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Galactomyces candidus diversity in the complex mycobiota of cow-milk bryndza cheese comprising antagonistic and sensitive strains

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ABSTRACT

Galactomyces candidus (orthographic variant: *Galactomyces candidum*) is a heterogeneous species of Saccharomycetales that comprises dimorphic yeasts described previously under various names (e.g. *Geotrichum*, *Dipodascus*). Its strains are common components of the cheese surface mycobiota. This study identified genetically and physiologically heterogeneous *G. candidus* strains in the complex mycobiota of artisanal cow-milk bryndza samples. The traditional Slovak bryndza is a cheese produced from ewe's milk in cooler mountainous regions and from cow's milk in warmer low-land regions. The taxonomic analysis of the culturable yeasts of the latter version carried out in this study revealed considerable differences from the yeast biota previously described for ovine bryndza. However, the conventional D1/D2- and ITS-based barcode analyses could not assign unanimously all isolates to species because of the intragenomic barcode diversity in certain groups and the discordance between the D1/D2 and ITS results in other groups. The identified species and groups of isolates had different abilities to utilise the carbon and energy sources (lactose, lactate, lipids and proteins) available in milk and ripening cheese. The *G. candidus* strains did not metabolise lactose and lactate, hydrolysed milk proteins with diverse, usually moderate efficiency and only could grow on certain amino acids as only energy sources. Their preferred substrate was lipid. Under aerobic conditions, its hyphae penetrated the lipid droplets and degraded their content from inside by developing a dense internal mycelium. Sporulation and different MLST (multilocus sequence typing) patterns indicated that the *Galactomyces* strains could sexually interact and their genomes could recombine. The *Galactomyces* and *Kluyveromyces* isolates had antagonistic effects against other members of the mycobiota.

1. Introduction

Cheese making has long traditions in many parts of the world where milk-producing animals (cow, buffalo, goat or sheep) are bred. Hundreds of traditional cheese varieties exist in the world and many of them are produced on industrial scale (Fox, 1993). Raw milk cheeses are produced with milk that has not been pasteurized or heat-treated. The first step of cheese making is coagulating the milk casein by reducing acidity and/or adding milk-clotting enzymes (rennet or bacterial enzymes). The solid curds are then separated from the liquid whey and pressed into raw cheese.

Milk and cheese are diverse microbial ecosystems. Micro-organisms present in milk come from the animal, the farm soil, and the dairy environment. Artisan producers using traditional cheese-making technologies rely on the existing natural microbial communities. Yeasts, generally considered to be adventitious environmental contaminants constitute an important part of these communities (for a recent review, see Bintsis, 2021). As the number of cheese types is high, the composition of the yeast communities is highly variable. *Galactomyces/Geotrichum* strains are regular components of the surface microbiota of almost all types of soft cheeses (Eliskases-Lechner et al., 2011).

Galactomyces is the most characteristic yeast of the bryndza cheese traditionally produced from unpasteurized ovine milk by shepherds in mountainous regions of Slovakia (Palo and Kaláb, 1984). However, the tradition of bryndza-making is not only confined to mountainous regions. After the collapse of the Turkish occupation of Eastern Europe in the seventeenth century, larger groups of ethnic Slovaks moved down to the plain regions abandoned by the Turks and took with themselves the tradition of bryndza-making to their new settlements. In the new environment, sheep farming became displaced by cow farming and cow's milk replaced ovine milk in the procedure. The cow's milk is rennetted to produce raw cheese. After a few days (sometimes weeks) of aging, the

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ripening cheese (hrudka) is ground up, salted, filled into a (traditionally wooden) barrel with a screw press (šaflík), pressed and aged further for up to several months. During aging, it gradually changes from mild soft (spreadable) cheese to sharp hard cheese. It can be consumed in any stage of aging and can be preserved for months in cool rooms.

The present study seeks to gain insight into the complexity of yeast populations of artisanal cow-milk bryndza. It investigates the taxonomic composition of the population by characterization of culturable yeasts instead of metabarcoding. While metabarcoding has major strengths and is widely used for diversity research, it still has limitations. The most commonly used second-generation taxonomic sequencing platforms (e.g. Illumina) generally do not allow species-level taxonomic identification, which precludes more precise characterizations of the population structures. Besides, metabarcoding can easily overestimate the diversity because it cannot discriminate the "relevant" sequences (of living organisms) from the "polluting" sequences (e.g. left behind by dead organisms) (Jagadeesan et al., 2019) and can misidentify organisms belonging to taxa of high intraspecies or intragenomic barcode diversity (e.g. Alper et al., 2011; Lachance et al., 2003; Paloi et al., 2022; Perkins et al., 2020; Sipiczki, 2022).

The investigation of the rDNA barcodes (segments of the repeats coding for rRNA) and the MLST analysis of the isolates revealed both phenomena in certain species found in the bryndza samples. The isolates were highly diverse in the ability and mode of the utilisation of carbon sources and some of them had antagonistic effects against other yeasts and moulds. The prevailing species was *Galactomyces candidus* (orthographic variant: *Galactomyces candidum*) a heterogeneous dimorphic species belonging to Saccharomycetales. It has both self-fertile and self-sterile but mating competent strains. The latter form two groups of different heterothallic mating types (e.g. Butler and Petersen, 1970; de Hoog et al., 1986; Groenewald et al., 2012). In mixed cultures of heterothallic strains, asci are formed where their hyphae meet. The asci contain one, rarely two ascospores that give rise to self-sporulating cultures (Butler and Petersen, 1972). The abundance of *G. candidus* in bryndza can be attributed to its favourable metabolic capacity in the specific environment (metabolism of lactate and lipids) and fast substrate colonisation by hyphal growth.

2. Materials and methods

2.1. Sampling and isolation of microorganisms

Roughly 500 mg (or 500 µl, in case of milk) of sample was added to 1 ml sterile water and homogenised. Various amounts of aliquots of the suspensions (in the case of cheese: soft cream-like material) were spread on YEA (0.5 % yeast extract, 2 % glucose, 2 % agar) plates. This medium preferentially supported the growth of yeasts and fungi. Yeast colonies (at least 20 for each sample) were randomly isolated from the plates regardless of their morphology and inoculated onto fresh YEA plates. From the plates, on which moulds also grew, representatives of each mould morphology were also isolated.

2.2. Molecular methods

Genomic DNA was extracted from overnight cultures of the isolates grown in YEL (YEA without agar) as described previously (Sipiczki, 2003). For the determination of the taxonomic affiliation of the isolates, the D1/D2 domains of the large subunit rRNA genes and the ITS1-5.8S-ITS2 segments of their DNA repeats coding for rRNAs were amplified with the primer pairs NL1-NL4 (O'Donnell, 1993) and ITS1-ITS4 (White et al., 1990), respectively. For the investigation of the intraspecies diversity of the *G. candidus* isolates by MLST analysis, the nuclear genes *ALA1* (alanyl-tRNA synthase), *CDC19* (pyruvate kinase), *GLN4* (glutaminyl-tRNA synthase), *PGI1* (phosphoglucoisomerase), *PGM2* (phosphoglucomutase) and *YSP3* (aspartic protease 3) were amplified with gene-specific primer pairs (Perkins et al., 2020). The amplicons were sequenced in both directions with the same primers.

2.3. Sequence analysis

The barcode sequences were compared to the corresponding sequences of the type strains of yeast and fungal species by performing similarity searches in the INSDC databases using the NCBI Blast service (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The same service was also used for finding database sequences similar to genes amplified and sequenced from the *Galactomyces* isolates (MLST analysis).

For the comparison of multiple sequences, the sequences were aligned with the MSA (multiple sequence alignment) algorithm Clustal W 1.7 (Thompson et al., 1994). Minimum free energy secondary structures were generated for the D1/D2 hairpin loops with Predict, a.

secondary structure webserver (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html) using default settings (Reuter and Mathews, 2010).

2.4. Physiological and growth tests

Utilisation (assimilation) of glucose, lactose, lactate and amino acids (alanine, glycine, lysine, tryptophane) as carbon sources was tested on Difco YNB (yeast nitrogen base) agar plates (2 % agar) supplemented with 0.5 % of the test compound (Kurtzman et al., 2011). For the investigation of lipid metabolism, YNB was supplemented with 2 % butter or 2 % sunflower oil. 10 µl of suspensions of cells (10^6 cells/ml) of late exponential-phase YEL cultures were dropped onto the plates and incubated at 25 °C. The growth of the *Galactomyces* isolates in oxygen-limited conditions was investigated in two ways: (a) on vacuum-packed YNB agar plates and (b) in YNB agar films sandwiched between glass slides and cover slips (Sipiczki, 2011).

The ability of the isolates to utilise proteins as carbon sources was examined in three ways: (a) by testing them for proteolytic activity on YEL solidified with 5 % gelatine (proteolysis liquefies the medium), by testing them for proteolytic activity and growth (b) on YNB agar supplemented with 5 % gelatine (proteolysis becomes visible as clear zone in the plate poured with acidic $HgCl_2$) and (c) on YNB agar supplemented with 3 % skim milk powder (proteolysis generates a clear halo around the colony). Salt tolerance was examined on YNB plates containing various amounts (5, 10, 15 and 20 %) of NaCl. In each test, 10 µl of suspensions of cells (10^6 cells/ml) of late exponential-phase YEL cultures were dropped onto the plates and incubated at 25 °C. Pulcherrimin-production was visualised by streaking of samples of yeast cultures onto YEA plates supplemented with 0.02 mg/ml $FeCl_3$. On this medium the pulcherrimin producers form reddish colonies and pigmented halos in the medium around the colonies (Sipiczki, 2020).

2.5. Sporulation test of Galactomyces isolates

Loop-full amounts of arthroconidia from each isolate were smeared on potato dextrose agar (PDA) (Difco Laboratories, Detroit) plates individually and in pairwise combinations with other isolates. After 10 days of incubation at room temperature, the mass of arthroconidia was removed with a spatula from the surface of the medium and a vertical slice of the invasive mycelium was mounted onto a glass slide for microscopic observation.

2.6. Antagonism tests

Interactions among the yeast isolates were investigated on YEA plates as described previously for grape yeasts (Sipiczki, 2016). Briefly, the plate was flooded with a dense suspension (~10^8 cells/ml) of cells of an isolate to obtain homogeneous lawns of cells on the surface of the medium. After removing the rest of the suspension and drying the surface of the plate, loopful amounts of other isolates were smeared on the lawn to form spots of ~5 mm in diameter. The growth of the spots and

the lawn around them was monitored at 20 °C for 7 days. If any of the latter isolates inhibited or reduced the growth of the lawn, it formed a (clear or a turbid) inhibition zone in the lawn around its colony. The involvement of iron in the inhibitory activity of Kluyveromes was tested by the supplementation of the medium with 0.04 mg/ml Fe Cl_3.

