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Starmerella lactis-condensi, a yeast that has adapted to the conditions in the oenological environment

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ABSTRACT

The yeast *Starmerella* (*Candida*) *lactis-condensi* is considered a food contaminant microorganism. The aim of our research was to determine why *St. lactis-condensi* could become the dominant species of Essences, the top sweet wine speciality of Tokaj wine region in Hungary. We investigated the physiological properties of these yeasts based on parameters that may influence their ability to selectively proliferate and persist during maturation in wines with very high sugar content. These include glucose and fructose, alcohol, and sulphur tolerance. Our studies have shown that *St. lactis-condensi* is a fructophilic yeast that is able to adapt quickly to very high sugar concentrations (up to 500 g/L) in the Essences. The high glucose concentration inhibits its growth, as well as that of the *St. bacillaris* (*Candida zemplinina*) strains tested. The type and amount of sugars in the Essences, together with the sulphur and alcohol content, influence the composition of the dominant yeast biota. Analysis of $(GTG)_5$ microsatellite in the nuclear genome and mtDNA-RFLP studies demonstrate that a diverse population of *St. lactis-condensi* occurs in the Tokaj wine region, in the Essences. This yeast species is characterised by both physiological and genetic biodiversity. GC–MS analysis of Essences colonised exclusively with these yeasts showed no deterioration in quality.

1. Introduction

The *Starmerella* genus currently contains 48 yeast species (Čadež et al., 2020; Santos et al., 2018). Over the past decade, numerous yeasts formerly belonging to the *Candida* genus have been reclassified as *Starmerella* (Kurtzman and Robnett, 2013; Shen et al., 2016). This was also the case with the former name *Candida lactis-condensi*.

A study by Stratford et al. (2002) found that *C. lactis-condensi* along with, *C. apicola*, *C. bombi*, *C. davenportii*, *C. etchellsii*, *C. floricola*, *C. stellata*, *C. batistae*, *C. powellii* and *C. bombicola* formed a statistically significant species group based on their 26S rDNA D1/D2 sequences. These yeasts, now belonging to the group of *Starmerella* species are often referred to as spoilage microbes in high-sugar foods like concentrated grape juice, concentrated citrus juice, concentrated fruit juice, soft drinks, tomato sauce, high-sugar vegetables, sugar syrups, chocolate pralines (Deák and Beuchat, 1993; Marvig et al., 2014; Recca and Mrak, 1952; Scarr and Rose, 1966; Spencer et al., 1970; Spencer et al., 1992). The xerophilic *St. lactis-condensi* strains were isolated from sweetened condensed milk in China (Hammer, 1919), sugar syrups (Scarr and Rose, 1966), traditional balsamic vinegar (Solieri et al., 2006), vinegar (Sengun and Karabiyikli, 2011), plant oil throughout the pomace-based ethanol production process in Spanish distilleries (Úbeda et al., 2014), "Manna" ash product extracted from *Fraxinus angustifolia* (Oleaceae) (Guarcello et al., 2019), and also from Gochujang (fermented red pepper paste) (Ramalingam et al., 2022). Tudor and Board (1993) classify *St. lactis-condensi* into 'Additional spoilage species' in the wine industry. *St. lactis-condensi* and its closest relative *St. vitis*, are believed to be specialized to niches with high sugar content such as grapes or floral nectar of different plant species (Čadež et al., 2020). *St. lactis-condensis* and the related species *St. bombi*, *St. vitis*, *St. davenportii* and *St. bombicola* are also associated with insects that can transmit them into high-sugar foods (Čadež et al., 2020; Lachance et al., 2001; Loureiro and Malfeito-Ferreira, 2003; Stratford et al., 2002).

Little is known about its oenological aspects. It was previously isolated from overripe, botrytised grape and was found in Tokaj Essences (Csoma and Sipiczki, 2003, 2007; Csoma et al., 2021; Magyar and Bene, 2006). It was one of the predominant species of sweet piquettes without ethanol in Spanish distilleries (Úbeda et al., 2014) and "mothers" of *Vino cotto*, Italy (Battistelli et al., 2021).

In our previous study (Csoma et al., 2021), we performed analytical

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and microbial analysis of 28 Tokaj Essences, the top Tokaj sweet wine speciality. Its rarity and uniqueness are because only every few years there are special environmental conditions in which quality noble rot occurs, with the intervention of *Botrytis cinerea*. The grapes that have undergone botrytis noble rot are sorted and collected separately and, without any external mechanical action, they squeeze out a juice, often with a sugar content of 500–700 g/L, by their own weight during storage. Tokaj is a closed wine region, the first in the world to be subject to specific legislation. The product specification for the Tokaj Protected Designation of Origin (TOKAJ product specification, 2020) contains descriptions of the Tokaj Essence, which legally regulate the method of production and the analytical and other parameters of these wines. The colour is described as light golden yellow to deep amber yellow. The bouquet is highly concentrated, with rich notes of botrytis grape honey and dried fruit, complemented by aromas from the nature of ageing. The taste has a dense, oily texture, complex with almost imperceptible alcohol content, blending the honeyed, dried fruit notes typical of botrytis grapes with the flavours that develop during ageing. It is characterised by a high residual sugar content (min. 450 g/L), balanced with lively acidity and an actual alcohol content of 1.2–8.0%vol. A basic sulphurisation is applied to the Essence at the beginning of storage. The sulphur has dual effect: on the one hand, it ensures microbiological stability, and on the other hand it inhibits the production of laccase and oxidases by *Botrytis cinerea*, which limits the wine browning (Dittrich and Großmann, 2011). In Hungary, the legal limit of total SO_2 is at most 400 mg/L and the free SO_2 is at most 60 mg/L in Tokaj botrytized wine specialties.

In three Essences with a 2017 vintage, we previously detected only *St. lactis-condensi* yeast strains, while for 11 wines it occurred mixed with *Z. rouxii* and *St. bacillaris* strains. Overall, they formed a very significant part of the yeast biota of the Tokaj Essences we examined. *St. lactis-condensi* seems to be the best-fitted yeast for propagation in and fermentation of the Essence wines (Csoma et al., 2021). Like *St. bacillaris*, *St. lactis-condensi* is an osmotolerant and psyhrotolerant yeast species, which makes it better adapted to growing in high sugar and low temperature environments (Sipiczki, 2003). These characteristics are particularly advantageous for growth in musts from botrytised Tokaj grapes, which have very high sugar contents and ferment at around 12 °C. The two main soluble sugars are glucose and fructose in grape berries. *Botrytis cinerea* can metabolise more than one-third of the sugar of the grape berries, glucose is metabolised more extensively than fructose, thus increasing the fructose/glucose ratio in the must (Gafner and Schütz, 1996). This can be a limiting factor for the yeasts involved in fermentation of botrytized musts. Gafner and Schütz (1996) reported that stuck fermentations are frequently caused by an unusually high fructose-to-glucose ratio. High residual fructose means a lower ethanol yield and a higher risk for microbial spoilage of the finished wine (Berthels et al., 2004).

