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Yeast strains with technological and probiotic traits isolated from Mihalic cheese

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Abstract

The present work investigated the technological and probiotic characteristics of 20 yeast strains originating from Mihalic cheese. All isolates exhibited lipolytic activities. Proteolytic activity was detected only in the strains *Geotrichum silvicola* and *Trichosporon asahii*. While all isolates assimilated glucose, 85% and 40% of isolates also assimilated galactose and lactose, respectively. Furthermore, 55% and 25% of the strains were positive for glucose and galactose fermentation, respectively. Lactate assimilation was detected in 60% of the isolates. Most isolates were able to grow at 37°C and 4°C, while 40% were also able to grow at 45°C. All yeast strains could grow at pH 5.4 and 4.0, and 65% were also able to grow at a pH of 2.5. All isolates could grow at 5% and 10% sodium chloride and 11 of them grew at 15% salt concentration. *Candida inconspicua* and *C. tropicalis* were found to be the species that were most resistant to 0.3% (w/v) Ox-Bile. Eight isolates exhibited weak inhibitory effects on *Staphylococcus aureus*. Three strains belonging to *C. famata* var. *famata*, *C. catenulata* and *G. silvicola* weakly inhibited the growth of *L. monocytogenes*. Based on the desired technological characteristics, *G. silvicola* M15 and *T. asahii* M1 isolates showed potential as adjunct starters.

Introduction

Fermented dairy products are widely valued as healthy components of human diet (Georgieva *et al.*, 2009). Currently, incorporation of lactic acid bacteria (LAB) and yeasts into various fermented milk products, as starters or adjuncts, is an important process used in industrial and commercial applications (Georgieva *et al.*, 2009; Padilla *et al.*, 2014). Biochemical traits of yeasts recovered in high numbers from cheeses (e.g., 10^6–10^9 CFU/g) are known to influence the organoleptic characteristics of cheese (Capece and Romano, 2009). *Debaryomyces hansenii* and *Yarrowia lipolytica* have been successfully used as components of starter cultures in cheese manufacturing, particularly to enhance flavour during cheese maturation (Golić *et al.*, 2013).

Composition of the microbial population plays a significant role in cheese maturation (Beresford *et al.*, 2001; Gardini *et al.*, 2006). Although starter lactic acid bacteria are responsible for acid production which contributes to the ripening process, secondary microbiota, consisting mainly of enterococci, micrococci, non-starter LAB and yeasts, also make an important contribution to cheese maturation. High counts of some yeast species seen in cheese have been attributed to their tolerance of low pH, reduced water activity and high salt concentrations, as well as to their ability to grow at low storage temperatures characteristic of the ripening environment (Ferreira and Viljoen, 2003; Gardini *et al.*, 2006). Reportedly, other important processes which occur in cheese were the assimilation/fermentation of lactose and galactose, and the assimilation of succinic, lactic and citric acids. In addition, the ability of dairy yeast strains to survive heat treatment and sanitising agents has been reported (Gardini *et al.*, 2006). Consumption of lactate, formation of alkaline metabolites, fermentation of lactose, lipolysis, proteolysis, and formation of aroma compounds are some other yeast activities, reported to be important for the development of typical characteristics of

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certain cheese varieties. On the other hand, yeasts may also act as spoilage organisms, causing typical defects such as yeasty off-flavour, loss of texture quality, excessive gas formation, and brown surface discoloration (Gardini *et al.*, 2006).

The biochemical and probiotic characteristics of yeasts occurring in various traditional cheeses have been previously reported (Karasu-Yalcin *et al.*, 2012; Golić *et al.*, 2013; Gkatzionis *et al.*, 2014; Ceugniez *et al.*, 2015). Some yeast strains originating from fermented products have shown probiotic potential as indicated by their ability to survive and colonise the gastrointestinal tract in different mammalian cell model assays, by resisting 37°C, low pH and the presence of bile salts. Furthermore, some probiotic yeasts may inhibit pathogenic bacteria (Silva *et al.*, 2011; Pedersen *et al.*, 2012).

Mihalic cheese, which in terms of production levels is among the top five in Turkey, is made from raw or pasteurised sheep's milk. It is widely produced in the provinces of Bursa, Balikesir and Canakkale (Cokal *et al.*, 2012). It is a hard, brined cheese, which is slightly acidic and very salty, and has regular openings and a 3-4 mm rind (Hayaloglu *et al.*, 2008). Although it is estimated that Mihalic cheese has a history of at least 200 years, studies on the microbiota of this cheese are scant. The objective of the present work was therefore to evaluate the technological characteristics of the yeast strains isolated from traditional Mihalic cheese based on their biochemical traits. Some probiotic characteristics of the yeasts were also investigated.