Since *Galactomyces* formed continuously extending (mould-like) hyphal colonies on the substrate, the yeast isolates were also tested for antagonism against its hyphal growth. In this test the isolates were smeared on the YEA plate as described above and the *Galactomyces* isolate was inoculated into the centre of the plate. The growth of its mycelium was then monitored at 20 °C for three weeks. It stopped growing near the colony of the antagonistic yeast but grew onto the colony of the non-antagonistic yeast.

The effect of the yeast isolates on the growth of moulds was tested in the same way. For testing of a *Galactomyces* and a mould isolate for interaction, the isolates were inoculated on a YEA plate at a distance of 5 cm from each other. Their interaction was also examined on bryndza plates. 10 g bryndza was layered in a Petri dish and sterilised by autoclaving at 105 °C. The isolates were smeared on the surface of the sterile bryndza 3 cm away from each other. Their growth was monitored at 20 °C for 4 weeks.

3. Results

3.1. Sample collection and isolation of yeasts and fungi

Samples were collected in three farmhouses (Table 1). In farmhouse V (Hrabovszki Farm) producing soft bryndza, samples were taken from raw milk, and from various stages of the technological procedure (V1 to V12 in Table 1). The bryndza from farmhouse P2 was soft being in the middle phase of ripening. In farmhouse P, crumbs were scraped off from hard ripe bryndza aging in a wooden barrel. All samples formed yeast colonies of various morphologies on the YEA plates with the exception of V9 and V10, which were taken from the interior of the ripening soft bryndza. However, because of the very fast growth of *Galactomyces*-type colonies and (in certain samples also) filamentous fungi, the exact number of yeast colonies and the relative abundance of the morphological types could not be determined. From the samples, a total of 168 yeast colonies representing all morphological types (glossy pinkish, glossy white, hairy-filamentous, rough, rugose, shiny and wrinkled) and 12 fungal colonies were randomly isolated.

3.2. Taxonomic identification

One to four representatives of each morphological category were selected for molecular taxonomic examination covering all samples (Supplementary Table A). Since the fast-growing *Galactomyces*-type colonies showed morphological heterogeneity, a larger number of them were examined to cover all morphological types.

Sequencing of the D1/D2 domains identified strains with 100 % identity with the type strains of [*Candida*] *zeylanoides*, *Kluyveromyces lactis*, *K. marxianus*, *Torulaspora delbrueckii* or *Wickerhamiella pararugosa* and strains whose sequences differed from all type-strain sequences deposited in databases.

The sequences of one morphological type were identical with sequences of the type strains of two species, *Debaryomyces hansenii* and *D. vindobonensis*. Surprisingly, the ITS sequences of these isolates were different from both species but showed 100 % identity with three other species of the genus: *D. fabryi*, *D. prosopidis* and *D. subglobosus*.

The D1/D2 sequences of the rugose colonies differed from all type-strain sequences. The most similar type-strain sequence was that of *Clavispora lusitaniae* CBS 6936^T (holotype of the species), from which they differed at 29 (9 isolates; Group A) or 30 (3 isolates; Group B) positions. A blast search in the INSDC databases identified one sequence (amplified from an Agave yeast isolated in Mexico) identical with the former group and dozens of sequences identical with the latter group (amplified from yeasts isolated from a broad spectrum of substrates in four continents, including raw milk in Algeria). One of these strains was the type strain CBS 4413^I of the former species *Candida lusitaniae* (now isotype of *C. lusitaniae*) (van Uden and Carmo-Sousa, 1959).

The *Wickerhamiella* sequences also formed two groups. Two of them were identical with the sequence of the type strain of the species *W. pararugosa*, whereas 6 sequences differed from that by 5 substitutions. Blast searches identified numerous identical sequences for both of them, among them sequences of strains isolated from dairy environments.

The D1/D2 sequences of the isolates that formed filamentous fast-extending colonies with hairy and farinose surface differed from the sequences of the type strains of all valid species. The most similar type-strain sequence was that of *G. candidus* (CBS 178.71). However, the calculation of differences was hampered by the presence of ambiguous

Table 1
Sampling and isolation of colonies.

Sample	Colony morphology[a]	Taxonomic affiliation[b]
V1: raw milk	1	*Galactomyces candidus*
	2	*Kluyveromyces marxianus*
	4	*Wickerhamiella pararugosa*
V2: milk before curdling	1	*Galactomyces candidus*
	2	*Kluyveromyces marxianus*
	3	*Wickerhamiella pararugosa*
V3: milk after curdling	1	*Galactomyces candidus*
	2	*Kluyveromyces marxianus*
	4	*Wickerhamiella pararugosa*
	5	*Clavispora lusitaniae*
V4: surface of raw cheese	1	*Galactomyces candidus*
	2	*Kluyveromyces marxianus*
V5: surface of ripening cheese	1	*Galactomyces candidus*
	2	*Kluyveromyces marxianus*
	5	*Clavispora lusitaniae*
V6: fresh salted bryndza	1	*Galactomyces candidus*
	6	*Debaryomyces* sp.
V7: surface of ripe bryndza	1	*Galactomyces candidus*
	3	*Kluyveromyces lactis*
	6	[*Candida*] *zeylanoides*
	PE8	*Penicillium cavernicola/ speluncae*
V8: beneath the surface of the ripe bryndza	1	*Galactomyces candidus*
V9: inside of ripe soft bryndza		No yeasts and fungi
V10: inside of ripe soft bryndza		No yeasts and fungi
V11: liquid seeping from ripening bryndza	1	*Galactomyces candidus*
	4	*Wickerhamiella pararugosa*
	5	*Clavispora lusitaniae*
V12: liquid seeping from ripening bryndza	1	*Galactomyces candidus*
	4	*Wickerhamiella pararugosa*
	5	*Clavispora lusitaniae*
Vm: ripe soft bryndza sold on the market	1	*Galactomyces candidus*
	2	*Kluyveromyces marxianus*
	3	*Kluyveromyces lactis*
	6	*Debaryomyces* sp.
	PE8	*Penicillium cavernicola/ speluncae*
P2: ripe soft bryndza produced in farm P2	1	*Galactomyces candidus*
	7	*Torulaspora delbrueckii*
	3	*Kluyveromyces lactis*
	PE8	*Penicillium cavernicola/ speluncae*
P: ripe hard bryndza produced in farm P	1	*Galactomyces candidus*
	2	*Kluyveromyces marxianus*
	6	*Debaryomyces* sp.
	7	*Torulaspora delbrueckii*
	PE6	*Aspergillus niger/foetidus/ welwitschiae*
	PE20	*Mucor racemosus*

[a] Colony morphology: 1: Hairy filamentous; 2: glossy pinkish; 3: glossy; 4: wrinkled; 5: rugose; 6: rough; 7: shiny; PE8: blue mould; PE6: yellow-black mould; PE20: brown mould.
[b] Based on the results presented in Supplementary Table B.

nucleotides both in the type-strain sequence available in GenBank (JN974262) and in the sequences of 16 isolates (superscript figures in Supplementary Table B). Within the *G. candidus* species, most isolates showed higher similarity to sequences of the type strains of two former species, *Geotrichum bryndzae* and *Geotrichum silvicola*, recently merged with *G. candidus* (Groenewald et al., 2012). The ITS sequences had no ambiguous positions and showed a somewhat different position in the species. All isolates differed from *G. silvicola* and shared an identical ITS sequence with the *G. candidus* and *G. bryndzae* type strains.

The taxonomic identification of the filamentous fungi was attempted by sequencing the ITS segments of the rDNA repeats. All isolates within each morphological category (*Aspergillus*, *Penicillium* and *Mucor*) had identical sequences. However, none of the groups could be unambiguously assigned to a single species (Supplementary Table B). The sequences of the *Aspergillus* isolates were identical with the sequences of the type strains of three *Aspergillus* species. The *Penicillium* isolates also proved to be identical in ITS sequence with three species. The *Mucor* isolates contained variable positions, indicating intragenomic heterogeneity of the repeats coding for rRNAs.

3.3. *Galactomyces* molecular diversity

The ambiguous nucleotides in the D1/D2 sequences indicated that the *Galactomyces* isolates had incompletely homogenised repeats of rRNA genes. Recent studies on *Metschnikowia* strains revealed that ambiguous nucleotides are not scattered randomly but occur in positions that are not essential for the stability of the secondary structure of the LSU rRNA molecules (Sipiczki, 2022; Sipiczki et al., 2013). To find out if this is the case in the *Galactomyces* isolates as well, all D1/D2 sequences were aligned. As presumed, the ambiguous nucleotides were not scattered randomly in these strains either. In the Clustal MSA only seven positions were variable (dimorphic; 2 A/G, 4 C/T transitions and one A/T transversion) (Fig. 1a). In the secondary structure of the transcript of the sequence of the isolate P-3 (OP142418) five sites were located in the D2 fold-back loop with two pairs at which compensatory substitutions can take place (Fig. 1b). On the neighbour-joining tree rooted by the *G. candidus* type strain, the *G. silvicola* type strain (UFMG-354-2; AY158042) had a marginal position in contrast to the type strain of *G. bryndzae* (CCY 16-2-1; EU186073) (Fig. 1c). This topology indicates that the isolates are more closely related to those previously isolated from ewe-milk bryndza. However, it has to be taken into consideration that the repeats coding for the rRNAs in the *G. bryndzae* and *G. silvicola* type strains may also be heterogeneous.

To further investigate the genetic diversity of the *Galactomyces* isolates, segments of six genes proposed in previous works for diversity studies (Perkins et al., 2020) were amplified and sequenced from 11 isolates covering milk, raw cheese and all bryndza samples (Table 2). None of the sequences had ambiguous nucleotides, demonstrating that the genomes were not heterozygous for these genes. The *ALA1*, *CDC19* and *GLN1* sequences were identical in all isolates. For *PGL1* and *PGM2* two and three alleles, respectively, were detected. *YAP3* could not be amplified from all examined strains, probably because of differences in the biding sites of one or both primers. All but the *YAP3* alleles were previously described in the literature, but in different combinations (Table 3). The *YAP3* sequences of the bryndza isolates differed from the most similar ones deposited in the databases by one substitution. Thus, all strains subjected to MLST examinations in this study differ from the strains for which MLST data are available.