The aim of our study was to test the tolerance of 43 yeast strains of *St. lactis-condensi* (Csoma et al., 2021) isolated from Tokaj Essences in different pH, sugar, alcohol, and sulphur levels under laboratory conditions to find out whether their presence in these wines is accidental or whether they were able to adapt to these wine specialties and selectively multiply to become the dominant yeast species. There is also the question of whether their population is homogeneous or diverse, whether in terms of specific oenological physiology or molecular characteristics. We do not have sufficient literature data on these. As Tokaj Essences have to meet specific organoleptic standards, we analysed the volatile components of those dominated by the three species.

2. Materials and methods

2.1. Yeasts

We characterised 43 *St. lactis-condensi*, 3 *Z. rouxii* and 2 *St. bacillaris* strains isolates which were previously identified in Csoma et al. (2021). We used as controls type materials of *St. bacillaris* (CBS 9494^T) and *St. lactis-condensi* (CBS 52^T), as well as *S. cerevisiae* and *S. uvarum* strains isolated from Tokaj Aszú wines. Yeast strains examined in this study are listed in Table 1. A modified YPGA (1 % yeast extract, 2 % peptone, 5 % glucose and 2 % agar; *w/v*; pH 6.8) solid medium was used for yeast maintenance.

2.2. Physiological characterisation via drop tests

Drop test were carried out as previously described in Csoma et al. (2021). The plates were incubated at 25 °C, and the growth of the isolates was evaluated after five and ten days of incubation. All tests were carried out in duplicate in two independent experiments. The growth vigour of the yeast strains was rated on a scale of 1 to 5.

2.2.1. Determination of tolerance to glucose, fructose, glucose:fructose (1:1), ethanol and potassium bisulfite ($K_2S_2O_5$)

Samples of the suspensions were dropped on the surface of YPA (pH 3.5) plates supplemented with various concentrations of glucose and fructose (30, 40, 50, 60 and 70 %; *w/v*), glucose:fructose (1:1) (30, 40, 50, 60, 70 and 80 %; w/v), and YPGA plates (5 % glucose; pH 3.5) containing various amounts of ethanol (3, 6, 8, 10, 12 and 14 *v*/v%) or $K_2S_2O_5$ (100, 150, 200, 250, 300, 350 and 400 mg/L). The modified YPGA (5 % glucose; pH 6.8) solid medium was used as control.

2.2.2. Test combining possible stress factors

In these studies, we have combined the major stress factors that can affect yeasts in the Essences. Four types of media were prepared, based on YPA (1 % yeast extract, 1 % peptone and 2 % agarose; all w/v):

I: YPA - 60 % sugar (30 % G + 30 % F), 100 mg/L sulphur, 3 v/v% alcohol, pH 3.5; II: YPA - 60 % sugar (30 % G + 30 % F), 200 mg/L sulphur, 3 v/v% alcohol, pH 3.5; III: YPA - 60 % sugar (30 % G + 30 % F), 100 mg/L sulphur, 5 v/v% alcohol, pH 3.5; IV: YPA - 60 % sugar (30 % G + 30 % F), 200 mg/L sulphur, 5 v/v% alcohol, pH 3.5.

The modified YPGA (5 % glucose; pH 6.8) solid medium was used as control.

Table 1
List of yeast strains examined in this study.

Species	Strains[#]	Source of isolation
Starmerella lactis-condensi	7-1, 7-10, G7-1, 8-60, G8-1, 9-1, 9-4, 9-24, G9-4, 11-33, 11-76, G11-4, 14-49, 14-50, 18-2, 18-5, G18-1, 19-1, 19-5, 19-15, 20-78, 20-85, 20-88, G20-4, 21-1, 21-25, G21-1, G21-7, 24-1, 24-8, G24-1, G24-10d, 25-1, 25-20, 25-25, G25-1, 26-2, 26-10, 26-13, G26-3, 27-39, 27-63, 27-75	Tokaj Essence, Hungary[*]
	11-1962 (CBS 52^T)	condensed milk
Zygosaccharomyces rouxii	19-6, 20-2	Tokaj Essence, Hungary[*]
Starmerella bacillaris	19-13, 20-18	Tokaj Essence, Hungary[*]
	10-372 (CBS 9494^T)	Tokaj Aszú wine, Hungary
Saccharomyces cerevisiae	10-489[a]	Botrytized grape must, Hungary
Saccharomyces uvarum	10-408[b]	Tokaj Essence, Hungary

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
[#] Strains are deposited in the Culture Collection of the Department of Genetics and Applied Microbiology, University of Debrecen, Hungary.
[a] Strain isolated by Zs. Antunovics (Antunovics et al., 2005).
[b] Strain isolated by Zs. Antunovics (Naumov et al., 2002).
[*] Strains isolated by Csoma et al. (2021). The numbering of these isolates refers to the serial number of the Essence sample, which in this case is the first number, while the second is the serial number of the yeast strain isolated from the wine.

2.2.3. Organic acid production and determination of pH optimum

The intensity of the acid production was examined by culturing the isolates on Custer's chalk plates. Samples of the suspensions of the isolates were dropped on the plates and incubated at 25 °C. On the tenth day, the width of the dissolution zones around the colonies was measured. The pH of YPGA (supplemented with 5 % glucose; *w/v*) medium was adjusted from 3.5 to 6.8 to test the growth vigour of the strains from acidic to neutral range.

