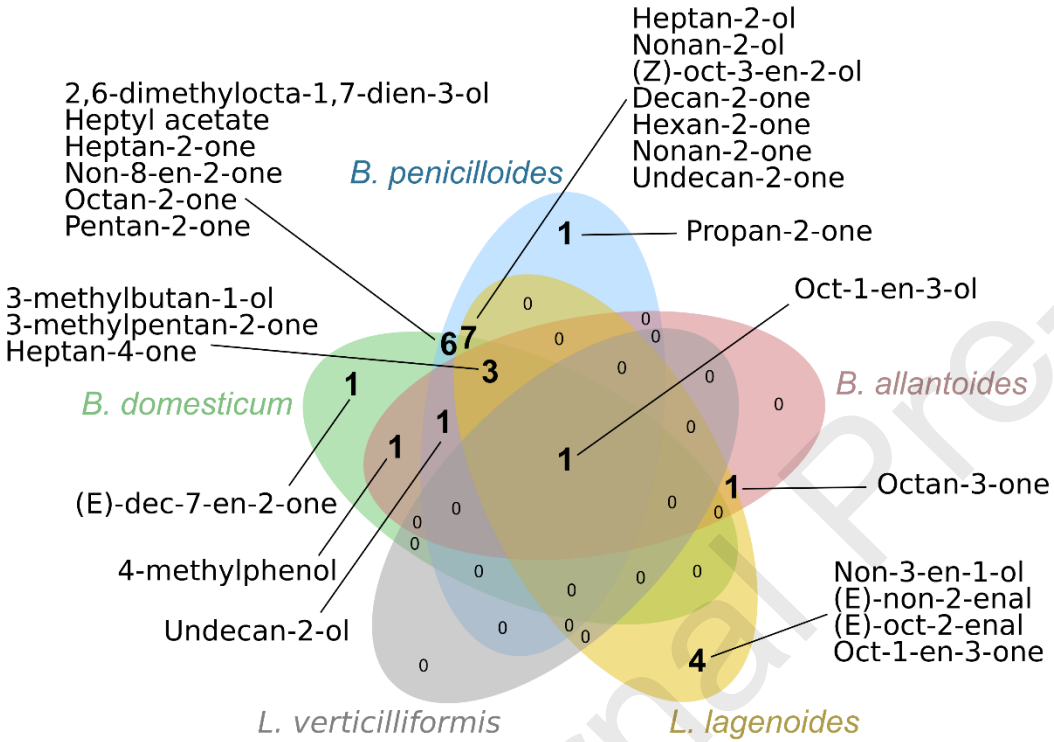

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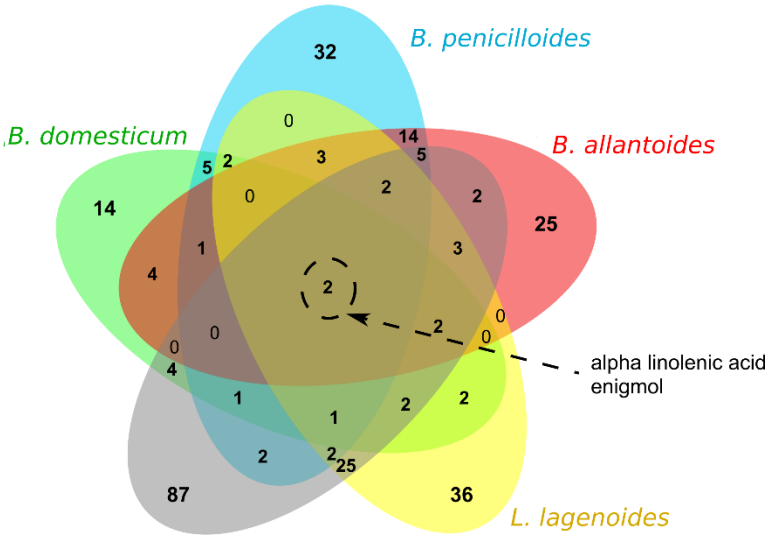
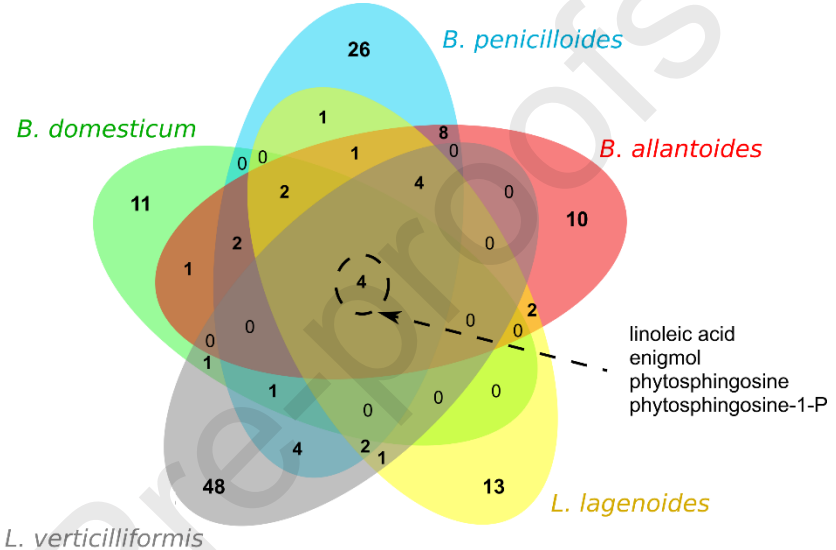
Figure 5. A