Materials and methods

Yeast strains and Identification by MALDI TOF/TOF MS

Twenty yeast strains, previously isolated from Mihalic cheese samples, were used in the present work. The yeast strains had been identified by API ID 32C (BioMerieux, France) and some additional identification tests conducted in our previous study (Karasu-Yalcin *et al.*, 2017). Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is a novel, high throughout identification method, based on the analysis of whole cell proteins, which is fast and cost-effective (Nacef *et al.*, 2017). MALDI-TOF analysis has been reported as a powerful tool that can be used for the identification of closely related yeast species which are difficult to distinguish using biochemical profiles (Guitard *et al.*, 2015). All yeast isolates were re-identified using MALDI TOF/TOF MS with tandem time-of-flight mass analysers. The identification based on these results was compared to those previously obtained via biochemical methods. The MALDI TOF/TOF mass spectrometer (Autoflex Speed, Bruker Daltonics, Germany) and the MALDI Biotyper 3.4 software package were used for identification based on the analysis of protein mass spectra. For sample preparation, the protein extraction method (ethanol - acetonitrile - formic acid) described by Schulthess *et al.* (2013) was used. Mass spectra were processed using Biotyper software (version 3.4; Bruker Daltonics) running the Biotyper database version DB-6903. Matching experimental profiles obtained from the unknown microorganism against reference profiles was conducted via Biotyper on a score basis. A score higher than 2.0 indicated identification at the species level, while a score between 1.7 and 2.0 implied a genus level identification. A score value under 1.7 indicated the absence of a significant similarity between the unknown profile and the database (Nacef *et al.*, 2017).

Determination of some technological characteristics of the yeast strains

The technological characteristics investigated were proteolytic and lipolytic activities, fermentation and assimilation of certain sugars, assimilation of some organic acids, growth at different pH and temperatures, and growth at different salt concentrations.

In order to determine proteolytic activities, activated yeast cultures were inoculated into 10% (w/v) milk agar media treated with reconstituted skim milk. Colonies surrounded by a clear zone following incubation at 28°C for 2-14 d were recorded as positive results (Harrigan, 1998). Lipolytic activities of the yeast strains were investigated according to Harrigan (1998). Activated cultures were inoculated on tributyrin agar and incubated at 28°C for 2-14 d. Formation of a clear zone as a result of tributyrin hydrolysis was considered as a positive result and recorded accordingly. Glucose, galactose and lactose fermentations were investigated using media with Durham tubes including 2% (w/v) of each tested sugar. Activated yeast cultures were inoculated into the media and incubated at 28°C for up to 28 d. Positive results were indicated by growth and gas formation in the Durham tubes (Yarrow, 1998). Assimilation of similar sugars was determined via assimilation test results obtained from API ID 32C strips (BioMerieux).

Assimilation of citric and succinic acid was investigated via the auxanographic method in yeast nitrogen base (YNB) agar (Deak and Beuchat, 1996;

Yarrow, 1998; Kurtzman *et al.*, 2003). Activated yeast cultures were suspended in distilled water and 0.5 mL of suspensions with a 2 McFarland turbidity were inoculated into YNB agar using the poured plate method, and following solidification, a small amount of granules (0.05 - 0.1 g) of the organic acid salt were aseptically deposited on the surface of the agar. Glucose granules were added onto each plate as a positive control. Media were incubated at 28°C for 2-3 d and growth around the organic acid granules was evaluated as positive. Assimilation profiles of API ID 32C (BioMerieux) test results were used for the assimilation of lactic acid (DL-lactate).

Growth of yeast strains at different pH values was investigated in malt extract (ME) broth (Psomas *et al.*, 2001). First, pH levels of the media were adjusted to 2.5 and 4.0. ME broth (pH 5.4) was used as the control medium. These pH values were selected based on cheese production conditions and the desired resistance of probiotics to gastric pH. In order to determine growth at different temperatures, activated cultures were inoculated into ME broth and then incubated at 4°C, 10°C, 28°C, 37°C, and 45°C for up to 7 d (Yarrow, 1998). Salt tolerance of the yeast strains was evaluated using ME broth including 5%, 10%, and 15% (w/v) NaCl (Yarrow, 1998). ME broth without salt was used as the control. Activated yeast cultures were inoculated into ME broth with NaCl and incubated at 28°C for 7 d. Salt concentrations were selected according to the properties of cheese. Water activity of the media was also determined.

Determination of some potential probiotic characteristics of yeast strains

Two probiotic properties, bile salt tolerance and inhibition of some pathogenic bacteria, were investigated. Bile salt tolerance was determined in ME agar including 0.5% and 1% (w/v) bile salt no.3 (Lab M, UK). For this purpose, 24-48 h old yeast cultures were inoculated into the media containing bile salt, by streaking on the agar medium surface, and incubated at 37°C for 72 h (Psomas *et al.*, 2001). Yeast strains resistant to a concentration of 1% (w/v) bile salt were activated in YM broth inoculated into ME broth with 0.3% (w/v) Ox-Bile (Merck, USA) at 10^5 CFU/mL and incubated at 37°C. Ox-Bile tolerance of the tested yeast strains were evaluated before and after 72 h of incubation, via yeast counts using ME agar. Strains unable to grow at 37°C could not be tested for bile salt tolerance.

The inhibition effects of the yeast strains on *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* were investigated via the modified agar-cup diffusion method (Izgü *et al.*, 1997). *E. coli* ATCC 35218, *S. aureus* ATCC 25523 and *L. monocytogenes* UVM 1462 strains were used for these experiments. *L. monocytogenes* UVM 1462 was obtained from Institute of Milk Hygiene, Milk Technology and Food Science, University of Veterinary Medicine (Vienna, Austria), coming from the culture collection of the Institute. Bacterial cultures were activated on brain heart infusion (BHI) agar at 37°C. Next, suspensions of bacterial cultures with 0.5 McFarland turbidity were prepared in 0.85% NaCl and spread on BHI agar in Petri dishes. Next, three wells were constructed on BHI agar, at the bottoms of which ME agar was placed. Yeast cultures were activated in ME broth at 30°C for 24 h. Subsequently, 0.2 mL yeast cultures were added to the prepared wells. Inhibition zones around the wells were examined following incubation at 30°C for 24-48 h. The probiotic yeast *Saccharomyces boulardii* CNCM I-745 (Biocodex, France) was used as the control strain in these experiments.