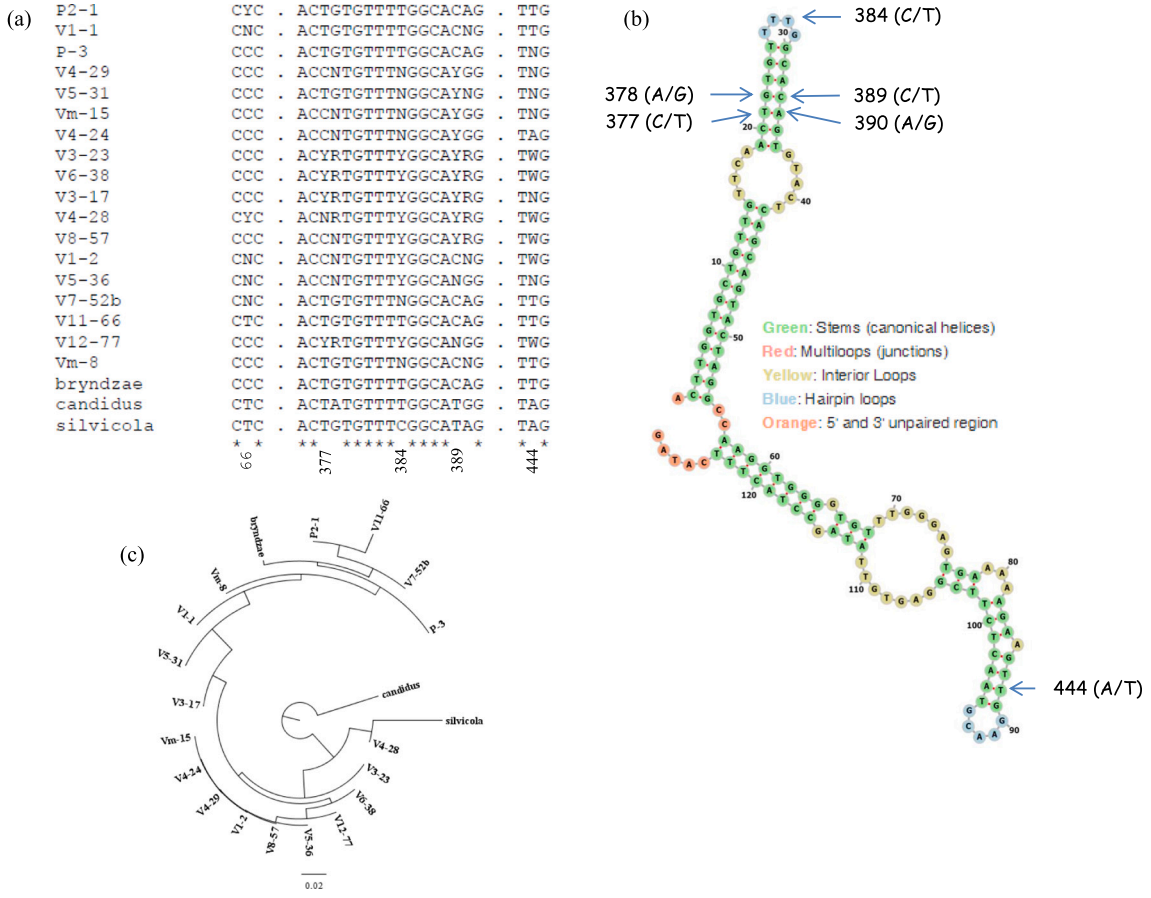

Fig. 1. Analysis of the D1/D2 domains of the 26S (large subunit) rRNA genes of the *Galactomyces* isolates. (a) Alignment of segments containing dimorphic sites. (b) Secondary structure of the D1/D2 domain showing the positions of the dimorphic sites. (c) Neighbour-joining tree of the sequences. bryndzae: *G. bryndzae* type strain; candidus: *G. candidus* type strain (outgroup); silvicola: *G. silvicola* type strain.

Table 2
Alleles of marker genes used in MLST analysis.

Gene	Alleles in *Galactomyces* isolates										
	P-3	Vm-8	Vm-15	P2-1	V1-1	V1-2	V4-28	V7-52B	V6-38	V8-57	V12-77
ALA1	A	A	A	A	A	A	A	A	A	A	A
CDC19	A	A	A	A	A	A	A	A	A	A	A
GLN41	A	A	A	A	A	A	A	A	A	A	A
PGL1	A	B	A	A	A	A	A	A	A	A	A
PGM2	A	B	A	A	C	A	A	C	A	A	A
YAP3	A	A	A	A	0	A	A	0	A	A	A

GenBank accession numbers of representative sequences: Alleles that differ from those of isolate P-3 are marked with colours. *ALA1*: OP210279 (A); *CDC19*: OP210280 (A); *GLN4*: OP210281 (A); *PGL1*: OP210282 (A), OP210285 (B), *PGM2*: OP210283 (A), OP210286 (B), OP210287 (C); *YAP3*: OP210284 (A).

Table 3
Identity of alleles of MLST markers with database sequences. Identity is marked with dark blue.

Gene	Allele	Identity with database sequences of LMA strains[a]															
		15	20	28	40	70	75	76	77	244	317	563	1028	1146	1024-26	1031-34	CLIB 918
ALA1	A	■		■	■		■		■			■	■	■			
CDC19	A	■			■							■	■	■			
GLN4	A	■			■			■				■	■	■			
PGL1	A	■		■	■			■	■			■	■	■			
	B		■			■		■		■	■						
PGM2	A	■							■					■			
	B			■	■							■					
	C										■						
YAP3	A[b]																

[a]Perkins et al. (2020).
[b]This allele differs from the most similar allele by one nucleotide.

3.4. Sporulation in Galactomyces isolates

The finding that the strains possessed different combination of MLST alleles and had heterogeneous D1/D2 repeats indicated that sexual interactions and recombination may shape their genomes. To test this possibility, the strains involved in the MLST analysis were tested individually and in combinations for sporulation on PDA. On this medium, most strains did not sporulate, which implies that they were either sterile or heterothallic. The latter possibility seems to be more likely because in certain mixed cultures sporogeneous branches and asci were seen on invasive filaments penetrating the culture medium (Fig. 2). The mature asci contained one, rarely two spores although the spores of many asci looked immature (or abortive). Thus, sexual interactions providing possibilities for exchange of genetic material can take place between certain isolates.

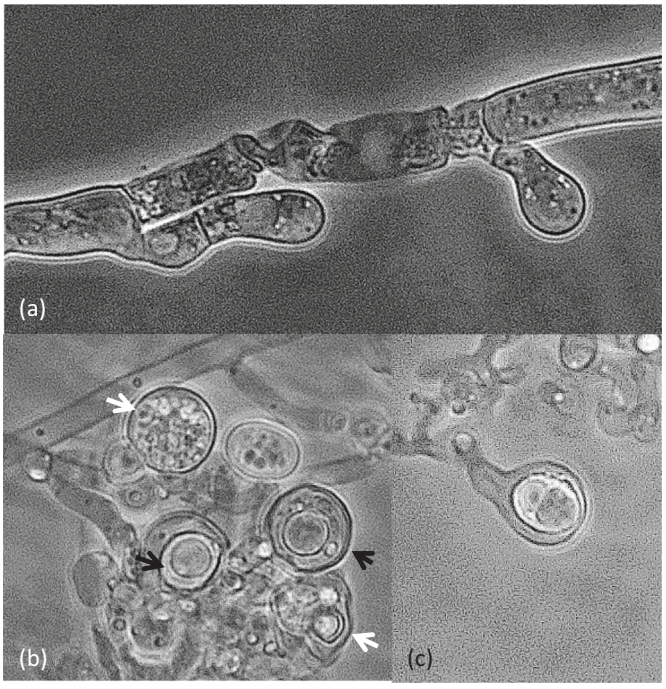

Fig. 2. Sexual processes in the mixed culture of the *Galactomyces* isolates Vm-8 and Vm-15. (a) Hypha with sporogeneous branches. (b) Single-spored asci (black arrowheads) among abortive asci (white arrowheads). (c) Ascus with two ascospores.

3.5. Utilisation of carbon sources

The carbon sources available for microorganisms in milk and cheese are lactose, lipids, proteins and their derivatives. Therefore, the isolates identified by sequencing (Supplementary Table B) were tested for the ability to grow on YNB plates supplemented with lactose, lactate, certain amino acids (alanine, glycine, lysine and tryptophane), butter, sunflower oil, gelatine and skim milk (Table 4).

The *Debaryomyces* and *Kluyveromyces* isolates grew on YNB plates containing lactose as a carbon and energy source. The *Clavispora*, *Galactomyces* and *Kluyveromyces* isolates grew on lactate. *Galactomyces* and *Wickerhamiella* also grew well on YNB plates supplemented with butter or sunflower oil (Fig. 3a and b). On butter, the [*C.*] *zeylanoides*, *Debaryomyces* sp. and *Kluyveromyces* isolates also grew, but their growth was

Table 4
Growth tests.