B



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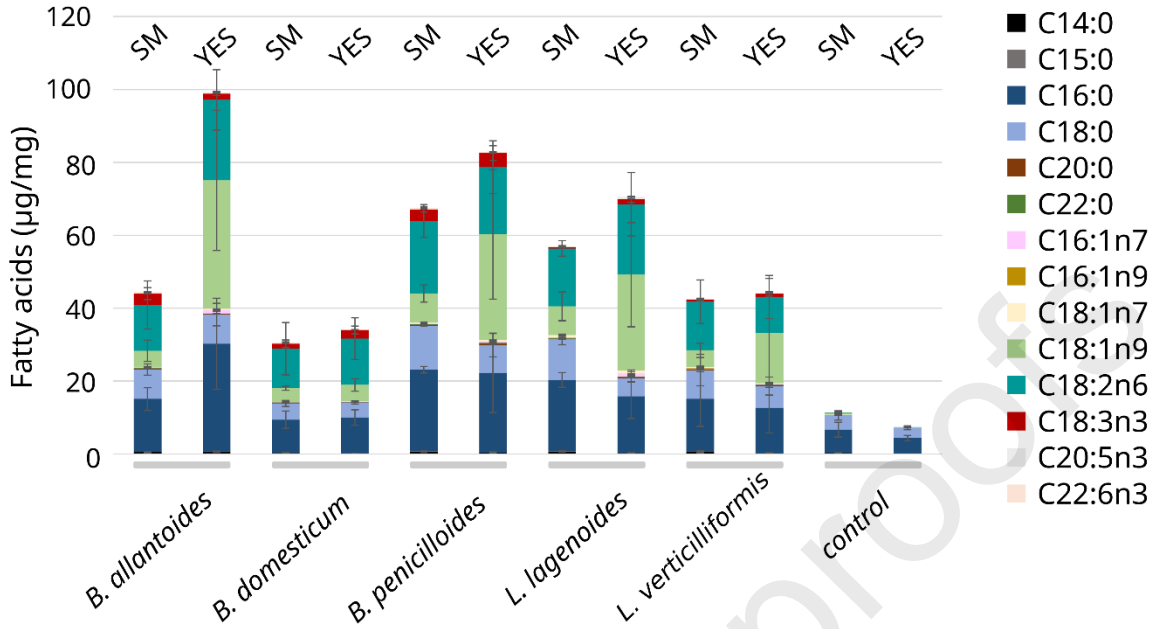