2.2.4. Detection of extracellular enzymatic activity

Seven enzymatic activities were evaluated using qualitative assays. β-glycosidase activity was tested as previously described by Arévalo-Villena et al. (2005). Yeast cells were grown in a medium containing 0.5 % cellobiose, 0.67 % yeast nitrogen base and 2 % agar, pH 3.5. Growth of the colonies after 5 days indicated the presence of β-glycosidase activity. Proteolytic activity was evaluated spotting yeast strains on skim milk agar medium. The pH was adjusted to 3.5. The appearance of a clear zone around the colony, after 5 days at 25 °C, is associated to protease activity (Englezos et al., 2015). The potential ability to produce H_2S was evaluated by growing yeast cells on modified Biggy agar (1 % yeast extract, 2 % glucose, 1 % glycine, 0.3 % sodiumm-sulfite, 0.5 % ammonium-bismute-citrate, 1.5 % agar; w/v; pH 6.8). The bismuth in the medium is used as an indicator. The colour of the colonies changes due to precipitated bismuth sulphide, which is thought to correlate with the level of sulfite reductase activity (Barbosa et al., 2018). To test the esterase activity of the yeast strains, the cells were grown in a medium composed of 1 % bacto peptone, 0.5 % NaCl, 0.4 % $CaCl_2$*$2H_2O$ and 1 % Tween 80; w/v. Esterase activity was indicated by a white precipitate around the colony (Carrasco et al., 2012). Qualitative detection of putrescine, histamine and tyramine was performed as described by Barbosa et al. (2018). A differential medium was used containing 3 % yeast extract, 1 % glucose, 2 % amino acid precursor (histidine, tyrosine or ornithine) and 0.015 g/L bromocresol lilac (alcohol soluble) (final pH adjusted to 5.2). As detailed in Csoma et al. (2021), 10 μL of the cell suspensions (OD 0.1) were dropped into plates incubated at 25 °C and growth of isolates was assessed after five days of incubation. While putrescine and histamine producing strains are detected by purple coloration around the colony, tyramine production is identified by the decolourization of the culture medium. The same differential medium without amino acid precursors served as a control.

2.2.5. Growth assay with microplates

Growth of the isolates in synthetic must (SM) supplemented with 20 % and 60 % glucose, fructose, or both glucose and fructose (10–10 % and 30–30 %) was examined in 96-well microplates as described in Csoma et al. (2021).

The growth of the yeast strains in the wells at 25 °C was monitored with a SPECTRO star Nano Microplate Reader (BMG Labtech, Offenburg, Germany) by measuring the absorbance at A_{590} (no. of flashes per well and cycle were 22) at regular time intervals (10,000 s) for 5 days. Three replicates of each measurement were done.

2.2.6. Growth competition tests

The relative growth rates (competition) tests were performed with modifications as previously described by Csoma et al. (2021). The isolates in the mixed populations were examined in synthetic must supplemented with 50 % glucose-fructose (1:1 proportion). For each pair, a mixed culture containing ~1×10^5 cells/mL of both strains and two pure control cultures of the strains containing ~1×10^5 cells/mL were set up in 30 mL $SM^{50\%\ G:F}$. The cultures were incubated on a gyratory shaker at 20 °C. The density of the $SM^{50\%G:F}$ cultures was determined after 72 h via cell counting in a Bürker chamber and diluted aliquots were spread on WL Nutrient Agar (Oxoid, Ltd., England) plates. On the WL selective medium, the *St. bacillaris* colonies tested are green, the *St. lactis-condensi* colonies are dark green, and the *Z. rouxii* yeast colonies are white or light green, so that they can be distinguished from each other based on their colony morphology (Fig. 2). To be on the safe side, the *St. bacillaris* and *St. lactis-condensi* colonies were also checked under the microscope. Based on our observations, under the conditions studied, the cells of the examined *St. bacillaris* strains tended to form cell aggregates, whereas the cells of *St. lactis-condensi* strains remained single. The experiment was carried out in triplicate for each strain in three independent experiments.

2.2.7. Molecular characterisation

For Microsatellite-Primed PCR (MSP-PCR) fingerprinting, genomic DNA was extracted from overnight YEL cultures. The microsatellite oligonucleotide primer $(GTG)_5$ was used for PCR reaction, as described by Baleiras-Couto et al. (1996). The method developed by Nguyen et al. (2000) was used to extract mtDNA, which was then digested with *Hae*III and *Mbo*I restriction endonucleases and analysed as described in Csoma et al. (2021). PyElph1.4 software for gel image analysis was used to determine the band size (Pavel and Vasile, 2012).

2.2.8. Cluster analysis of molecular and physiological patterns

Distance matrices were constructed from binary matrices of MSP-PCR and physiological patterns with using the Dice coefficient (Dice, 1945). These were then analysed with the average-linkage hierarchical clustering algorithm UPGMA (Unweighted Pair Group Method with Arithmetic mean) using the service available at http://genomes.urv.es/UPGMA (Garcia-Vallve et al., 1999). The FigTree programme (http://tree.bio.ed.ac.uk) was used to visualise dendrograms.

2.2.9. Analysis of volatile components of Tokaj Essences by GC–MS

VOC (Volatile Organic Compound) profiling of the Essence samples headspace was carried out using a Bruker Scion 456-gas chromatograph equipped with Bruker SHS-40 Headspace Sampler coupled to a Bruker SQ mass spectrometer. Measurements were prepared and conducted as described in Rakonczás et al. (2020). Identification of the VOCs was based on mass spectrometric data obtained from the National Institute of Standards and Technology (NIST) (Version 2005) mass spectral library.

3. Results

3.1. Physiological characterisation

The osmotolerance of the examined *St. lactis-condensi* strains was first tested under laboratory conditions. Testing was performed on solid medium (YPGA), in the presence of glucose and fructose, both separately and together, as these are the two most important hexoses found in these wines. The osmotolerance of the tested control strains at different levels of glucose and fructose is shown in Table A, while that of the tested *St. lactis-condensi* strains is shown in Table B. Fig. S1 shows the results obtained for some isolates, which are also representative of the growth scale (up to 5–1+ and no growth with "-") used for all tests in the evaluations. The yeast strains tested still grew at 30 % glucose, but 40 % limited their growth. There were strains that needed longer time to adapt and were able to produce minimal growth by day 10, but strains from samples 25, 26, 27 were not able to grow at all. At 50–60 % glucose, a quarter of the strains showed minimal viability. No strain of *St. lactis-condensi* was able to tolerate a glucose concentration of 70 %. Of the control strains, *Z. rouxii* could grow at 70 %. When the medium was supplemented with fructose, both the *St. lactis-condensi* isolates and the control strains (even *S. cerevisiae* and *S. uvarum*) grew at 70 % (Table B).

We nuanced the sugar experiment by also preparing media containing both glucose and fructose up to 40–80 %. At 40 % mixed sugar, the strains grew better than in the separate tests. At 70 % mixed sugar content, minimal growth was detected, similar to that observed on the 70 % fructose medium.

In the sulphur tolerance tests (Table C), we found that the *St. lactis-condensi* strains were able to grow at 150 mg/L total sulphur, but above this level the difference was obvious. At 200 mg/L, 18 isolates from 6

Essences, at 250 mg/L 15 isolates from 5 Essences and at 300 mg/L total sulphur, 7 strains of *St. lactis-condensi* from 3 Essences were able to grow. Above this level, the isolates were no longer able to form colonies. The type strain *St. lactis-condensi* (CBS 52^T) was not able to grow at all at a sulphur concentration of 100 mg/L, the inhibitory concentration for the *St. bacillaris* type strain (CBS 9494^T) was 250 mg/L (Table C), which was also observed in our previous studies with *St. bacillaris* strains isolated from Essences (Csoma et al., 2021). For the control *S. cerevisiae* (10–498) strain, 300 mg/L was the inhibitory concentration, while *S. uvarum* (10–408) and *Z. rouxii* (19–6) proved to be the most tolerant to sulphur, with 400 mg/L not preventing their growth (Table C).