Results and discussion

Identification of yeast strains by MALDI TOF/TOF MS

Identification results obtained by MALDI TOF/TOF MS are shown in Table 1. Re-identification was achieved for 90% of the yeasts. Fifteen of the isolates were identified at species level with score values higher than 2.0. The species identification of seven isolates; M1, M25, M57, M2, M43, M22 and M16, fully matched those previously obtained via API ID 32C and additional identification tests. In addition, the strain M54 was identified as *Kodamaea ohmeri* (at genus level), which is a teleomorph of *Candida guilliermondii* var. *membranefaciens*. However, the ascospore formation test indicated that this strain lacked ascospores and was therefore incapable of sexual reproduction (Karasu-Yalcin *et al.*, 2017). Thus, the identification of M54 as *C. guilliermondii* var. *membranefaciens* was confirmed at the genus level by MALDI TOF/TOF MS. The isolate M3 was identified as *Pichia cactophila*, which is the teleomorph of *Candida inconspicua*. Since M3 was also incapable of sexual reproduction, according to Karasu-Yalcin *et al.* (2017), it was identified as *C. inconspicua*. Two isolates, M6 and M15, previously identified as *Geotrichum candidum*, were identified as *Geotrichum silvicola* by MALDI TOF/TOF MS. Reportedly *G. silvicola* is phylogenetically and phenotypically close to *G. candidum* (Groenewald *et al.*, 2012). The isolates M73 and M77 were identified as *C. catenulata* while M22, M83, M32, and M63 were identified as *C. famata* by MALDI

Table 1. Identification results obtained using MALDI TOF/TOF MS in comparison with previous biochemical identifications.

Isolate no.	API ID 32C and additional identification tests (Karasu-Yalcin *et al*., 2017)	MALDI TOF/TOF MS	
		Result	Score value
M1	*Trichosporon asahii*	*Trichosporon asahii*	2.181
M25	*Candida catenulata*	*Candida catenulata*	2.548
M57	*Candida krusei*	*Candida krusei*	2.506
M54	*Candida guilliermondii* var. *membranefaciens*	*Kodamaea ohmeri*[a]	1.991
M8	*Candida robusta*	*Candida famata*	1.897
M2	*Candida tropicalis*	*Candida tropicalis*	2.223
M43	*Candida tropicalis*	*Candida tropicalis*	2.294
M3	*Candida norvegensis*	*Pichia cactophila*[b]	2.294
M22	*Candida famata* var. *famata*	*Candida famata*	2.327
M81	*Candida famata* var. *famata*	NRI[c]	1.533
M18	*Candida famata* var. *famata*	*Candida famata*	1.948
M89	*Candida famata* var. *famata*	NRI[c]	1.488
M6	*Geotrichum candidum*	*Geotrichum silvicola*	2.163
M15	*Geotrichum candidum*	*Geotrichum silvicola*	2.186
M16	*Candida zeylanoides*	*Candida zeylanoides*	2.559
M73	*Candida cylindracea*	*Candida catenulata*	2.346
M77	*Candida cylindracea*	*Candida catenulata*	2.376
M83	*Candida cylindracea*	*Candida famata*	2.134
M32	*Candida paludigena*	*Candida famata*	2.354
M63	*Candida bertae*	*Candida famata*	2.255

[a]Identified as *Candida guilliermondii* var. *membranefaciens* (anamorph form).

[b]Identified as *Candida inconspicua* (anamorph form).

[c]No reliable identification.

TOF/TOF MS (Table 1). The variety of the isolate M22 was determined as *C. famata* var. *famata* due to its inability to grow at 37°C. The identification of M54, M8 and M18 could be confirmed at the genus level via MALDI TOF/TOF MS. The isolates M81 and M89 could not be identified in a reliable manner, probably due to the absence of reference spectra in the database.

Technological characteristics of the yeast strains

Results related to proteolytic and lipolytic activities, assimilation/fermentation of certain sugars and assimilation of certain organic acids are shown in Table 2. It was found that all isolates showed lipolytic activities while only *T. asahii* (M1) and *G. silvicola* (M15) exhibited proteolytic activities. Although the secondary flora of cheese is reportedly proteolytic during ripening (Sousa *et al.*, 2001), the yeast isolates of Mihalic cheese were noteworthy due to their lipolytic activities rather than their proteolytic activities. Lipolysis results in the formation of free fatty acids, which are precursors of flavour compounds such as methylketones, alcohols and lactones (de Wit *et al.*, 2005). De Freitas *et al.* (2009) reported the enhancement of lipolysis in Cantalet cheese due to the activity of *Y. lipolytica* and suggested that the use of yeast may enhance the modulation of cheese flavour. Padilla *et al.* (2014) demonstrated that *D. hansenii* possibly contributed to lipolysis and the liberation of free fatty acids during ripening of traditional ewe and goat cheese. *C. famata* (anamorph of *D. hansenii*) was the predominant yeast in Mihalic cheese (Karasu-Yalcin *et al.*, 2017). Although it is known as a potential adjunct having both proteolytic and lipolytic activities, none of the *C. famata* isolates in the present work displayed proteolytic activity. This characteristic of *D. hansenii* was also reported by Atanassova *et al.* (2016), who investigated proteolytic and lipolytic activities of yeasts isolated from short-ripened, starter-free, raw cow milk cheese, made in Galicia. They reported that *Y. lipolytica*, *K. lactis*, and *D. hansenii* strains displayed high lipolytic activity. In the present work, *G. silvicola* stood out in regard to its proteolytic activity in addition to lipolytic activity, which may be a reason for its selection as an adjunct starter in cheese production. *G. silvicola* is reported to be phylogenetically very close to *G. candidum*, a typical dairy yeast (Groenewald *et*