Group of yeast isolates	Growth on YNB supplemented with					Degradation of gelatine	Growth on YEA supplemented with NaCl (%)			
	Lactose	Lactate	Butter	Sunflower oil	Skim milk		5	10	15	20
[*Candida*] *zeylanoides*	–	–	(+)	(+)	–	–	++	++	–	–
Clavispora lusitaniae group A	–	+	–	–	–	–	+++	++	–	–
Clavispora lusitaniae group B	–	+	–	–	–	–	++	+	–	–
Debaryomyces sp.	+	–	(+)	(+)	+	+	+++	+++	++	+
Galactomyces candidus MLST type I	–	+	+	+	(+),+	+	++	–	–	–
Galactomyces candidus MLST type II	–	+	+	+	+	(+)	++	–	–	–
Galactomyces candidus MLST type III	–	+	+	+	–	(+)	++	–	–	–
Galactomyces candidus rest	–	+	+	+	–, (+)	–, (+)	++	–	–	–
Kluyveromyces lactis	+	+	(+)	–	+	+	++	+	–	–
Kluyveromyces marxianus	+	+	(+)	–	+	+	++	+	–	–
Torulaspora delbrueckii	–	–	–	–	–	–	++	–	–	–
Wickerhamiella pararugosa group A	–	–	+	+	–	–	++	+	–	–
Wickerhamiella pararugosa group B	–	–	+	+	–	–, +	++	+	–	–

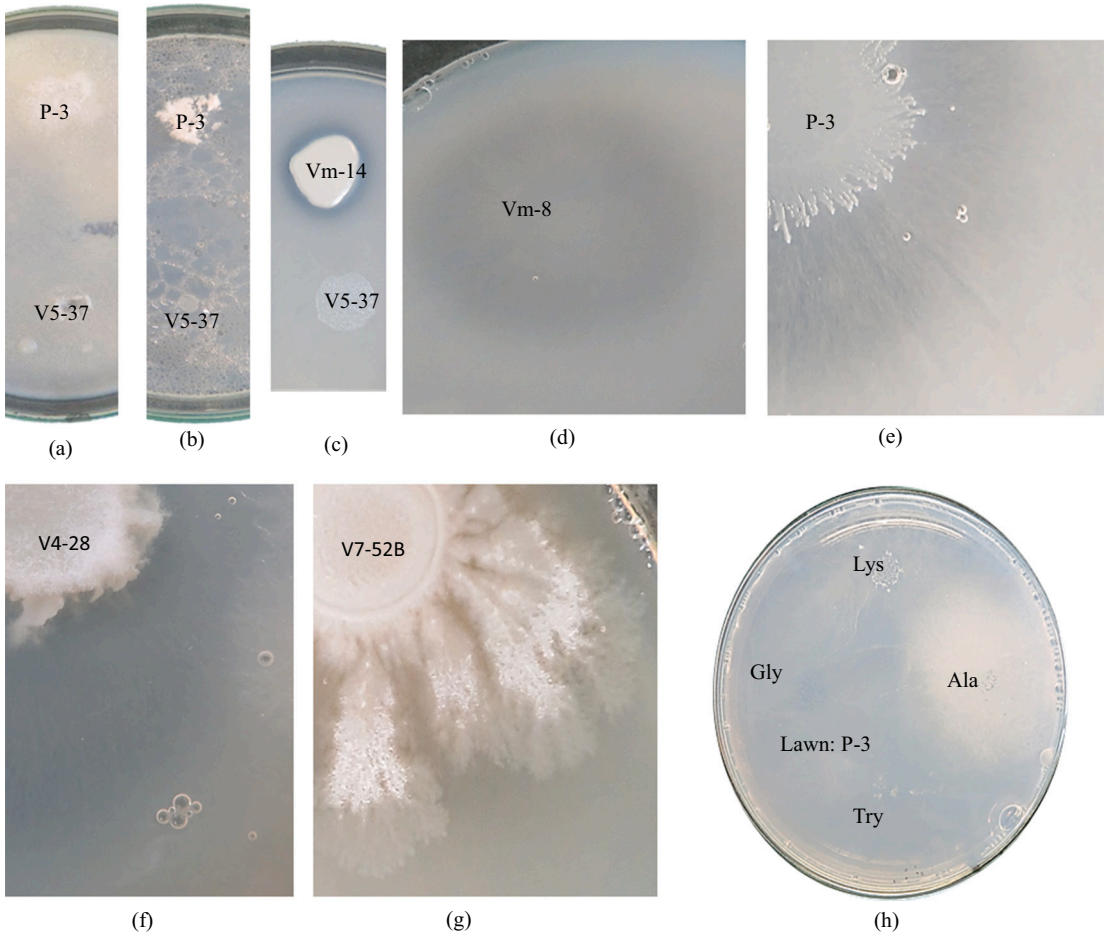

Fig. 3. Growth tests on YNB medium supplemented with carbon sources. (a) Butter. (b) Sunflower oil. (c–g) Skim milk. (h) Amino acids. After 5 days (a–c and h) and 4 weeks of incubation. P-3, V4-28, V7-52B and Vm-8: *G. candidus*. Vm-14: *K. marxianus*. V5-37: *C. lusitaniae*.

poor and thus was not necessarily due to lipid metabolism (butter also contains other potential carbon sources).

All *Debaryomyces*, *Kluyveromyces* and *Galactomyces* isolates degraded gelatine. Six out of the nine tested *Wickerhamiella* isolates also dissolved gelatine albeit with much lower efficiency. *Debaryomyces* and *Kluyveromyces* also dissolved the proteins of skim milk and grew well on YNB plates, in which milk proteins were the only carbon source (Fig. 3c). On this medium, the *Galactomyces* isolates were highly heterogeneous. All dissolved milk proteins to some extent, but the dissolution zones appeared with considerable delay and were vague. Five isolates (P2-1, Vm-8, Vm-15, V5-31, V12-77) did not form visible colonies at all, and only developed a very thin mycelium on the surface of the medium (Fig. 3d). The rest (14 isolates) formed colonies albeit with various efficiency (examples are shown in Fig. 3e–g). The delayed and slow growth could be due to the selective metabolism of amino-acids (Fig. 3h). The [*C.*] *zeylanoides*, *T. delbrueckii* and *Clavispora* isolates had no proteolytic activity on the test media.

3.6. Substrate colonisation

The *Galactomyces* isolates formed a dense tissue of hyphae extending on the surface of the glucose-containing YEA medium (Fig. 4a and b) and also penetrating into the medium. Hyphae were also formed in low oxygen environment in a YEA film sandwiched between a glass slide and a cover slip (Fig. 4c and d) or on vacuum-wrapped plates but the mycelium was loose and the hyphae usually died without forming arthrospores (Fig. 4e and f). The latter observation indicates that *Galactomyces* can colonise both the surface and the inside of glucose-containing substrates but its growth is sensitive to low level of oxygen.

In the butter medium and the plant-oil medium lipid droplets were formed. When the *Galactomyces* isolate was inoculated on a thin film of the medium, the growth of its hyphae could be monitored by microscopic examination. When a hypha run into a droplet, penetrated it and developed a mesh of filaments (mycelium) inside it (Fig. 5a). From the colonised droplets many short hyphae grew out to form chains of arthroconidia (Fig. 5b). They also formed a few non-sporulating long hyphae that invaded other droplets (Fig. 5a). No invasion of droplets was observed when the film was covered with a cover slip or on medium in a Petri dish vacuum-packed in a plastic bag (Fig. 5c). These observations imply that the *Galactomyces* isolates could utilise lipids under aerobic conditions and colonise the surface of the lipid-containing substrate. The *Clavispora* and *Wickerhamiella* isolates could also penetrate the agar medium but they only formed slowly extending pseudohyphae.

The growth of two groups of isolates was highly salt-sensitive. Of all yeasts isolated from bryndza, the growth of *Galactomyces* and *Torulaspora* was most inhibited by the supplementation of the growth medium with NaCl (Table 4).

3.7. Antagonistic interactions

During the isolation of yeasts and moulds we noticed that certain yeast colonies reduced the growth of other yeast colonies and/or inhibited the extension of the fungal mycelia. This observation hinted at antagonistic interactions within the colonising mycobiota. To explore these interactions, representatives of the yeast species were tested for antagonism against each other and against the isolated moulds.

To detect interactions between yeasts, each isolate was inoculated onto dense lawns of other isolates on agar plates. If it inhibited the growth of the cells of the lawn, an inhibition zone appeared around its colony (Fig. 6a, g–k). No inhibition was detected in most combinations (Table 5). Clear zones were seen around the *Kluyveromyces* colonies on the lawns of the isolates of all but one species. The exceptions were the lawns of the *Galactomyces* isolates in which only turbid inhibition zones were visible around the *Kluyveromyces* colonies (Fig. 6b). The inhibition zones were associated with the ability to produce pink halos in the medium. Supplementation of the medium with 0.04 mg/ml $FeCl_3$ prevented the formation of both the inhibition zone and the pigmented halo and turned the *Kluyveromyces* colony red (Fig. 6c). On *Galactomyces* lawn, the elevated iron concentration not only prevented growth inhibition but even allowed the hyphae to grow onto the *Kluyveromyces* colonies (Fig. 6d). The hyphae also overgrew the non-antagonistic isolates (Fig. 6f).

The yeast isolates were also tested for effects on the growth of *Galactomyces* and mould mycelia by inoculating them ahead of the growing hyphae. In these tests, the *Galactomyces* isolates proved to be both sensitive and antagonistic. As shown in Fig. 7a, the *Kluyveromyces* isolates drastically reduced the growth of the *Galactomyces* hyphae. On the other hand, the *Galactomyces* colony stopped the growth of *Mucor* (Fig. 7b)

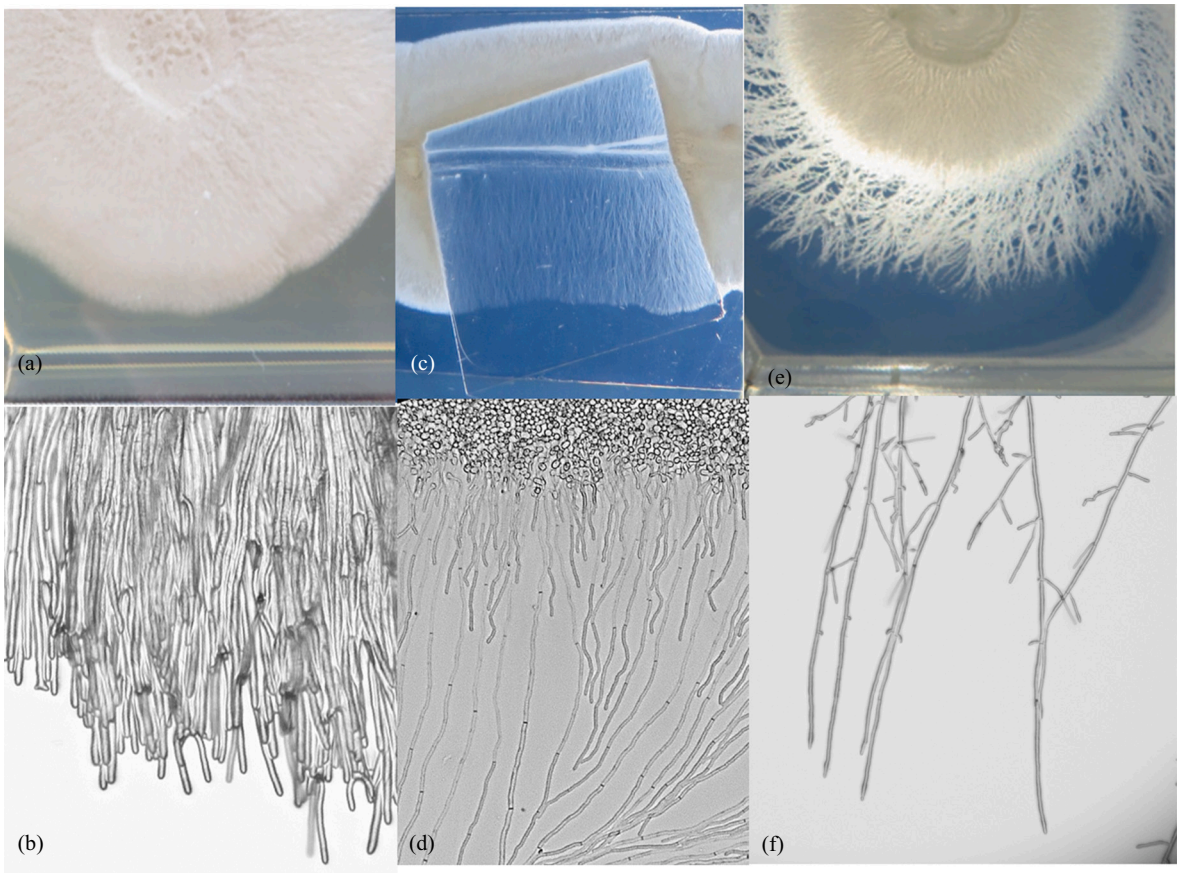

Fig. 4. Growth of the *Galactomyces* isolate P-3 under oxygen-limited conditions. (a and b) On the surface of a YEA plate. (c and) In a YEA film sandwiched between a glass slide and a cover slip. (e and f) On the surface of a vacuum-packed YEA plate.