The alcohol tolerance tests showed (see Table D) that the *St. lactis-condensi* strains were able to tolerate 6 *v*/v% alcohol. Of the control strains, *S. uvarum* and *St. bacillaris* formed colonies at 8 v/v% alcohol, with the most tolerant strain being *S. cerevisiae* at 12 v/v%.

The possible stress factors (acidic pH, high sugar, alcohol, and sulphur content) that can occur in an Essence-type wine were combined in four different types of YPA medium (Table 2). On culture medium I. (YPA, 60 % sugar (G/F, 30 + 30 %), pH 3.5) supplemented with 100 mg/L sulphur and 3 v/v% alcohol, all *St. lactis-condensi* isolates of wine origin were able to grow. The same medium composition with 200 mg/L sulphur (Medium II.) already selected the isolates, some of which needed longer time for colony formation. Increasing the alcohol content to 5 v/v% at 100 mg/L sulphur (Medium III) further reduced the growth vigour and there were isolates that were no longer able to grow at all. The most drastic negative effect was the increase of sulphur to 200 mg/L at 5 v/v% alcohol content (Medium IV.). Only isolates from samples 7., 8., and 11. showed minimal growth on day 5, followed by isolates from samples 21., 24., 25., 26. on day 10. *S. cerevisiae* (10–498) and *S. uvarum* (10–408) strains showed no growth at all. The limiting factor was the sugar content of 60 %.

The strains isolated from Essences 7 (7-1, 7-10, G7-1), 8 (8-60, G81) and 11 (11-33, G11-4) stand out from the other *St. lactis-condensi* in terms of their sulphur, hexose, and alcohol tolerance. They belong to the same physiological group (I. Tolcsva; Fig. 1). These wines have significant alcohol content (3.8–5.11 v/v%), average reducing sugar content (above 500 g/L) in terms of Tokaj Essences, an acid pH (3.14–3.52), and high total sulphur content (210–384 mg/L) (Csoma et al., 2021).

The dendrogram (Fig. 1) based on the tolerance tests (Method 2.2.1., 2.2.2.), clearly shows that the isolates are divided into several groups. The pattern shows that the clusters are mainly grouped according to the production area (I. Tolcsva, III. A. Bodrogolaszi clusters), the latter being linked to a particular winery. The origins of the isolates in 'II. Mix' cluster can be traced back to the areas of Mád, Tarcal and Tolcsva, which form a triangle within the Tokaj wine region.

In the pH optimum test, the growth vigour of the isolated *St. lactis-condensi* strains reached 4+ in the pH range 3.5–4.5 and 5+ in the control strains. In the pH range 5.0–6.8, all yeast strains tested showed maximum growth vigour (5+).

Finally, compared to the control strains, the tested isolates did not produce significant amounts of organic acids in the Custer's chalk plate tests. A dissolution zone of 0.5 mm was measured for 30 isolates, while the others varied between 1.0 and 2.0. Dissolution zones of 3.0 mm were detected for *Saccharomyces* strains and *St. bacillaris*, and 1.5 mm for *Z. rouxii*.

Table 2
Results of combined stress tolerance of *St. lactis-condensi* isolates.

Strains	I.		II.		III.		IV.	
	a	b	a	b	a	b	a	b
7-1, 7-10, G7-1, 8-60, G8-1, 11-33								
9-1					-		-	-
9-4, 9-24, 11-76, 14-50, G18-1			-		-		-	-
G9-4			-				-	-
14-49, 18-2					-		-	-
18-5					-		-	-
19-1, 19-5, 19-15, 20-78, 20-85, 20-88, G20-4			-		-		-	-
21-1							-	
21-25, G21-1, G21-7, 24-8, G24-1, G24-10d, 25-1							-	
24-1			-	-	-		-	-
25-20, 25-25, G25-1, 26-10, 26-13, G26-3							-	
26-2							-	-
27-39							-	
27-63							-	-
27-75			-				-	-
St. l - CBS 52^T	-		-	-	-	-	-	-
St. b -			-		-	-	-	-
S. u - 10-408	-	-	-	-	-	-	-	-
S. c -	-	-	-	-	-	-	-	-
Z. r - 19-6								

a: growth detected on the 5th day; b: growth detected on the 10th day; -: no growth; +: growth. The Roman numerals refer to the four types of media used in the experiment, the composition of which is given in Method 2.2.2. Scale of growth ability: 5+□ 4+□ 3+□ 2+□ 1+□ -□

3.2. Detection of extracellular enzymatic activity

We investigated the activity of some extracellular enzymes of interest from an oenological point of view. These may be important in the release of primary aromatic and volatile components, in wine stabilisation, and from a health point of view. For the *St. lactis-condensi* strains isolated from the Tokaj Essences we tested, as well as for the type strain (CBS 52^T), we could not identify β-glucosidase (release of primary aromatic compounds of grape origin) and protease (wine stabilising enzyme) activity at acidic pH. The strains of *St. lactis-condensi* were not able to grow on the medium used for detecting esterase activity and their enzyme activity could not be detected. The control strains *St. bacillaris* (CBS 9494^T), *Saccharomyces* sp. (10-408, 10-489) strains and the *Z. rouxii* (19-6) were able to grow on the medium but did not show esterase enzyme activity. Qualitative tests also did not detect biogenic

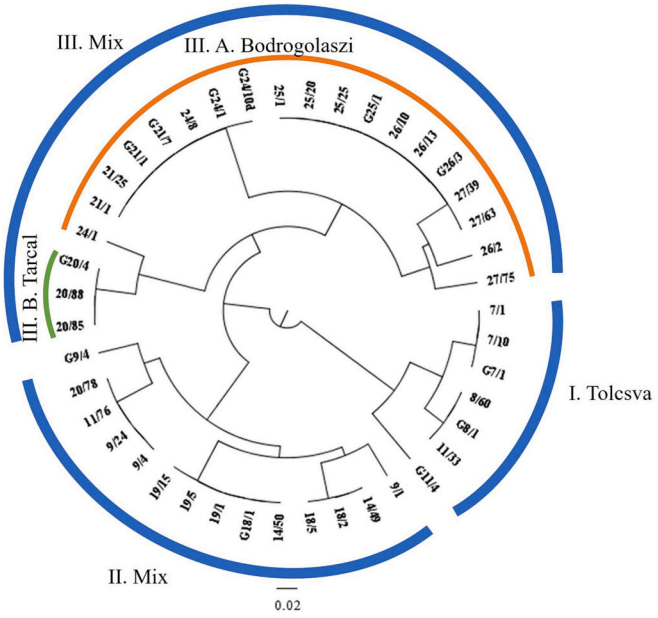

Fig. 1. Physiological diversity of the *St. lactis-condensi* isolates based on tolerance tests. The main clusters are marked with a Roman number, next to which the production area associated with the commune within the Tokaj wine region is indicated.