Table 2. Results of proteolytic and lipolytic activities, assimilation/fermentation of some sugars and assimilation of some organic acids.

Isolate no.	Yeast species	Proteolytic activity	Lipolytic activity	Glucose A/F	Galactose A/F	Lactose A/F	DL-lactate	Citric acid	Succinic acid
M1	*Trichosporon asahii*	+	+	+/-	+/-	+/-	+	-	+
M25	*Candida catenulata*	-	+	+/+	+/-	-/-	+	-	-
M57	*Candida krusei*	-	+	+/+	-/-	-/-	+	+	+
M54	*Candida guilliermondii* var. *membranefaciens*	-	+	+/+	+/+	-/-	-	+	-
M8	*Candida robusta*	-	+	+/+	-/-	-/-	-	-	-
M2	*Candida tropicalis*	-	+	+/+	+/+	-/-	-	+	+
M43	*Candida tropicalis*	-	+	+/+	+/+	-/-	-	+	+
M3	*Candida inconspicua*	-	+	+/+	-/-	-/-	+	+	+
M22	*Candida famata* var. *famata*	-	+	+/-	+/-	+/-	+	-	-
M81	*Candida famata* var. *famata*	-	+	+/-	+/+	+/-	-	-	-
M18	*Candida famata* var. *famata*	-	+	+/-	+/-	+/-	+	-	+
M89	*Candida famata* var. *famata*	-	+	+/-	+/+	+/-	-	-	-
M6	*Geotrichum silvicola*	-	+	+/-	+/-	-/-	-	-	+
M15	*Geotrichum silvicola*	+	+	+/-	+/-	-/-	+	-	+
M16	*Candida zeylanoides*	-	+	+/+	+/-	-/-	-	-	-
M73	*Candida catenulata*	-	+	+/+	+/-	-/-	+	-	-
M77	*Candida catenulata*	-	+	+/+	+/-	-/-	+	-	-
M83	*Candida famata*	-	+	+/+	+/-	+/-	+	+	-
M32	*Candida famata*	-	+	+/-	+/-	+/-	+	-	-
M63	*Candida famata*	-	+	+/-	+/-	+/-	+	-	-

A: assimilation, F: fermentation.

al., 2012). It was reported that *G. candidum* may improve the organoleptic properties of cheese by its action on protein metabolism, causing the hydrolysis of β-casein (Boutrou and Guéguen, 2005). *T. asahii* was the other species exhibiting proteolytic activity. Suzzi *et al.* (2003) reported that *T. asahii* was the most frequently encountered yeast in Manteca cheese and that caseinolytic activity was displayed by all *T. asahii* isolates, some of which produced very high amounts of free amino acids in milk.

All isolates assimilated glucose, while 9 (45%) did not exhibit fermentation ability (Table 2). Galactose assimilation was also a common property among the yeast isolates. All isolates except *C. krusei* (M57), *C. robusta* (M8) and *C. inconspicua* (M3) assimilated galactose. Only five isolates belonging to *C. guilliermondii* var. *membranefaciens* (M54), *C. tropicalis* (M2, M43) and *C. famata* var. *famata* (M81, M89) fermented galactose. Lactose assimilation was displayed by eight isolates belonging to the species of *T. asahii* (M1), *C. famata* var. *famata* (M22, M81, M18, M89) and *C. famata* (M83, M32, M63). None of the isolates were able to ferment lactose. Residual sugars, such as lactose and galactose, are reported to be commonly present in cheeses at levels ranging from trace amounts to 5% (Lee *et al.*, 2014). It has been reported that lactose is partially hydrolysed to glucose and galactose during cheese production (Ferreira and Viljoen, 2003). Ferreira and Viljoen (2003) also reported that glucose content of cheese was in trace amounts, since it was rapidly fermented by starter bacteria. They further reported that *D. hansenii* and *Y. lipolytica* contributed to galactose metabolism in Cheddar cheese when used as adjunct starters. Narvhus and Gadaga (2003) reported that galactose utilisation by yeasts in some naturally fermented milks occurred as a part of their interaction with galactose-exporting lactic acid bacteria. Lactose assimilation and fermentation are considered as important characteristics for cheese-originated microorganisms. Kagkli *et al.* (2006) investigated lactose assimilation properties of three *K. lactis*, 1 *K. marxianus*, and two *S. cerevisiae* strains isolated from Camembert cheese, in a cheese-based medium. It was reported that all *Kluyveromyces* strains rapidly used lactose, while *S. cerevisiae* strains could not use this sugar. In another study, some yeast strains belonging to *K. lactis*, *Y. lipolytica*, and *P. fermentans* were used as adjunct starters in Cantalet cheese production. Reportedly,

Table 3. Growth at different temperatures, pH and NaCl concentrations.