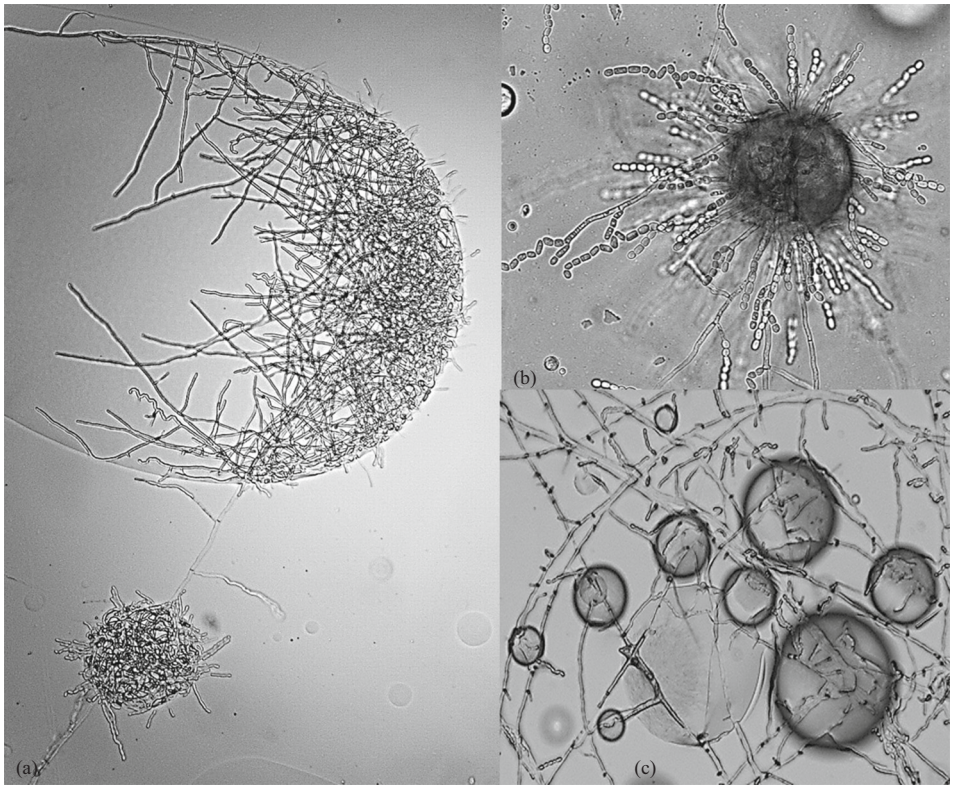

Fig. 5. Growth by "drop-invasion" on a medium containing butter as carbon source (*Galactomyces* isolate P-3). (a) Mycelium formation within lipid droplets. (b) Hyphae grow out from the colonised lipid droplet and form arthrospores. (c) No drop-invasion takes place in the agar film sandwiched between a glass slide and a cover slip.

and *Penicillium* (Fig. 7d and f) both on agar plates and on the surface of sterilised bryndza.

4. Discussion

The investigation of the culturable mycobiota identified strains of 11 ascomycetous yeast and fungal genera in artisanal cow-milk bryndza. Four morphological groups of isolates could be unanimously assigned by the analysis of the rRNA barcodes to [*Candida*] *zeylanoides*, *Kluyveromyces lactis*, *K. marxianus* or *Torulaspora delbrueckii*. Other groups either considerably differed in barcode sequence from the type strains of known species or were heterogeneous.

4.1. Taxonomic identification is hampered by intragenomic and intraspecies barcode diversity

Yeasts showing the characteristic dimorphism of *Galactomyces* (*Geotrichum*) were found in most samples. *Galactomyces* is a ubiquitous dimorphic yeast genus which occurs commonly on nutritionally rich, semisolid or liquid substrates, such as decaying plant material, and in a wide variety of foods (for a review, see Eliskases-Lechner et al., 2011). Their strains are common and important components of the microflora of raw milk and soft cheeses (for a review, see Boutrou and Guéguen, 2005). The cheese strains are usually classified as *Geotrichum candidum*, the anamorph of *Galactomyces candidus* (orthographic variant: *Galactomyces candidum*).

The D1/D2 sequences of the strains isolated in this study differed from that of the *G. candidus* type strain and were much more similar to the type-strain sequences of *G. silvicola* and *G. bryndzae*. On the Neighbour-Join tree all but one isolate formed a separate cluster which also included the *G. bryndzae* sequence, indicating that they are closer to *G. bryndzae* than to the type strain of *G. candidus*. However, a recent taxonomic revision of the genus merged the three species, and reduced the taxonomic rank of *G. silvicola* and *G. bryndzae* to synonyms of the species name *G. candidus* (Groenewald et al., 2012). Consequently, these bryndza isolates belong taxonomically to *G. candidus*. The application of the D1/D2 domains for the taxonomic identification was hampered by the presence of ambiguous nucleotides in the sequences of the isolates and in the database sequence of the *G. candidus* type strain. Therefore, the ITS regions of the rDNA of certain isolates were also sequenced. All ITS sequences were free of variable positions and identical with those of the *G. candidus* and *G. bryndzae* type strains. These results confirm their conspecificity with *G. candidus* and very close relationship with the former species *G. bryndzae*. The name *G. bryndzae* was originally proposed for strains isolated from sheep-milk bryndza (Sulo et al., 2009).

A group of isolates could be assigned to *Debaryomyces*, another genus whose strains are common in dairy products. The dairy strains identified in previous studies were usually assigned to *D. hansenii* (mostly) by rRNA barcode sequencing without presenting the underlying similarity data. Further, it was ignored that the D1/D2 of the type strain of *D. hansenii* is identical with that of the type strain of *D. vindobonensis*. For example, the cheese strain identified by D1/D2 sequencing as *D. hansenii* by Haastrup et al. (2018) could also be assigned to *D. vindobonensis*. On the other hand, the *D. vindobonensis* D1/D2 barcode ASV (Amplicon Sequence Variant) found in a goat cheese could have been a *D. hansenii* sequence (Biolcati et al., 2022). The D1/D2 sequences of the bryndza isolates of this study were also identical with the corresponding sequences of both type strains. In order to find out which species the bryndza isolates belonged to, ITS segments were also sequenced. Their analysis did not solve the dilemma either, but caused even more confusion. All isolates showed 100 % ITS identity with the *D. fabryi*, *D. prosopidis* and *D. subglobosus* type strains. Thus, these isolates cannot be assigned unambiguously to a single species by rRNA barcode sequencing. Therefore we refrain from designating them *D. hansenii*. It is possible

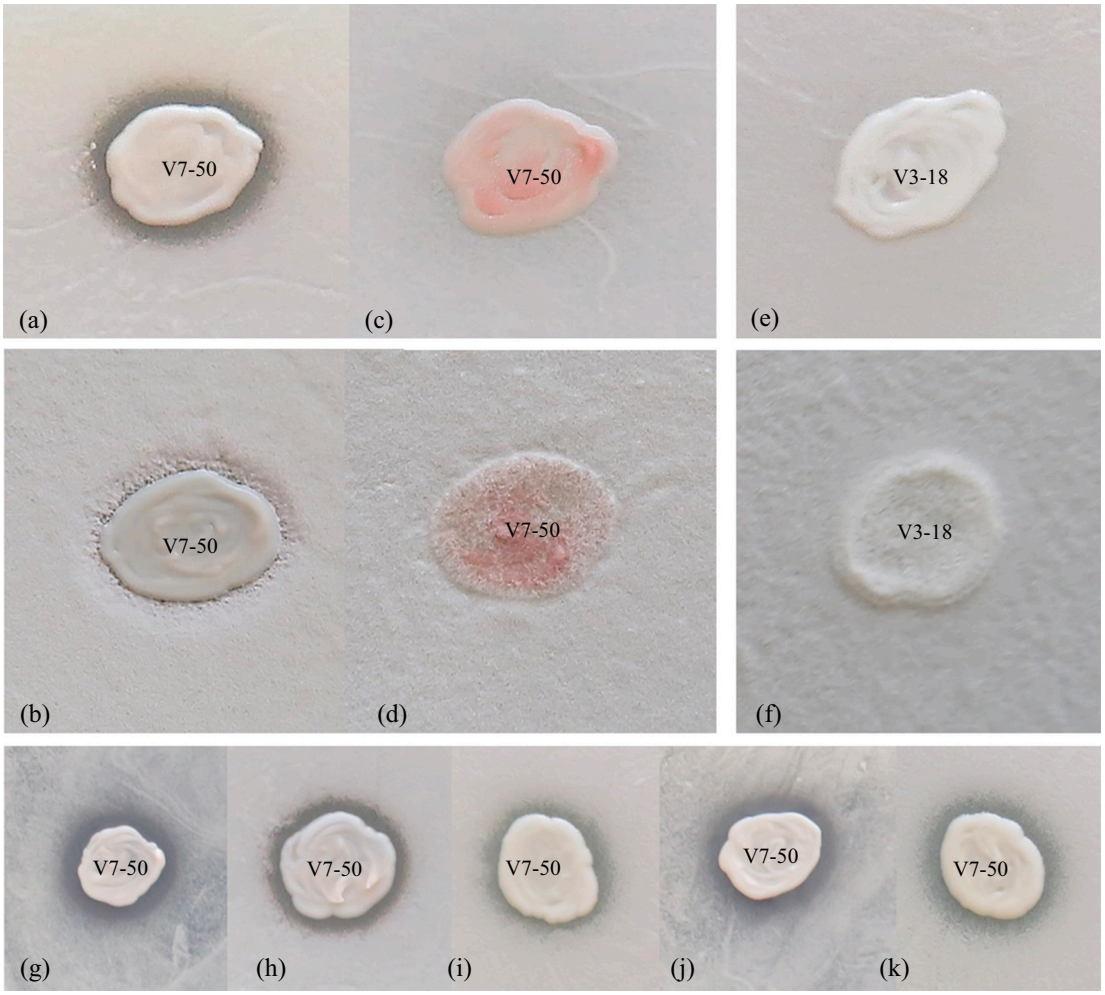

Fig. 6. Antagonism tests on yeast lawn. The lawn is *Debaryomyces* Vm-7 (a, c and e), *Galactomyces* P-3 (b, d and f), [*C*] *zeylanoides* V7-13 (g), *C. lusitaniae* V3-18 (h), *K. marxianus* Vm-5 (i), *T. delbrueckii* P2-2 (j) and *W. pararugosa* V12-74 (k). V7-50: *K. lactis*. V3-18 *C. lusitaniae*. The medium is supplemented with $FeCl_3$ in (c) and (d).