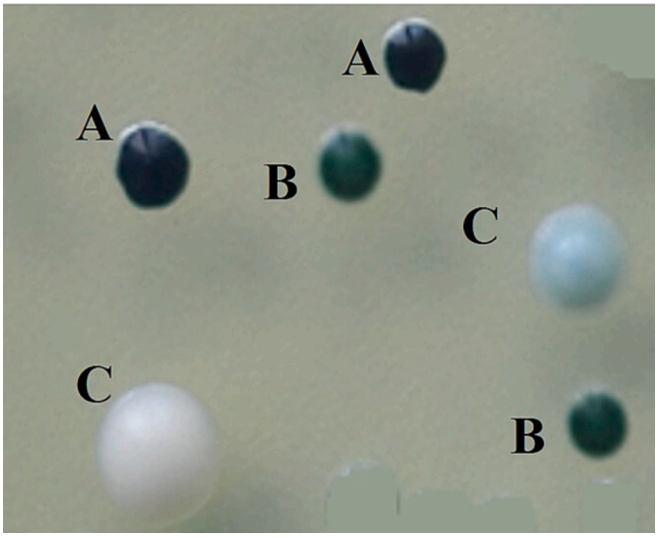

Fig. 2. Morphological differences of yeast strains on WL Nutrient Agar. (A) *St. lactis-condensi* (19–15), deep green; (B) *St. bacillaris* (CBS 9494^{T}), green; (C) *Z. rouxii* (19–6), white or light green.

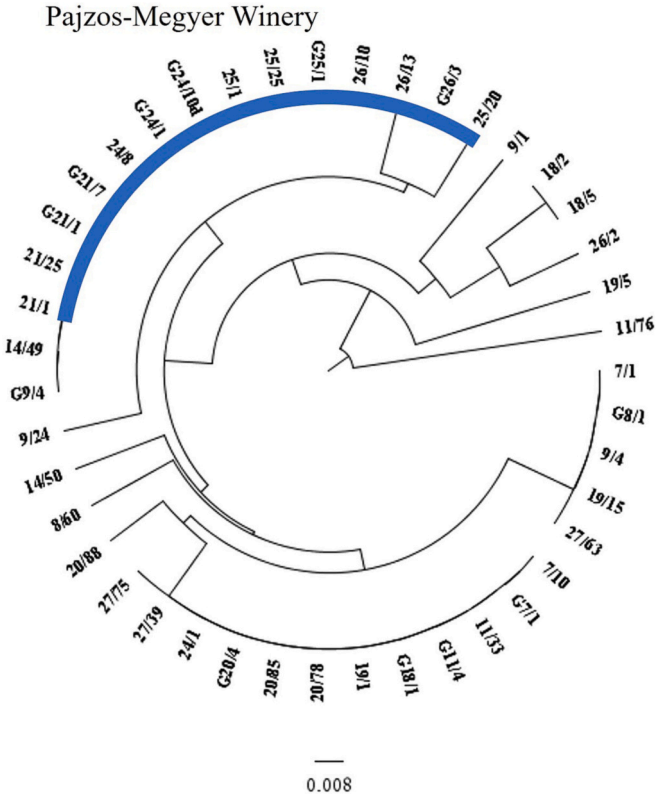

Fig. 3. Molecular diversity of the examined *St. lactis-condensi* strains. Dendrogram from UPGMA clustering analysis, based on Dice coefficient of MSP-PCR fingerprinting. The strains, which are also grouped close to each other according to their physiological characteristics, are highlighted in the figure (Pajzos-Megyer Winery).

amine production. Only moderate hydrogen sulphide production is characteristic of *St. lactis-condensi* strains. On the tenth day, it was a 4 on a scale of 6. The modification of the Biggy medium was necessary because the *St. lactis-condensi* strains were not able to grow on the original medium. This meant that the yeast extract was increased by a factor of 10 and the glucose content by a factor of two compared to the base medium. β-glucosidase activity was detected in the other strains tested as controls, as well as H_2S production, with *Z. rouxii* (19-6) having the least (a 2 on a scale of 6) and *St. bacillaris* (CBS 9494^{T}) the highest (a 5 on a scale of 6). The other control strains scored 4.

3.3. Growth competition tests

In the competition tests, *St. lactis-condensi* (19-15, 20-78), *St. bacillaris* (19-13, 20-18) and *Z. rouxii* (19-6, 20-2) strains were grown separately and together in SM$^{50\%G:F}$ medium for three days. With this medium, we tried to simulate the high osmolality also observed in the Tokaj Essences. This is a factor that presumably influences the viability of yeasts in the wines. The *St. bacillaris* strains we tested produced higher colony numbers when grown alone than *St. lactis-condensi* and *Z. rouxii* strains (Table 3). The *Z. rouxii* strains alone could not reproduce at the same rate as the other strains under the same conditions at the same time. In mixed cultures, *Z. rouxii* strain 19-6 was undetectable, while colonies of strain 20-2 were identifiable alongside the other species. Surprisingly, all but one of the *St. lactis-condensi* strains tested was detectable in dominant amounts in the mixed cultures. For strains of *Starmerella* species, in both mixed cultures, *St. lactis-condensi* strains were represented in considerably higher colony numbers.

3.4. Growth assay with microplates

In order to get an overview of the growth kinetics of our isolates in high-sugar liquid, we tested their growth in 60 % synthetic must containing different sugar sources. For comparison, we also tested them in a synthetic must with an average sugar content of 20 %. Tables 4 and 5 summarizes the growth kinetics of the *St. lactis-condensi* isolates we tested, and the control strains used for comparison, showing the end of the lag phase, the half exponential phase, and the beginning of the stationary phase, also the absorbance measured after 120 h. As shown in Table 4, the tested *St. lactis-condensi* strains and the *St. bacillaris* type strain reached the onset of the stationary phase in SM $^{60\%G:F}$ medium faster, on day 3, than the other control strains.

As can be seen in Table E, there were differences between our

Table 3
Reproductive vigour of representative strains of yeast species dominant in Essences in single and mixed cultures.