Isolate no.	Yeast species	Temperature (°C)					pH			NaCl (%)		
		4	10	28	37	45	2.5	4.0	5.4	5	10	15
M1	*Trichosporon asahii*	+	+	+	+	+	+	+	+	+	+	-
M25	*Candida catenulata*	+	+	+	-	-	+	+	+	+	+	-
M57	*Candida krusei*	+	+	+	+	+	+	+	+	+	+	-
M54	*Candida guilliermondii var. membranefaciens*	+	+	+	+	+	+	+	+	+	+	+
M8	*Candida robusta*	+	+	+	+	-	-	+	+	+	+	+
M2	*Candida tropicalis*	-	+	+	+	+	+	+	+	+	+	-
M43	*Candida tropicalis*	-	+	+	+	+	+	+	+	+	+	-
M3	*Candida inconspicua*	+	+	+	+	+	+	+	+	+	+	-
M22	*Candida famata* var. *famata*	+	+	+	+	-	-	+	+	+	+	+
M81	*Candida famata* var. *famata*	+	+	+	-	-	-	+	+	+	+	+
M18	*Candida famata* var. *famata*	+	+	+	-	-	-	+	+	+	+	+
M89	*Candida famata* var. *famata*	+	+	+	-	-	+	+	+	+	+	+
M6	*Geotrichum silvicola*	+	+	+	+	+	+	+	+	+	+	+
M15	*Geotrichum silvicola*	+	+	+	+	+	+	+	+	+	+	+
M16	*Candida zeylanoides*	+	+	+	+	-	+	+	+	+	+	-
M73	*Candida catenulata*	+	+	+	+	-	-	+	+	+	+	-
M77	*Candida catenulata*	+	+	+	+	-	-	+	+	+	+	-
M83	*Candida famata*	+	+	+	-	-	-	+	+	+	+	+
M32	*Candida famata*	+	+	+	-	-	+	+	+	+	+	+
M63	*Candida famata*	+	+	+	+	-	+	+	+	+	+	+

residual lactose concentration did not change with the use of yeasts, indicating that the yeasts did not use lactose (De Freitas *et al.*, 2009). Although it has been reported that lactose fermentation by yeasts influences aroma formation due to the formation of ethanol and acetaldehyde (Welthagen and Viljoen, 1999), lactose fermentation was not found to be a common property among yeasts originating from cheeses (Ferreira and Viljoen, 2003). In the present work, none of the Mihalic cheese isolates exhibited lactose fermentation ability. Narvhus and Gadaga (2003) reported that most yeast species found in non-fermented milk were lactose-negative. Ferreira and Viljoen (2003) reported that although yeasts are traditionally associated with fermentation, nearly half of the presently known yeast species lacked the ability to ferment sugars, and that lactose-fermenting species were not typically represented in the cheese environment. This was substantiated by the results obtained in the present work.

Twelve isolates (60%) were detected as positive for lactate assimilation (Table 2). Of these, however, only six (30%) could assimilate citrate. Eight isolates (40%) displayed succinic acid assimilation ability. These were *C. famata* var. *famata* (M18), *G. silvicola* (M6, M15), *T. asahii* (M1), *C. krusei* (M57), *C. tropicalis* (M2, M43) and *C. inconspicua* (M3). It is known that organic acids are formed in dairy products during the course of normal metabolism and the breakdown of milk proteins, fat, lactose and citrate during manufacture and storage (Manolaki *et al.*, 2006). Among organic acids found in cheese, lactic acid is present in higher concentrations than those found in the others (Beresford *et al.*, 2001). It is known that yeasts contribute to the ripening process by consuming lactate and increasing pH, thereby supporting the growth of lactic acid bacteria (Ferreira and Viljoen, 2003). Petersen *et al.* (2002), investigated lactate assimilation by *D. hansenii* strains in Danish surface-ripened cheeses during ripening. They reported that the ability of *D. hansenii* to grow on lactate varied according to the strain, which was similar to the observations made in regard to *C. famata* (anamorph of *D. hansenii*) in the present work. Kagkli *et al.* (2006), compared deacidification capacities of *K. lactis* and *S. cerevisiae* strains isolated from cheeses, in a cheese-like medium. It was reported that the strains of both species grew well in a lactate medium and caused an increase in pH. Citrate assimilation has been cited as a reason for the survival of some yeast strains during cheese ripening (Ferreira and Viljoen, 2003). Citrate assimilation could also contribute to deacidification via lactate formation during the ripening process (Kagkli *et*

al., 2006). Van Den Tempel and Jakobsen (1998) investigated technological properties of *D. hansenii* and *Y. lipolytica* strains isolated from Danablu cheese. They reported that all strains assimilated lactate and citrate, except for one, the *D. hansenii* strain. Reportedly, succinate can be produced in cheese during metabolism of citrate by lactic acid bacteria (Skeie *et al.*, 2008). Boutrou and Guéguen (2005) reported that lactate, citrate and succinate were among the carbon sources for *G. candidum* during cheese ripening, but only some strains of *G. candidum* could assimilate these organic acids. The present work found that *G. silvicola* M15 originating from Mihalic cheese was capable of using lactate and succinate but could not use citrate.