that similar uncertainties could also be revealed by more careful molecular analysis of other dairy strains described in the literature as *D. hansenii*. From the analysis of the rRNA barcode sequences, it is impossible to determine whether the bryndza isolates are conspecific with the *D. hansenii* strains found in other cheeses or represent a different, perhaps unique type. Nevertheless, it is pertinent to mention here that *D. vindobonensis* was first described from municipal wastewater (Lopandic et al., 2013) and later also detected (together with *D. hansenii*) among endophytic yeasts in rice leafs (Tantirungkij et al., 2015), garlic field soil (Ji-Yoon et al., 2020), marine plastic debris (Marsay et al., 2022) and goat cheese (Biolcati et al., 2022). It is necessary to mention here that *Debaryomyces hansenii* is a highly heterogeneous species and its taxonomic delineation is still subject to debate. Numerous attempts have been made to explore its intraspecies diversity and its relationship to other species of the genus by sequencing of segments of the rRNA cistrons and the *ACT1* genes, investigation of DNA-DNA reassociation kinetics, comparing of the G + C contents and the ascus morphology, fingerprinting of mini- and microsatellite regions (Groenewald et al., 2008) and the rRNA gene intergenic spacers (Nguyen et al., 2009), investigation of the intron sequences found in certain housekeeping genes (Jacques et al., 2009) and nuclear mitochondrial DNA insertions (Jacques et al., 2010), etc.

The third group of isolates, whose taxonomic position determined by rRNA barcode sequencing seems to be poorly supported by sequence similarity, differs from the nearest type-strain to an extent (29–30 substitutions in the D1/D2 domains and 8 substitutions in the ITS region) that significantly exceeds the usual difference separating species. The most similar type-strain (holotype) sequence was that of *C. lusitaniae*. This species is also ubiquitous ascomycetous yeast. It has been detected in various substrates such as soils, waters, plants, and gastrointestinal tracts of many animals (Lachance, 2011). The differences of the bryndza isolates from the type strain might be used as arguments for considering them as strains of a distinct species. However, they are identical or almost identical in the D1/D2 sequence with *Candida lusitaniae*, an isotype of *C. lusitaniae*. Since the holotype and the isotype were declared conspecific on the basis of the level of DNA reassociation between them (Lachance et al., 2003), the bryndza isolates can be classified as *C. lusitaniae*, despite the considerable sequence difference from the type strain of the species.

The isolates, for which the most similar type-strain sequences were those of *Wickerhamiella pararugosa*, formed two groups. In one group both the D1/D2 and the ITS sequences were identical with those of the type strain. In the other group both sequences were different. The presence of both types in the samples infers that genetically different *Wickerhamiella* clones participated in the process. Both types have been identified by previous studies in diary environments (e.g. MW587735. MZ491090).

4.2. *The yeast biota of bovine bryndza differs from that of ovine bryndza*

The composition of the yeast biota of the cow-milk bryndza investigated in this study differed significantly from that of the ovine bryndza.

Table 5
Antagonism.

Strain inoculated on lawn			Strain of lawn						
Species	Isolate		*G. candidus* P-3	*Debaryomyces* sp. Vm-7	*K. lactis* P2-8	*K. marxianus* Vm-5	*C. lusitaniae* V5-32	[*C.*] *zeylanoides* V7-43	*W. pararugosa* V1-8
G. candidus	P-3	Grows on		+	+	+	+	+	+
		Inhibits		–	–	–	–	–	–
	Vm-8	Grows on	+	+	+	+	+	+	+
		Inhibits	–	–	–	–	–	–	–
	V12-77	Grows on	+	+	+	+	+	+	+
		Inhibits	–	–	–	–	–	–	–
Debaryomyces sp.	P-2	Grows on	+	+	+	+	+	(+)	(+)
		Inhibits	–	–	–	–	–	–	–
	Vm-7	Grows on	+		+	+	+	(+)	(+)
		Inhibits	–		–	–	–	–	–
K. lactis	P2-8	Grows on	(+)	+		+	+	+	+
		Inhibits	–	z		–	(z)	tz	z
	V7-50	Grows on	–	+	+	+	+	+	+
		Inhibits	tz	z	–	z	z	z	z
K. marxianus	Vm-5	Grows on	(+)	+	+		+	+	+
		Inhibits	–	z	–		(z)	tz	z
	V1-6	Grows on	–	+	+	+	+	+	+
		Inhibits	–	tz	–	–	z	–	z
C. lusitaniae	V5-32	Grows on	+	+	+	+		+	+
		Inhibits	–	–	–	–		–	–
	V3-18	Grows on	(+)	+	+	+	+	+	+
		Inhibits	–	–	–	–	–	–	–
[*C.*] *zeylanoides*	V7-43	Grows on	+	+	+	+	+		+
		Inhibits	–	–	–	–	–		–
	V7-52	Grows on	+	+	+	+	+	+	+
		Inhibits	–	–	–	–	–	–	–
W. pararugosa	V1-8	Grows on	(+)	+	+	+	+	+	
		Inhibits	–	–	–	–	–	–	
	V12-74	Grows on	(+)	+	+	+	+	+	+
		Inhibits	–	–	–	–	–	–	–

+: growth; (+) slow growth; –: no growth, no inhibition.
z: clear inhibition zone.
tz: turbid inhibition zone.

The differences can partially be attributed to the use of different taxonomic names for certain species. For example, *Dipodascus candidum*, *D. silvicola* (Kačániová et al., 2019, 2021), *G. bryndzae* (Sulo et al., 2009), *G. geotrichum*, *G. candidum* (Šaková et al., 2015) can be considered synonyms of *G. candidus*, and all strains affiliated with these names can be conspecific. Based on this assumption, it can be concluded that *G. candidus* is a major yeast in both types of bryndza. The *Candida lusitaniae* isolates identified by MALDI-TOF MS (Kačániová et al., 2021) and the *Clavispora lusitaniae* isolates identified in this study by barcode sequencing can also be conspecific. However, species of the yeast/yeast-like genera *Diutina* (e.g. *C. catenulata*, *C. rugosa*), *Pichia* (e.g. *C. crusei*, *P. cactophila*), *Suhomyces* (e.g. *C. xylopsoci*), *Trichosporon*, *Yarrowia* found in ewe-milk bryndza (Chebeňová-Turcovská et al., 2011; Kačániová et al., 2019, 2021; Pangallo et al., 2014; Šaková et al., 2015) were not detected in the cow-milk bryndza samples. Their absence can be due to the different environment in the cow-milk bryndza, the different technology and/or the different stringency and accuracy of the identification methods applied by the different laboratories (rRNA barcode sequencing versus MALDI-TOFF MS or metabarcoding). The examination of more strains isolated from more samples collected in more farms will shed more light on the differences between the yeast biota compositions of the two types of bryndza.

4.3. The G. candidus isolates are genetically diverse

The presence of ambiguous nucleotides in the D1/D2 sequences of the *Galactomyces* isolates hinted at intragenomic diversity of the repeats coding for rRNA. These units (cistrons) encode three ribosomal RNAs (18S, 5.8S, and 26SrRNA, separated by the internal transcribed sequences ITS1 and ITS2) and are repeated many times in the genome. Despite their high copy numbers, the repeats have essentially identical sequences in most yeast species due to the process referred to as concerted evolution or homogenisation (for a review, see Naidoo et al., 2013). If homogenisation is insufficient, different repeats coexist in the genome and the amplification of their barcode segments results in amplicons containing ambiguous nucleotides (SNPs) at positions where the units differ from each-other. Insufficient repeat homogenisation has been observed in a few yeast species including *Geotrichum* (Alper et al.,