Species	Isolates	CFU (10^5/mL) of isolates after 72 h of incubation
Z. rouxii	19–6	4.5 ± 1.32
St. bacillaris	19–13	9.9 ± 2.55
Mixed cultures	19–6 + 19–13	- / 7.53 ± 5.52
Z. rouxii	19–6	2.06 ± 0.96
St. lactis-condensi	19–15	4.1 ± 3.01
Mixed cultures	19–6 + 19–15	- / 1.66 ± 0.80
St. bacillaris	19–13	29.3 ± 0.91
St. lactis-condensi	19–15	20.4 ± 1.55
Mixed cultures	19–13 + 19–15	0.23 ± 0.05 / 25.7 ± 1.05
Z. rouxii	20–2	6.63 ± 1.12
St. lactis-condensi	20–78	16.4 ± 6.57
Mixed cultures	20–2 + 20–78	3.83 ± 0.70 / 2.06 ± 1.85
St. bacillaris	20–18	34.73 ± 3.04
St. lactis-condensi	20–78	16.7 ± 7.9
Mixed cultures	20–18 + 20–78	0.26 ± 0.20 / 17.85 ± 3.60
Z. rouxii	20–2	88.3 ± 1.27
St. bacillaris	20–18	22.5 ± 1.31
Mixed cultures	20–2 + 20–18	5.36 ± 0.8 + 1.63 ± 1.45

-: no growth detected.
±: the standard deviation values.

Table 4
Growth kinetic parameters of the yeast strains studied in SM$^{60\%sugar}$.

Strains	SM $^{60\%G:F}$ medium				SM $^{60\%G}$ medium				SM $^{60\%F}$ medium			
	End Lag[a]	Half Exp.[a]	Start Stac.[a]	Max Abs.	End Lag[a]	Half Exp.[a]	Start Stac.[a]	Max Abs.	End Lag[a]	Half Exp.[a]	Start Stac.[a]	Max Abs.
St. lactis-condensi isolates[b]	16.0 ± 4.38	28.372 ± 5.15	51.697 ± 9.88	0.739 ± 0.11	n.d.	n.d.	n.d.	0.262 ± 0.16	17.372 ± 2.11	27.697 ± 2.97	46.418 ± 6.55	0.771 ± 0.11
St. l - CBS 52^T	11	22	39	0.826 ± 0.04	n.d.	60	n.d.	0.255 ± 0.03	17	31	69	0.837 ± 0.10
St. b - CBS 9494^T	11	28	56	0.189 ± 0.09	n.g.	60	n.g.	0.126 ± 0.06	22	58	89	0.427 ± 0.01
S. u - 10-408	19	39	120	0.036 ± 0.00	n.g.	60	n.g.	0.146 ± 0.00	n.g.	n.g.	n.g.	0.016 ± 0.00
S. c - 10-489	19	38	106	0.349 ± 0.07	11	60	75	0.553 ± 0.01	n.g.	n.g.	n.g.	0.015 ± 0.01
Z. r - 19-6	8	44	89	0.803 ± 0.07	8	25	50	1.015 ± 0.06	8	28	58	0.618 ± 0.02

Data in hours and Absorbance (A_{590}) are averages of three measurements.
n.g.: no growth.
n.d.: phases not distinct.
[a] These values are in hours.
[b] Average of the total values measured for the 43 *St. lactis-condensi* isolates.

Table 5
Growth kinetic parameters of the yeast strains studied in SM$^{20\%sugar}$.

Strains	SM $^{20\%G:F}$ medium				SM $^{20\%G}$ medium				SM $^{20\%F}$ medium			
	End Lag[a]	Half Exp.[a]	Start Stac.[a]	Max Abs.	End Lag[a]	Half Exp.[a]	Start Stac.[a]	Max Abs.	End Lag[a]	Half Exp.[a]	Start Stac.[a]	Max Abs.
St. lactis-condensi isolates[b]	5.860± 0.64	13.120± 3.31	22.380± 8.34	1.300± 0.26	5.119 ± 1.47	13.880± 2.01	33.260± 3.36	1.532± 0.16	5.142± 1.37	12.666± 1.60	28.095± 5.29	1.377± 0.22
St. l - CBS 52^T	6	14	22	1.157 ± 0.08	6	17	31	1.572 ± 0.06	6	12	25	1.105± 0.10
St. b - CBS 9494^T	6	14	25	1.703 ± 0.07	3	14	33	1.673± 0.02	6	14	39	1.753± 0.03
S. u - 10-408	6	12	22	1.412 ± 0.02	3	12	25	1.393± 0.03	6	14	28	1.304± 0.01
S. c - 10-498	6	12	22	2.021 ± 0.10	3	14	36	2.001± 0.10	3	17	47	1.968± 0.08
Z. r - 19-6	6	14	28	1.962 ± 0.04	6	19	42	1.858± 0.05	6	19	47	1.877± 0.02

Data in hours and Absorbance (A_{590}) are averages of three measurements.
[a] These values are in hours.
[b] Average of the total values measured for the 43 *St. lactis-condensi* isolates with standard deviations (±).

isolates, which were also reflected in the Absorbance values measured at hour 120, with the lowest being 0.533 (14-49) and the highest 0.93 (G9-4), with an average of 0.739.

In the SM medium supplemented with 60% glucose, some phases of the growth curve were identifiable in *Z. rouxii* (19-6) and *S. cerevisiae* (10-498) control strains. Of the two strains, *Z. rouxii* clearly showed greater osmotic tolerance. In the case of *St. lactis-condensi* isolates, it was not possible to identify the individual growth phases at 120 h. Except for isolates 7-1, 7-10, G7-1, 8-60, G8-1, 11-33, G11-4 (Table E), they were not able to utilize or tolerate such amounts of glucose as only carbon source, as were the control strains *S. uvarum* (10-408) and *St. bacillaris* (CBS 9494^T). In the SM medium supplemented with 60 % fructose, *St. lactis-condensi* strains reached the stationary phase in approximately two days after an average lag phase of 17 h. Also, in comparison with control strains, they prefer fructose over glucose (Table E).

A control medium containing 20 % sugars, be it glucose or fructose, was tolerated by all yeast strains. In terms of growth kinetics, they were able to reproduce and enter each phase more rapidly than with 60 % sugar concentrations (Table 5). Our isolates of *St. lactis-condensi* reached the stationary phase faster in SM medium containing 20 % fructose than in 20 % glucose. Although the deviation values here also show that the strains from Essences did not behave uniformly.

3.5. Molecular characterisation

As the yeast strains studied appeared to be diverse in terms of their physiological properties, we investigated their mtDNA and the $(GTG)_5$ microsatellite profile of their genomic DNA. As shown in Fig. S2, a slight difference was detected between the mtDNA profiles of the strains, with three biotypes being distinguished.