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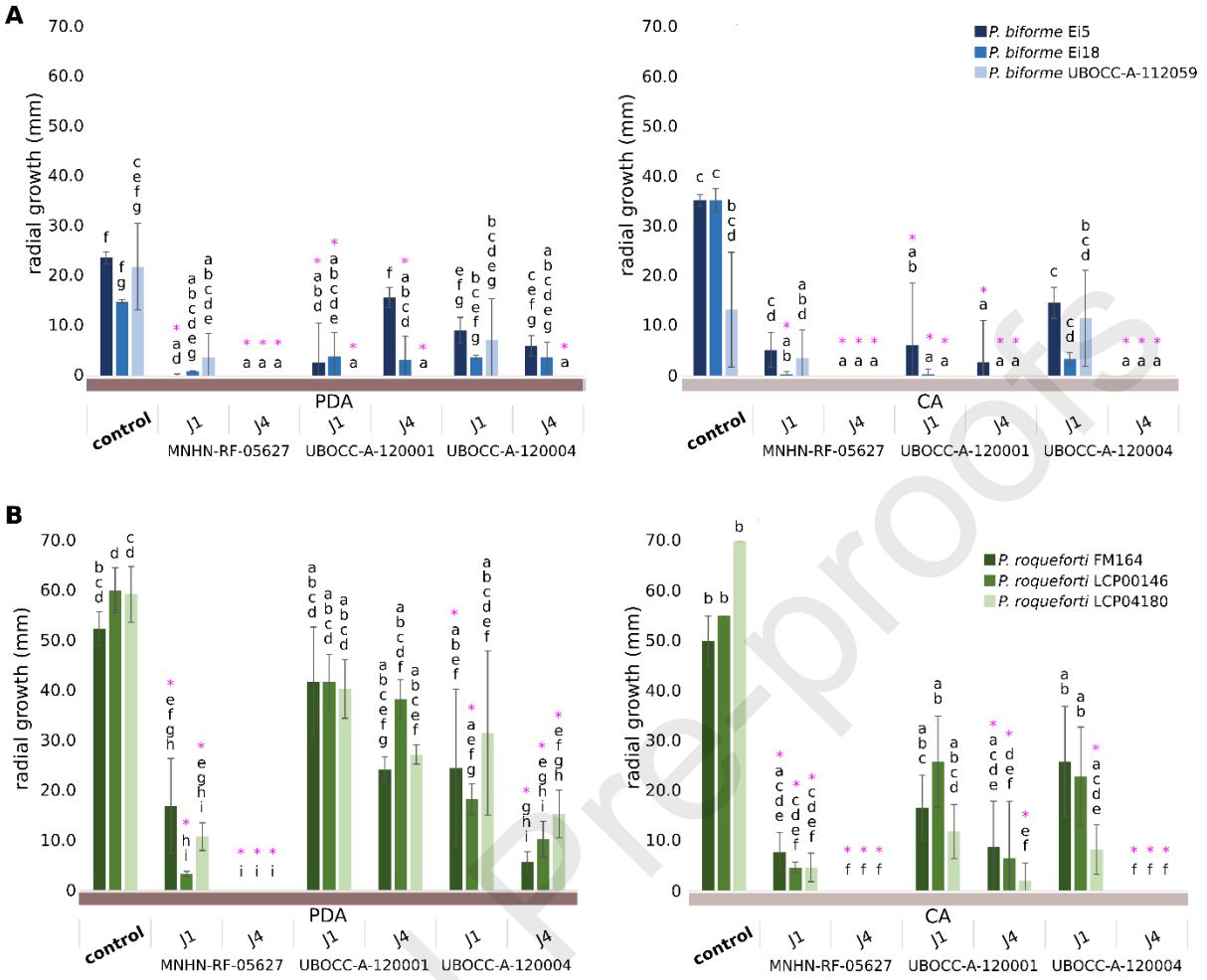