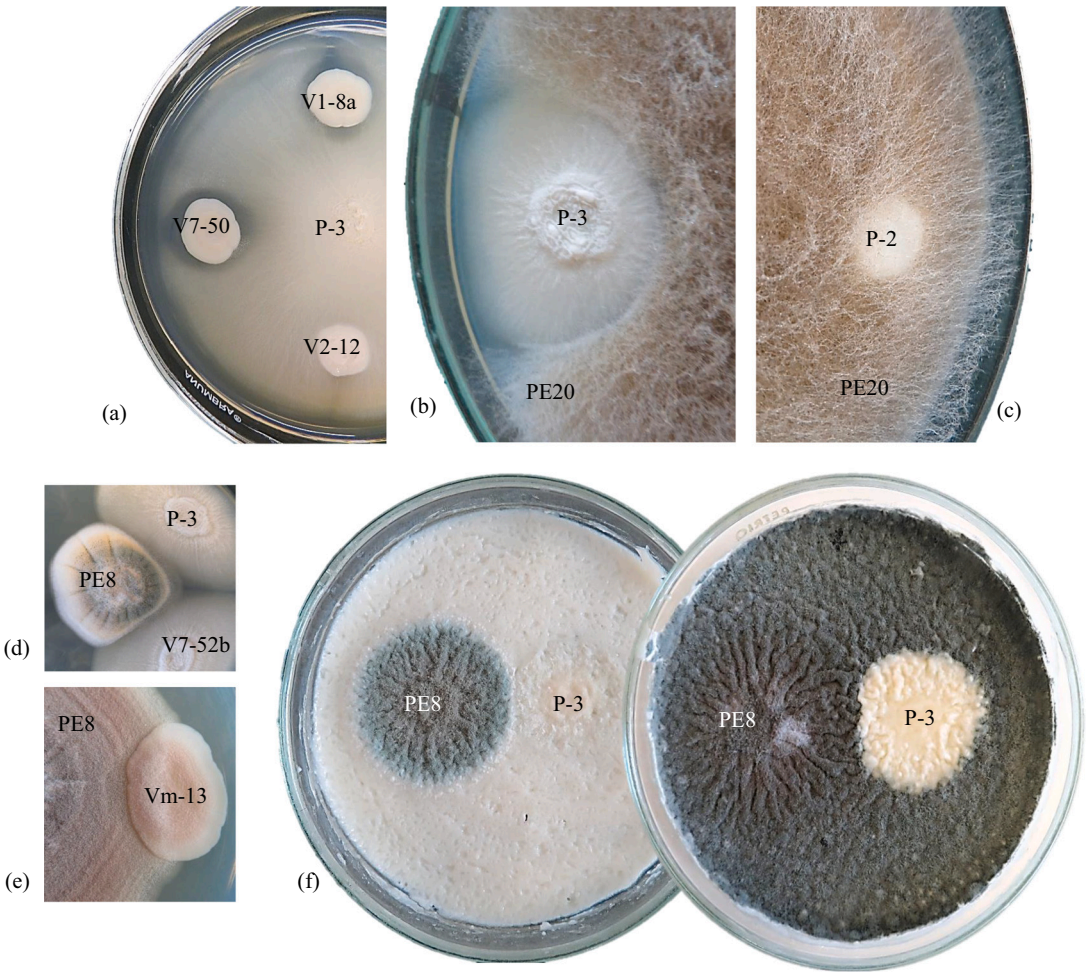

Fig. 7. Antagonism tests by inhibition of mycelium growth. (a) *Kluyveromyces* inhibits the growth of the *Galactomyces* mycelium, but *Wickerhamiella* does not affect it. (b) Inhibition of *Mucor* by *Galactomyces*. (c) *Mucor* is not inhibited by *Debaryomyces*. (d) *Galactomyces* stops the growth of the *Penicillium* colony. (e) *Debaryomyces* does not affect it. The *Penicillium* mycelium grows on the yeast colony. (f) Inhibition of *Penicillium* by *Galactomyces* on bryndza. Medium: YEA in (a to e) and bryndza in (f). P-2 and Vm-13: *Debaryomyces*; P-3 and V7-52b: *G. candidus*; V1-8a: *K. marxianus*; V2-12: *W. pararugosa*; V7-50: *K. lactis*; PE8: *Penicillium*; PE20: *Mucor*.

2011; de Hoog and Smith, 2004; Groenewald et al., 2012) and was investigated in detail in *Metschnikowia* (e.g. Sipiczki, 2022; Sipiczki et al., 2013). Like in *Metschnikowia*, the variable (di- or polymorphic) sites of the *Galactomyces* repeats were located in functionally less critical positions of the D2 fold-back loop. Interestingly, the ITS segments were not diverse.

No intragenomic diversity was detected in the protein-encoding genes proposed by Perkins et al. (2020) for MLST analysis either. However, three of them were diverse at the population level. Two *PGL1* alleles and three *PGM2* alleles were found in the isolates, and from three isolates no *YAP3* sequence could be amplified. The combinations of the alleles of the five genes differed from those found in cheese-borne *Galactomyces* strains in previous studies (Jacques et al., 2017; Perkins et al., 2020).

The different allele combinations of these genes and the rRNA barcode heterogeneity in the isolates indicate that exchange and recombination of genetic material can take place in the *Galactomyces* populations propagating in the cow-milk bryndza. Since numerous tested isolates formed asci in mixed cultures with other isolates, sexual interactions can be assumed to provide possibilities for combining their genetic material via hybridisation and meiotic recombination.

4.4. The yeast species differ in the utilisation of carbon sources available in milk and cheese

Milk and cheese are highly specific environments that can be colonised by yeasts and fungi capable of generating energy for growth from rather unusual carbon sources. The major saccharide as potential carbon source is lactose. Only the *K. marxianus*, *K. lactis* and *Debaryomyces* isolates grew by lactose assimilation. Since both *Kluyveromyces* species can also ferment this sugar (Snoek and Steensma, 2006), they might actively participate in the ripening of the cheese by CO_2 production. However, the contribution of *K. lactis* cannot be significant because, in contrast to its ability to ferment, it cannot grow under anaerobic conditions (Snoek and Steensma, 2006). The close phylogenetic relationship of the *Debaryomyces* isolates to *Debaryomyces hansenii* which has a very low fermentative activity (Sánchez et al., 2006) suggests that they may not play a role in CO_2 production either. The rest of the isolates could only grow on carbon sources that are derivatives of lactose fermentation (lactate), lipids or proteins and can be metabolised by oxygen-requiring processes. Consistent with the aerobic carbon-source metabolism of *Galactomyces*, its hyphae only grew very poorly in semi-anaerobic conditions.

All *Galactomyces* and *Wickerhamiella* isolates grew well on the medium containing lipids as carbon sources. From the intense growth of the *Galactomyces* isolates on lipids and their poor growth on skim-milk medium, it can be inferred that their preferred carbon and energy

source is the lipid of the milk. The *Geotrichum/Galactomyces* yeasts are known to produce extracellular lipolytic enzymes (e.g. Charton and Macrae, 1992; Diks and Lee, 1999) of high hydrolytic activity. We found that the bryndza isolates decomposed the lipid droplets from inside by forming dense mycelia in them. This growth morphology indicates that the lipases are not released into the environment but reside on the outer surface of the cell wall after being secreted from the cytoplasm. No vesicle invasion was observed under cover slips, indicating that the process required oxygen.

The *Kluyveromyces* isolates could use lactate and the proteins of skim milk for growth. *Debaryomyces* also grew well on skim milk. Lactate and amino acids can efficiently be metabolised in aerobic conditions.

In the light of these observations, it is not surprising that we were unable to isolate yeasts from the interior of the cheese. The yeasts seem to preferentially colonise the surface of the cheese and the lipid-containing liquid leaking from it. Being strictly aerobic microorganisms, the moulds found in the samples are also components of the surface microbiota.

4.5. The Galactomyces and Kluyveromyces strains antagonise other yeasts and fungi in different ways

The microorganisms of mixed communities usually interact in a variety of ways. Their interactions affect the population dynamics and shape the composition of the community. Some microorganisms can be inhibited or killed by metabolic substances or antimicrobial compounds released by other members of the microbiota. In this study, the methods applied to test the isolates for antagonisms revealed that *Galactomyces* halted the extension of the *Mucor* and *Penicillium* colonies. Due to this antifungal effect, its strains may act as bioprotective agents that protect the surface of the bryndza from fungal invasion. Similar contact inhibition was observed between *Galactomyces* and *Penicillium* strains isolated from a Danish cheese (Nielsen et al., 1998). The *Kluyveromyces* species also had antagonistic activities. Their strain reduced the growth intensity of the *Galactomyces* mycelium and inhibited the growth of all other yeasts involved in the tests by an iron-suppressible, non-killer mechanism. Since their colonies produced a reddish pigment, the antagonism of the *Kluyveromyces* isolates can be attributed to the secretion of pulcherriminic acid that produces a red complex (pulcherrimin) with ferric ions (Sipiczki, 2020). Interestingly, none of the *Debaryomyces* isolates proved to be antagonistic. Previous studies described dairy *D. hansenii* strains that were antagonistic against various dairy moulds (Liu and Tsao, 2009).

5. Conclusion

The composition of the culturable yeast mycobiota of the bovine bryndza partially differs from that previously described for ovine bryndza. The taxonomic identification is hampered by intragenomic diversity of the D1/D2 barcodes (*Galactomyces*), inconsistency between the results of the analyses of the D1/D2 and ITS barcodes (*Debaryomyces*), and/or low level of sequence identity with type strains (*Clavispora*). The yeast species have diverse abilities to use lactose, lactate, lipids or proteins as carbon and energy sources available in milk and preferentially grow under aerobic conditions (no viable yeasts in the interior of the ripening bryndza). The *G. candidus* strains are genetically diverse, hydrolyse milk protein with diverse, usually moderate efficiency and preferentially utilise lipids by invading the interior of the lipid droplets with a dense mycelium. *G. candidus* can halt the extension of moulds by contact inhibition, and the *Kluyveromyces* isolates can inhibit the growth of all other yeasts and moulds by secretion of pulcherriminic acid.

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CRediT authorship contribution statement

Matthias Sipiczki: Conceptualization, Methodology, Investigation, Formal analysis, Funding acquisition, Project administration, Visualization, Writing – review & editing. **Valéria Hrabovszki:** Methodology, Investigation.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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References