The MSP-PCR profiles of the *St. lactis-condensi* strains we have studied are highly variable (Fig. 3). They cannot be correlated with the geographical origin of the Essences, their analytical profile or their vintage, except for a narrow group. Fourteen isolates from the Pajzos-Megyer Winery in the Bodrogolaszi area are found near each other, similar to what was seen in the physiological analyses (Fig. 1, Cluster III. A. Bodrogolaszi). Among the physiological assays, the combined stress factor tests showed a similar clustering of these isolates. In other respects, the GTG_5 microsatellite marker patterns do not correlate with the UPGMA groups generated from physiological studies.

3.6. Profiles of volatile components in Tokaj Essences

Analyses of the volatile components of Tokaj Essences (Csoma et al., 2021) dominated by the yeast *St. lactis-condensi* were carried out. For comparison, we also included wines in which the other two species were

dominant and took into account samples of the same but different vintage and storage conditions. This was done to exclude ingredients that might be present not because of the yeasts but, for example, because of the ageing process. We also wanted to verify that the wines dominated by *St. lactis-condensi* meet the requirements for their volatile components.

The results of the semi-quantitative GC–MS analysis revealed the presence of various volatile compounds, which could be divided into four categories: esters, alcohols, terpenes, and other compounds. 36 components from 43 GC–MS peaks could have been identified in the wine extracts dominated by *St. lactis-condensi* (Essences 7, 8, and 25) (Table F). Esters with sweet, ethereal, fruity, alcoholic, fusel, rummy, cognac, pineapple, banana, overripe fruit aromas, were most abundantly detected. Ethyl acetate (ethereal, fruity, sweet, grape, and rum-like aromas) accounted for 67–68 % of the peak areas. These were followed by higher alcohols, notably 7.8–9.4 % 3-methyl-1-butanol (fusel, alcoholic, whiskey, fruity, banana aromas), 5–6 % (*S*)-2-methyl-1-butanol (ethereal, fresh odour), and 3–7 % 2-methyl-1-propanol (fusel, whiskey odour). Other VOCs that can be highlighted are isocaryophyllene, humulene and β-copaene, which are sesquiterpenoid components of plant origin with woody, spicy fragrance notes. The proportion of these (0.4–1 %) was higher in the barrel aged Essence. The identified VOCs include propanoic acid, 2,2-dimethyl-, ethyl ester, and 1,3-dioxolane, 4-ethyl-4-methyl-2-pentadecyl- are present in wines dominated by *St. lactis-condensi* regardless of storage conditions and other parameters compared with the other wines. The first has fruity fragrance notes, the second is unidentifiable.

For the other wines, fewer volatile components were detected, regardless of the vintage and storage methods (see Table F). Comparing wines with the same vintage (2017), grape variety (Furmint), storage method (bottle) and locality (Tolcsva), the one dominated by *St. lactis-condensi* (Essence 7) showed a more complex aroma profile than one dominated by *St. bacillaris* (Essence 10).

4. Discussion

Since the occurrence of *St. lactis-condensi* is so significant in Tokaj botrytised wine specialities, we wanted to know more about its physiological nature, which could help its survival in high-sugar wines, especially in Tokaj Essence. Also, analyse what the relationship might be between the three dominant yeast species detected previously. Several factors could shape the composition of the microbiota in Tokaj Essence. These might include sugar content, pH, alcohol, sulphur content, and storage temperature. We analysed these wine-related characteristics of 43 *St. lactis-condensi* isolated from Tokaj Essences and other yeast species, including strains of *St. bacillaris*, *S. cerevisiae*, *S. uvarum* and *Z. rouxii*.

The osmotolerance to glucose of the *St. lactis-condensi* isolates we tested was weaker (generally 40 % tolerated), except for some strains, than that of the control *Z. rouxii* strain. *Z. rouxii* has previously been reported to have excellent sugar tolerance (Marvig et al., 2014; Wang et al., 2015), surviving above 70–75 % glucose (Csoma et al., 2021; Dakal et al., 2014). However, 60 % extremely high sugar stress was reported to inhibit the growth of *S. cerevisiae* (Dakal et al., 2014; Silva et al., 2005). Dakal et al. (2014) have identified *S. cerevisiae* as moderately osmotolerant yeast. Our results confirm the same observation, with the addition that our *S. cerevisiae* strain (10-498, isolated from Tokaj sweet wine) was able to colonize at 70 % fructose concentration. However, it is well known that most of the yeasts including *Saccharomyces* species prefer fermenting glucose to fructose if both sugars are present in the medium (Berthels et al., 2004; Magyar and Tóth, 2011) as well as *S. uvarum* and *S. bayanus* (Magyar and Tóth, 2011). Our growth kinetics studies with synthetic must have already shown a glucose preference in case of *Saccharomyces* strains. Our physiological studies have shown that the presence of fructose enhances osmotic tolerance (up to 60 %). The co-presence of the two hexoses showed a similar trend, with *St. lactis-condensi* isolates showing better osmotolerance (around 70 %). Of the three yeasts dominant in the Essences, the osmotolerance of *Z. rouxii* showed no difference in the three conditions, with *St. bacillaris* and *St. lactis-condensi* appearing to be more osmotolerant to fructose. Previous literature has shown that *Z. rouxii* (Leandro et al., 2014) and *Z. bailii* (Pina et al., 2004; Sousa-Dias et al., 1996) consume fructose faster than glucose (Emmerich and Radler, 1983). *St. bacillaris* (Duarte et al., 2012; Magyar and Tóth, 2011; Mills et al., 2002; Tofalo et al., 2012) and *St. stellata* (García et al., 2018; Magyar and Tóth, 2011; Soden et al., 2000) are also considered to be fructophilic yeasts.

The alcohol tolerance of our isolates was 6 v/v% and the sulphur tolerance reached 300 mg/L. Similar or lower values have been detected in Tokaj Essences (Csoma et al., 2021). In the combined stress tolerance, the vast majority of strains were able to tolerate 60 % sugar content, 5 v/v% alcohol and 200 mg/L sulphur. *Z. rouxii* showed a higher tolerance, *St. bacillaris* a lower one. This may provide an answer to how these yeasts can become dominant. Similar conclusions were reached from growth competition studies and growth kinetics studies. *St. lactis-condensi* yeast strains can overcome the *St. bacillaris* strains by outgrowing them and can adapt more rapidly to higher sugar medium.

We also performed a qualitative analysis of other physiological traits that may be of relevance from a wine point of view, such as extracellular enzyme activity, biogenic amine production or organic acid production capacity.