The growth of yeast isolates at different pH, temperatures and NaCl concentrations are shown in Table 3. All isolates grew well at 10°C and 28°C. Most isolates could grow at 37°C except the strains *C. catenulata* (M25), *C. famata* var. *famata* (M81, M18, M89) and *C. famata* (M83, M32). Growth at 45°C was positive for only eight isolates. All isolates were able to grow at 4°C, except two *C. tropicalis* (M2, M43) strains, which were able to grow at all other temperatures. Growth of Mihalic cheese isolates at 4 and 10°C could be attributed to their resistance to ripening and storage conditions. Ripening temperature was reported to be 16°C for Pecorino cheese (Vannini *et al.*, 2008), 15-18°C for White pickled cheeses of Serbia (Golić *et al.*, 2013), 4-6°C for Erzincan Tulum cheese (Hayaloglu *et al.*, 2008), 5-10°C for Xinomyzithra cheese and 14-16°C for Kefalotyri cheese (Litopoulou-Tzanetakis and Tzanetakis, 2011). Kamber (2007) reported that the ripening temperature of Mihalic cheese fluctuated between 15-25°C. The growth of yeast isolates at high temperatures (37 and 45°C) may provide information regarding their resistance to cooking conditions. It was reported that cheese curd cut into small pieces may be cooked at temperatures ranging from 32°C to 54°C. The temperature usually used for cooking Mihalic cheese curd ranges between 40-45°C (Kamber, 2007). Growth ability of 40% of the Mihalic cheese isolates at 45°C indicates their potential to survive during cheese production. Reported cooking temperatures were; 50-55°C for Pecorino cheese (Vannini *et al.*, 2008), 38°C for Kasseri cheese and 43-45°C for Kefalotyri cheese (Litopoulou-Tzanetakis and Tzanetakis, 2011).

All yeast strains isolated from Mihalic cheese were able to grow at pH 5.4 and 4.0, while 65% of them could grow at pH 2.5 (Table 3). The pH of different traditional cheeses changes according to variety but is usually between 3.7-5.4 (Litopoulou-Tzanetakis and Tzanetakis, 2011). Reportedly, the changes in pH range between 3.7-4.0 for Galotyri cheese, 3.8-4.2 for Katiki cheese, 4.7-5.4 for Touloumissio cheese (Litopoulou-Tzanetakis and Tzanetakis, 2011) and 4.8-5.2 for Erzincan Tulum cheese (Hayaloglu *et al.*, 2008), while higher pH values were seen for some traditional cheeses such as Kefalotyri cheese with a pH usually higher than 5.0 and Manouri cheese with a pH between 6.8-7.3. The strains used in the present work were isolated from Mihalic cheese samples with pH values between 3.6-4.6 (Karasu-Yalcin *et al.*, 2017). The ability of the isolates to grow at a low pH is considered to be a result of their adaptation to Mihalic cheese, which could be valuable for their evaluation as adjunct starters, as it enables survival during the ripening of many cheeses. Resistance to a pH of 2.5 could be also attributed to the probiotic potential of these yeast strains, which enhances their survival in the gastrointestinal tract. Gotcheva *et al.* (2002) investigated the probiotic characteristics of some yeast strains isolated from a fermented drink, which were able to grow between pH 2.0-3.0.

All isolates were able to survive 5% and 10% sodium chloride (Table 3). Eleven were resistant to a salt concentration of 15%. All *C. famata* and *G. silvicola* strains displayed ability to grow at this concentration. Water activities (a_w) of the media having 0-15% sodium chloride were also determined. It was found that a_w of a medium without sodium chloride was 0.999. Water activities were measured as 0.969, 0.934 and 0.887 for media with 5, 10 and 15% sodium chloride, respectively. The salt content of Mihalic cheese was reported to change between 7.5-9.3% (Kamber, 2007; Cokal *et al.*, 2012). In the present work, strains were isolated from Mihalic cheese samples having 3.46-13.99% sodium chloride (Karasu-Yalcin *et al.*, 2017). Resistance of these yeast isolates to high salt concentrations, which may be associated with their origin, enhances their potential as adjunct starters in different cheeses, especially brined cheeses with a high salt concentration. It has been reported that brine salt concentrations required for different traditional pickled cheeses may change from 5.56 to 20% (Vannini *et al.*, 2008; Litopoulou-Tzanetakis and Tzanetakis, 2011). Cosentino *et al.* (2001) reported that among isolates originating from Sardinian ewe cheese; only some *D. hansenii* and *K. lactis* strains were able to grow at 10% NaCl concentrations. Resistance of *C. famata* strains to 15% NaCl, seen in the present work, was expected since *D. hansenii*, which is the teleomorph of *C. famata*, is one of the most halotolerant yeast species which is commonly found in salty environments (Breuer and Harms, 2006; Kumar *et al.*, 2012). *D. hansenii* was

defined as a biochemically interesting, extremophilic yeast, which could grow in media containing up to 4 M NaCl (Breuer and Harms, 2006). Gori *et al.* (2012) reported that ability of *D. hansenii* to grow at high salt concentrations has led to its prominence in the production of salty foods including different types of cheeses and meat products. The same may also be applied to *C. famata* strains described in the present work.