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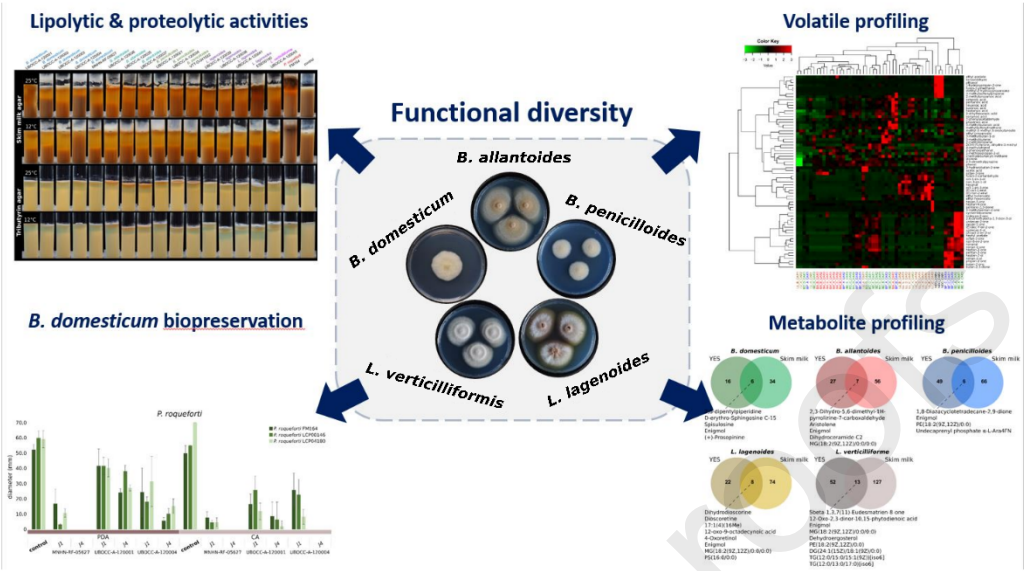
Table 1. *Nectriaceae* spp. strains used for the inter- and intra-specific studies.

Species	Strain code	Substrate/Origin
Bisifusarium allantoides	UBOCC-A-120035	Cheese (Manchego, Spain)
	UBOCC-A-120036[HT]	Soft cheese type (France)
	UBOCC-A-120037	Soft cheese type (France)
Bisifusarium domesticum	MNHN RF-05627[NT]	Food (Belgium)
	UBOCC-A-120001	Commercial adjunct culture (France)
	UBOCC-A-120002	Commercial adjunct culture (France)
	UBOCC-A-120003	Cheese (Raclette d'alpage, Switzerland)
	UBOCC-A-120004	Commercial adjunct culture (France)
Bisifusarium penicilloides	UBOCC-A-120021[HT]	Cheese (Mont d'Or, France)
	UBOCC-A-120034	Cheese (Mont d'Or, France)
	VTT-D-041022	Surface of sandstone building, Arbroat Abbey (Scotland)
Longinectria lagenoides	ESE 00140	Cheese (Tilsit, France)
	UBOCC-A-120038	Cheese (Raclette d'alpage, Switzerland)
	UBOCC-A-120039[HT]	Cheese (Vacherin Fribourgeois, Switzerland)
	UBOCC-A-120041	Raw cow's milk cheese (Switzerland)
Longinectria verticilliformis	UBOCC-A-120043[HT]	Cheese (Alpeggio, Italy)

[HT]holotype, [NT]neotype strains.

UBOCC: Université de Bretagne Occidentale Culture Collection, France; ESE: Laboratoire Écologie, Systématique et Évolution UMR 8079 culture collection, France; MNHN: Muséum National d'Histoire Naturelle culture collection, France; VTT Culture Collection, Finland

Graphical abstract



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Highlights

- Higher proteolytic activity at 12°C for several *B. domesticum* isolates
- Volatile compounds associated with cheese odor descriptors identified
- Higher volatile compound production by *B. domesticum* and *B. penicilloides*
- *B. domesticum* bioprotective activity shown against *P. roqueforti*

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Authors' contributions

EC, J-LJ, FG and MC obtained the funding, supervised the study, edited and proofread the manuscript. EC, J-LJ, MC and OS designed the experiments. OS performed the experimental work, analysed the data and drafted the manuscript. AT and M-BM performed GC-MS analyses and AT and MC provided assistance for statistical and correlation analyses. OS and MC performed all extrolite profiling experiments by LC-Q-TOF and data analyses. JF provided extrolite profiling methodology and performed extrolite identifications. All authors contributed and proofread the article.

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Declaration of interests

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

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