Extracellular enzyme activity can also be detected in non-*Saccharomyces* yeast strains. Specifically, *Candida* spp. have been described as extracellular enzymes producer (Andorrà et al., 2010; García et al., 2017; Merín et al., 2015). There are no literature data on *St. lactis-condensi*, *St. bacillaris* and *Z. rouxii* strains with protease activity, and we were unable to detect any within the scope of the present study. β-glucosidase activity has been detected among yeast strains *St. bacillaris* (Di Maio et al., 2012) and *Z. rouxii* (Manzanares et al., 2000), but no literature data are available for *St. lactis-condensi*. We could not detect β-glucosidase activity in any of the *St. lactis-condensi* strains we studied.

The amount of volatile sulphur components in wines is another important quality parameter. Their formation can be traced back, among other things, to the production of hydrogen sulphide (H_2S) by the metabolism of the yeasts during fermentation. Most strains of *St. bacillaris* were found to be medium to high producers of H_2S (Di Maio et al., 2012; Pfliegler et al., 2014), while *Z. rouxii* strains were found to be low producers (Csoma et al., 2021). The *St. lactis-condensi* strains previously (Csoma et al., 2021) and now tested by us were found to be medium H_2S producers under laboratory conditions and are not expected to pose a threat in this respect. During alcoholic fermentation of wines, even yeasts can contribute to the production of biogenic amines. These are basic nitrogen compounds which, above a certain limit, can cause health problems in sensitive individuals. Within the *Candida* genus, *C. stellata*, *C. zemplinina* and *C. versatilis* have been reported to be able to produce biogenic amines in wine and in soy sauce (Russo et al., 2019, 2020). There are no literature data on the biogenic amine-producing capacity of *St. lactis-condensi*. We could not detect histamine, putrescine, and tyramine production in the strains we tested. It can therefore be said that this yeast does not pose a risk in this respect.

The ability of *St. lactis-condensi* to produce organic acid was lower than that of the other strains tested. Among the organic acids, acetic acid can cause serious quality problems in wines with its pungent, sour, vinegary smell and odour. Acetic acid can be produced by both bacteria and yeasts. The maximum limit for volatile acids in Tokaj Essences is 2.1 g/L (TOKAJ product specification, 2020). Of the Essences we tested, Essence 8 had the highest limit, 1.2 g/L (Csoma et al., 2021), so none of the Essences were close to the limit. VOC analysis also confirmed this previous measurement.

The suitability of yeasts for fermentation is partly determined by their genetic composition, which is a species and strain-dependent factor. Yeast species, even at strain level, may differ in these characteristics. Under stress conditions, these differences are even more apparent, and

strains may respond differently depending on their adaptability. Therefore, we performed mtDNA-RFLP and MSP-PCR analyses to assess the degree of genetic diversity. Molecular intraspecific diversity is observed in both the mitochondrial genome and the nuclear genome of the *St. lactis-condensi* population we studied. Previously, Battistelli et al. (2021) was able to identify one biotype among strains isolated from "mother" of *Vino cotto* vines by RAPD-PCR analysis with primer M13. Physiological characteristics and microsatellite profiles of the examined *St. lactis-condensi* strains are very diverse, which is remarkable considering that we have studied isolates from a single wine region and a single wine variety. One of the groups detected in the MSP-PCR analyses had strains that showed similarly high tolerance in the combined tests.

Certain strains isolated from the Essences 7, 8 and 11 stood out from the other *St. lactis-condensi* strains in several aspects. Their relatively high sugar, sulphur and alcohol tolerance placed them in cluster I. Tolcsva and only these strains were able to grow in Synthetic must supplemented with 60 % glucose. If we had a larger number of samples, we could confirm our hypothesis that the *St. lactis-condensi* strains of oenological origin are grouped according to the winery or, rather, according to the general wine-making practices used in wineries.

The unique aroma characteristics of Tokaj botrytised sweet wines are fruity, floral-honey, dried fruit, roasted, caramel notes (Miklósy et al., 2000; Miklósy et al., 2004; TOKAJ product specification, 2020). These are formed by a variety of fragrance components, which include esters, higher alcohols, furanoids, pyranoids, volatile acids, volatile phenols, terpenoids, carbonyls, and pyrroles (Furdíková et al., 2020; Machyňáková et al., 2021; Miklósy et al., 2000; Miklósy et al., 2004). Their occurrence and quantity depend on a number of factors, including the grape variety, the growing area, the winemaking technique, the yeast strain, the fermentation and ageing conditions of the must. Most of the volatile aroma components detected by us in the Essences are from the ester group. It is known that the largest amount of the esters develop during the fermentation, but their precursors, e.g. amino acids are influenced by *B. cinerea* (Miklósy et al., 2004). The most abundant ester in the Essences was ethyl acetate, which was previously detected in Aszú wines (Miklósy et al., 2000). Below 80 mg/L it contributes to the positive organoleptic properties of wines and complex aromas (Ribereau-Gayon, 1978), above which they have a diluting odour. The other most abundant group was higher alcohols, also common in Aszú wines (Machyňáková et al., 2021; Miklósy et al., 2000; Miklósy et al., 2004). These aroma components are produced by yeasts during amino acid metabolism. Excessive concentrations are considered undesirable in wine, but moderate concentrations contribute to the desirable complexity of the wine aroma (Lilly et al., 2006). Our studies revealed a more complex aroma profile in the *St. lactis-condensi*-dominated Essences.

5. Conclusion

The yeast *St. lactis-condensi* is considered to be a wine spoilage agent, which at first sight is associated with some negative effects. However, our studies have clarified this statement. One of the reasons why the yeast *St. lactis-condensi* is one of the dominant representatives of the yeast biota of the Tokaj Essences is that it is well adapted to the environment characteristic of these wine specialities more efficient at utilising fructose than the *St. bacillaris* and grows in the simultaneous presence of glucose and fructose in a similar way to *Z. rouxii* strains. It can easily compete with *St. bacillaris*, as it tolerates the high sugar content of wine, combined with alcohol and sulphur, better. At the strain level, they are selected by higher sulphur and alcohol content. *St. lactis-condensi* does not produce harmful substances such as biogenic amines, large amounts of hydrogen sulphide and unpleasant odorous components that would negatively affect organoleptic qualities or health. In addition, the yeast species *St. lactis-condensi* isolated from the Tokaj wine-growing region has both physiological and genetic diversity.

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Hajnalka Csoma: Conceptualization, Methodology, Validation, Writing – original draft, review & editing. **Zoltán Kállai:** Methodology, Validation, Writing – original draft. **Kinga Czentye:** Methodology. **Matthias Sipiczki:** Conceptualization, Funding acquisition, Supervision, Writing –critical review.

Declaration of competing interest

There are no conflicts to declare.

Data availability

Data will be made available on request.

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