Potential probiotic characteristics of the yeast strains

Bile salt tolerance of these yeasts was tested for potential probiotic characteristics and ten of them were able to grow in a medium with 0.5% (w/v) bile salt. Nine of the isolates showed resistance to 1% (w/v) bile salt (data not shown). Growth and survival of the eight strains that grew well in the medium containing 1% (w/v) bile salt were further investigated in a medium containing 0.3% (w/v) Ox-Bile. *C. catenulata* M73 was the most sensitive isolate, followed by *C. catenulata* M77. After 72 h of incubation, no growth was observed for the strain M73, while 2.3 logarithmic units of decrease were recorded for M77. The most resistant isolate was found to be *C. inconspicua* M3, as its cell count increased by 3 logarithmic units. It was followed by *C. tropicalis* M43, with a 2.75 logarithmic unit cell count increase. For the other four isolates, *C. krusei* M57, *T. asahii* M1, *C. guilliermondii* var. *membranefaciens* M54 and *C. tropicalis* M2, the increase in cell count altered between 1.55-2.66 logarithmic units, while it was 2.62 for the control strain, *S. boulardii*. Bile salt resistance is considered as an important issue, because an ingested microorganism, which is unable to resist bile in the duodenum, will not survive to reach the intestinal tract in viable form (Silva *et al.*, 2011). García-Hernández *et al.* (2012), investigated potential probiotic traits of nine yeast strains isolated from broilers and reported that tolerance to bile salts was an *in vitro* selection criterion for probiotic candidates. In their study, resistance of yeasts to 0.3% and 0.6% bile salt were examined and a *Williopsis anomalus* strain was reported to show the highest resistance to bile salt. In another study performed by Silva *et al.* (2011), resistance of yeasts isolated from Portuguese brined olives to 3 g/L Ox-Gall was investigated. It was reported that most of the tested yeast strains showed growth potential, when incubated under bile-like conditions analogous to those prevailing in the duodenum, and that *P. membranifaciens* and *C. oleophila* appeared to be the most promising candidates for eventual inclusion in tailor-made probiotic starter/adjunct cultures. Gil-Rodríguez *et al.* (2015) screened 130 yeast strains isolated from different foods for probiotic potential. They reported that 50% of the isolates could grow at 37°C and among those, 95% could survive exposure to conditions simulating gastrointestinal transit. It was reported that most yeasts were able to resist conditions containing 3 g/L bile salts in models which were used to simulate gastrointestinal digestion. Pedersen *et al.* (2012), investigated the probiotic potential of yeasts isolated from Fura, a spontaneously fermented West African cereal. Survival and growth under gastrointestinal conditions were examined via incubation at 37°C in pH 2.5 and 0.3% (w/v) Ox-Gall, where all examined yeast isolates survived and grew under these conditions. In another study, some probiotic traits of 18 yeast strains isolated from Altamura sourdough were investigated. It was reported that a significant inhibition effect of 0.3% bile salt was found only on two isolates which were *S. cerevisiae* and *C. humilis* (Perricone *et al.*, 2014). The resistance of most yeasts from Mihalic cheese to 1% bile salt as well as 0.3% (w/v) Ox-Bile seen in the current study indicate that such resistance to bile salt may be a characteristic of a probiotic trait.

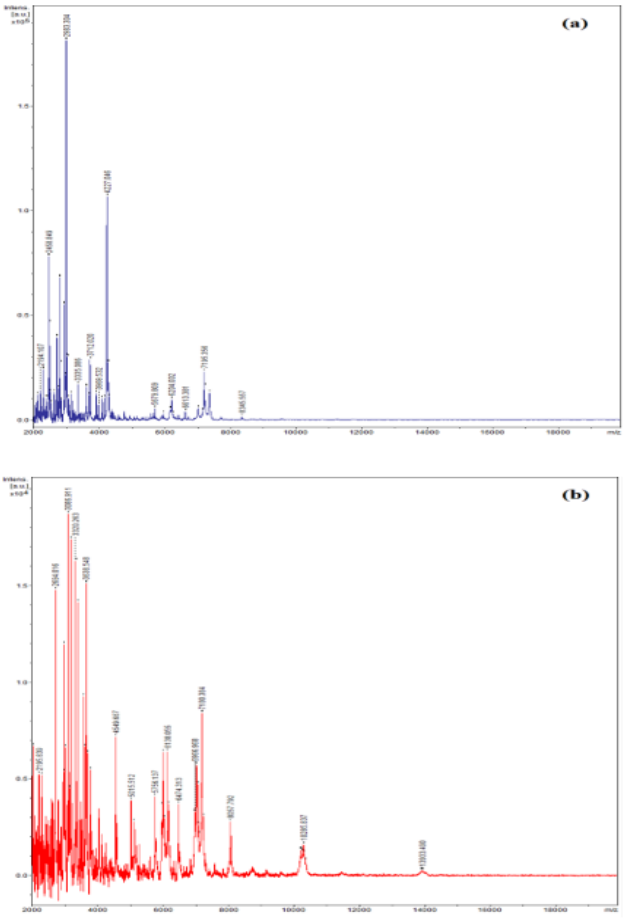

Figure 1. MALDI-TOF/TOF mass spectra of *T. asahii* M1 (a) and *G. silvicola* M15 (b).

Table 4. Inhibition effects of tested yeast strains on selected pathogenic bacteria.