Alper, I., Frenette, M., Labrie, S., 2011. Ribosomal DNA polymorphisms in the yeast geotrichum candidum. Fungal. Biol. 115, 1259–1269.
Bintsis, T., 2021. Yeasts in different types of cheese. AIMS Microbiol. 7, 447–470.
Biolcati, F., Ferrocino, I., Bottero, M.T., Dalmasso, A., 2022. The bacterial and fungal microbiota of “Robiola di roccaverano” protected designation of origin raw milk cheese. Front. Microbiol. 12, 776862.
Boutrou, R., Guéguen, M., 2005. Interests in Geotrichum candidum for cheese technology. Int. J. Food Microbiol. 102, 1–20.
Butler, E.E., Petersen, L.J., 1970. Sexual reproduction on Geotrichum candidum. Science 169, 481–482.
Butler, E.E., Petersen, L.J., 1972. Endomyces geotrichum a perfect state of Geotrichum candidum. Mycologia 64, 365–374.
Charton, E., Macrae, A.R., 1992. Substrate specificities of lipases a and B from Geotrichum candidum CMICC 335426. Biochim. Biophys. Acta 1123, 59–64.
Chebeňová-Turcovská, V., Zenišová, K., Kuchta, T., Pangallo, D., Brežná, B., 2011. Culture-independent detection of microorganisms in traditional Slovakian bryndza cheese. Int. J. Food Microbiol. 150, 73–78.
de Hoog, G.S., Smith, M.T., 2004. Ribosomal gene phylogeny and species delimitation in geotrichum and its teleomorphs. Stud. Mycol. 50, 489–515.
de Hoog, G.S., Smith, M.Th., Guého, E., 1986. A revision of the genus geotrichum and its teleomorphs. Stud. Mycol. 29, 1–131.
Diks, R.M.M., Lee, M.J., 1999. Production of a very low saturate oil based on the specificity of Geotrichum candidum lipase. J. Am. Oil Chem. Soc. 76, 455–462.
Eliskases-Lechner, F., Guéguen, M., Panoff, J.M., 2011. Geotrichum candidum. In: Fuquay, J.W., Fox, P.F., Mcsweeney, P.L.H. (Eds.), Encyclopedia of Dairy Sciences, Volume 4. Academic Press, London, pp. 765–771.
Fox, P.F., 1993. Cheese: an overview. In: Fox, P.F. (Ed.), Cheese: Chemistry, Physics and Microbiology. Springer, Boston, pp. 1–36.
Groenewald, M., Coutinho, T., Smith, M.T., van der Walt, J.P., 2012. Species reassignment of geotrichum bryndzae, geotrichum phurueaensis, geotrichum silvicola and geotrichum vulgare based on phylogenetic analyses and mating compatibility. Int. J. Syst. Evol. Microbiol. 62, 3072–3080.
Groenewald, M., Daniel, H.-M., Robert, V., Poot, G.A., Smith, M.T., 2008. Polyphasic re-examination of Debaryomyces hansenii strains and reinstatement of D. hansenii, D. fabryi and D. subglobosus. Persoonia 21, 17–27.
Haastrup, M.K., Johansen, P., Malskær, A.H., Castro-Mejía, J.L., Kot, W., Krych, L., Arneborg, N., Jespersen, L., 2018. Cheese brines from danish dairies reveal a complex microbiota comprising several halotolerant bacteria and yeasts. Int. J. Food Microbiol. 285, 173–187.
Jacques, N., Mallet, S., Casaregola, S., 2009. Delimitation of the species of the Debaryomyces hansenii complex by intron sequence analysis. Int. J. Syst. Evol. Microbiol. 59, 1242–1251.
Jacques, N., Mallet, S., Laaghouiti, F., Tinsley, C.R., Casaregola, S., 2017. Specific populations of the yeast geotrichum candidum revealed by molecular typing. Yeast 34, 165–178.
Jacques, N., Sacerdot, C., Derkaoui, M., Dujon, B., Ozier-Kalogeropoulos, O., Casaregola, S., 2010. Population polymorphism of nuclear mitochondrial DNA insertions reveals widespread diploidy associated with loss of heterozygosity in Debaryomyces hansenii. Eukaryot. Cell 9, 449–459.

Jagadeesan, B., Gerner-Smidt, P., Allard, M.W., Leuillet, S., Winkler, A., Xiao, Y., Chaffron, S., Van Der Vossen, J., Tang, S., Katase, M., McClure, P., Kimura, B., Ching Chai, L., Chapman, J., Grant, K., 2019. The use of next generation sequencing for improving food safety: translation into practice. Food Microbiol. 79, 96–115.

Ji-Yoon, K., Sang-Min, H., Seon-Jeong, P., Ji-Eun, J., Jong-Soo, L., 2020. Isolation and characterization of unrecorded wild yeasts obtained from soils of spice fields and mountains. Kor. J. Mycol. 48, 151–160.

Kačániová, M., Kunová, S., Štefániková, J., Felšöciová, S., Godočíková, L., Horská, E., Nagyová, L., Haščík, P., Terentjeva, M., 2019. Microbiota of the traditional slovak sheep cheese "Bryndza". J. Microbiol. Biotechnol. Food Sci. 9, 482–486.

Kačániová, M., Terentjeva, M., Kunová, S., Haščík, P., Kowalczewski, P.Ł., Štefániková, J., 2021. Diversity of microbiota in slovak summer ewes' cheese "Bryndza". Open Life Sci. 16, 277–286.

Kurtzman, C.P., Fell, J.W., Boekhout, T., 2011. Methods for isolation, phenotypic characterization and maintenance of yeasts. In: Kurtzman, C.P., Fell, J.W., Boekhout, T. (Eds.), The Yeasts: A Taxonomic Study. Elsevier, Amsterdam, pp. 87–110.

Lachance, M.-A., 2011. Chapter 21 - Clavispora Rodrigues de Miranda (1979). In: Kurtzman, C.P., Fell, J.W., Boekhout, T. (Eds.), The Yeasts: A Taxonomic Study. Elsevier, Amsterdam, pp. 349–353.

Lachance, M.-A., Daniel, H.M., Meyer, W., Prasad, G.S., Gautam, S.P., Boundy-Mills, K., 2003. The D1/D2 domain of the large-subunit rDNA of the yeast species Clavispora lusitaniae is unusually polymorphic. FEMS Yeast Res. 4, 253–258.

Liu, S.-Q., Tsao, M., 2009. Biocontrol of dairy moulds by antagonistic dairy yeast Debaryomyces hansenii in yoghurt and cheese at elevated temperatures. Food Control 20, 852–855.

Lopandic, K., Rentsendorj, U., Prillinger, H., Sterflinger, K., 2013. Molecular characterization of the closely related debaryomyces species: proposition of D. vindobonensis sp. Nov. From a municipal wastewater treatment plant. J. Gen. Appl. Microbiol. 59, 49–58.

Marsay, K.S., Koucherov, Y., Davidov, K., Iankelevich-Kounio, E., Itzahri, S., Salmon-Divon, M., Oren, M., 2022. High-resolution screening for marine prokaryotes and eukaryotes with selective preference for polyethylene and polyethylene terephthalate surfaces. Front. Microbiol. 13, 845144.

Naidoo, K., Steenkamp, E.T., Coetzee, M.P.A., Wingfield, M.J., Wingfield, B.D., 2013. Concerted evolution in the ribosomal RNA cistron. PLoS ONE 8, e59355.

Nguyen, H.V., Gaillardin, C., Neuveglise, C., 2009. Differentiation of Debaryomyces hansenii and Candida famata by rRNA gene intergenic spacer fingerprinting and reassessment of phylogenetic relationships among D. hansenii, C. famata, D. fabryi, C. flareri (=D. subglobosus) and D. prosopidis: description of D. vietnamensis sp. nov. closely related to D. nepalensis. FEMS Yeast Res. 9, 641–662.

Nielsen, M.S., Frisvad, J.C., Nielsen, P.V., 1998. Colony interaction and secondary metabolite production of cheese-related fungi in dual culture. J. Food Prot. 61, 1023–1029.

O'Donnell, K., 1993. Fusarium and its near relatives. In: Reynolds, D.R., Taylor, J.W. (Eds.), The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics. CAB International, Wallingford, pp. 225–233.

Palo, V., Kaláb, M., 1984. Slovak sheep cheeses. Milchwissenschaft 39, 518–521.

Paloi, S., Jennifer Luangsa-ard, J., Mhuantong, W., Stadler, M., Kobmoo, N., 2022. Intragenomic variation in nuclear ribosomal markers and its implication in species delimitation, identification and barcoding in fungi. Fungal Biol. Rev. In press.

Pangallo, D., Šaková, N., Koreňová, J., Puškárová, A., Kraková, L., Valík, L., Kuchta, T., 2014. Microbial diversity and dynamics during the production of may bryndza cheese. Int. J. Food Microbiol. 170, 38–43.

Perkins, V., Vignola, S., Lessard, M.-H., Plante, P.-L., Corbeil, J., Dugat-Bony, E., Frenette, M., Labrie, S., 2020. Phenotypic and genetic characterization of the cheese ripening yeast geotrichum candidum. Front. Microbiol. 11, 737.

Reuter, J.S., Mathews, D.H., 2010. RNAstructure: software for RNA secondary structure prediction and analysis. BMC Bioinformatics 11, 129.

Šaková, N., Sádecká, J., Lejková, J., Puškárová, A., Koreňová, J., Kolek, E., Valík, Ľ., Kuchta, T., Pangallo, D., 2015. Characterization of may bryndza cheese from various regions in Slovakia based on microbial, molecular and principal volatile odorants examinations. J. Food Nutr. Sci. 54, 239–251.

Sánchez, N.S., Calahorra, M., González-Hernández, J.C., Peña, A., 2006. Glycolytic sequence and respiration of Debaryomyces hansenii as compared to Saccharomyces cerevisiae. Yeast 23, 361–374.

Sipiczki, M., 2003. Candida zemplinina sp. nov., an osmotolerant and psychrotolerant yeast that ferments sweet botrytised wines. Int. J. Syst. Evol. Microbiol. 53, 2079–2083.

Sipiczki, M., 2011. Dimorphic cycle in Candida citri sp. nov., a novel yeast species isolated from rotting fruit in Borneo. FEMS Yeast Res. 11, 202–208.

Sipiczki, M., 2016. Overwintering of vineyard yeasts: survival of interacting yeast communities in grapes mummified on vines. Front. Microbiol. 7, 212.

Sipiczki, M., 2020. Metschnikowia pulcherrima and related pulcherrimin-producing yeasts: fuzzy species boundaries and complex antimicrobial antagonism. Microorganisms 8. E1029.

Sipiczki, M., 2022. When barcoding fails: genome chimerization (admixing) and reticulation obscure phylogenetic and taxonomic relationships. Mol. Ecol. Resour. 22, 1762–1785.

Sipiczki, M., Pfliegler, W.P., Holb, I.J., 2013. Metschnikowia species share a pool of diverse rRNA genes differing in regions that determine hairpin-loop structures and evolve by reticulation. PLoS One 8, e67384.

Snoek, I.S., Steensma, H.Y., 2006. Why does kluyveromyces lactis not grow under anaerobic conditions? Comparison of essential anaerobic genes of Saccharomyces cerevisiae with the kluyveromyces lactis genome. FEMS Yeast Res. 6, 393–403.

Sulo, P., Laurencík, M., Poláková, S., Minárik, G., Sláviková, E., 2009. Geotrichum bryndzae sp. Nov., a novel asexual arthroconidial yeast species related to the genus galactomyces. Int. J. Syst. Evol. Microbiol. 59, 2370–2374.

Tantirungkij, M., Nasanit, R., Limtong, S., 2015. Assessment of endophytic yeast diversity in rice leaves by a culture-independent approach. Antonie Van Leeuwenhoek 108, 633–647.

Thompson, J.D., Higgions, D.G., Gibson, T.J., 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

van Uden, N., Carmo-Sousa, L., 1959. Further studies on the significance of temperature relations and vitamin deficiency patterns in yeast taxonomy. Port. Acta Biol. Série B 6, 239–256.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols. A Guide to Methods and Applications. Academic Press, San Diego, pp. 315–322.