Isolate no.	Yeast species	Inhibition effect (zone formation)		
		E. coli	*S. aureus*	*L. monocytogenes*
M1	*Trichosporon asahii*	-	-	-
M25	*Candida catenulata*	-	+ (w)	-
M57	*Candida krusei*	-	-	-
M54	*Candida guilliermondii var. membranefaciens*	-	+ (w)	-
M8	*Candida robusta*	-	-	-
M2	*Candida tropicalis*	-	+ (w)	-
M43	*Candida tropicalis*	-	+ (w)	-
M3	*Candida inconspicua*	-	-	-
M22	*Candida famata* var. *famata*	-	-	-
M81	*Candida famata var. famata*	-	-	-
M18	*Candida famata* var. *famata*	-	+ (w)	-
M89	*Candida famata* var. *famata*	-	-	+ (w)
M6	*Geotrichum silvicola*	-	+ (w)	-
M15	*Geotrichum silvicola*	-	-	+ (w)
M16	*Candida zeylanoides*	-	-	-
M73	*Candida catenulata*	-	+ (w)	+ (w)
M77	*Candida catenulata*	+ (w)	+ (w)	-
M83	*Candida famata*	-	-	-
M32	*Candida famata*	-	-	-
M63	*Candida famata*	-	-	-
Reference yeast	*Saccharomyces boulardii*		-	-

+: inhibition zone positive, w: weak inhibition zone (< 1 mm), -: inhibition zone negative.

The inhibition effects of yeast isolates against *E. coli*, *S. aureus* and *L. monocytogenes* are presented in Table 4. Only *C. catenulata* M77 exhibited a weak inhibition effect against *E. coli*. Results indicated that eight isolates, *C. tropicalis* (M2, M43), *C. catenulata* (M25, M73, M77), *C. guilliermondii* var. *membranefaciens* (M54), *C. famata* var. *famata* (M18) and *G. silvicola* (M6), showed inhibition effects against *S. aureus*. The strains, *C. famata* var. *famata* M89, *C. catenulata* M73 and *G. silvicola* M15, weakly inhibited the growth of *L. monocytogenes*. In general, the inhibitory potential exhibited by yeasts against pathogenic bacteria is considered as an important probiotic characteristic. In addition, the inclusion of these yeasts in adjunct cultures to enhance the hygienic quality of fermented products, confers an added advantage with obvious favourable effects in terms of public health (Silva *et al.*, 2011). Ceugniez *et al.* (2015) studied the antimicrobial effects of some yeasts isolated from a traditional French cheese "Tomme d'orchies". They reported that *K. marxianus* and *K. lactis* isolates showed inhibition effects on *L. monocytogenes*, *Bacillus subtilis* and *Bacillus thuringiensis*. Silva *et al.* (2011) investigated the inhibition effect of yeasts isolated from Portuguese brined olives on some pathogenic bacteria and reported strong inhibition of *L. monocytogenes* by *P. membranaefaciens*. It was also reported that one *S. cerevisiae* isolate inhibited the growth of *E. coli*. Georges *et al.* (2006) investigated anti-listerial potential of 404 foodborne yeast strains, 304 of which were isolated from smear-ripened cheeses. It was reported that only 4% of red smear cheese isolates clearly inhibited the growth of *L. monocytogenes*. Yeast strains showing high inhibitory effects were mainly species of *C. intermedia* and *K. marxianus*. In another study by Georges *et al.* (2011), 175 yeast strains isolated from different sources were screened for anti-listerial activities. It was reported that one *Pichia norvegensis* strain inhibited *L. monocytogenes* by 7 log units while 14% of the strains had anti-listerial activities. The yeast, *G. candidum*, is also known to have potential antimicrobial activities. Wouters *et al.* (2002) reported that *G. candidum* had the ability to excrete D-3-phenyllactic acid which inhibited the growth of *L. monocytogenes*. It also played an important role by competing with undesirable contaminants in cheese (Fadda *et al.*, 2004). The present work demonstrated the antimicrobial activity by *G. silvicola* as well as some other species, such as *C. catenulata*, *C. famata* and *C. guilliermondii*, which have not been frequently mentioned in other reported studies screening antimicrobial activities.

Strain selection upon technological and probiotic traits

When all technological and probiotic properties of these isolates were compared, *G. silvicola* M15 was the strain which exhibited most of the desired technological characteristics, such as proteolytic and lipolytic activities, assimilation and fermentation of glucose and galactose, assimilation of lactate and succinate as well as growth at all tested temperatures, pH and salt concentrations. Therefore, it may be recommended for potential use in adjunct starter studies. The next strain with most potential was *T. asahii* M1, which yielded positive results for most tests, except for sugar fermentation ability, citrate assimilation and growth at 15% NaCl. Mass spectra obtained by MALDI TOF/TOF MS for these two isolates are shown in Figure 1. In regard to probiotic traits, it was determined that *G. silvicola* M15 did not exhibit bile salt tolerance but demonstrated antimicrobial activity against *S. aureus*, while *T. asahii* M1 showed strong tolerance to bile salts but did not exhibit antimicrobial activity. Furthermore, it is felt that the isolate, *C. tropicalis* M2 warrants further investigation into its potential probiotic characteristics although its technological traits did not appear to be promising.

Conclusion

The use of multifunctional microorganisms such as starter cultures that exhibit both superior technological characteristics and probiotic activity is being recognised as a new trend in food microbiology (Perricone *et al.*, 2014). Ours is the first study demonstrating technological and probiotic characteristics of yeast strains isolated from Mihalic cheese. Strains such as *G. silvicola* M15 and *T. asahii* M1 showed potential as adjunct starters according to their superior technological characteristics. Determination of some probiotic traits provided additional information that may be helpful for future studies.

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