

Monitoring of qualitative parameters of red wine from the Nitra winegrowing region in Slovakia using the method of simultaneous sugar and malic acid fermentation

Štefan Ailer¹, Lucia Ailer ^{2,*}, Dana Urminská³, Zuzana Chlebová², and Violeta Ivanova-Petropulos ⁴

¹Faculty of Horticulture and Landscape Engineering, Institute of Horticulture, Slovak University of Agriculture in Nitra, Nitra, Slovak Republic

²AgroBioTech Research Centre, Slovak University of Agriculture in Nitra, Nitra, Slovak Republic

³Faculty of Biotechnology and Food Sciences, Institute of Biotechnology, Slovak University of Agriculture in Nitra, Nitra, Slovak Republic

⁴Faculty of Agriculture, University "Goce Delčev"-Štip, Krste Misirkov 10-A, 2000 Štip, North Macedonia

*Corresponding author: AgroBioTech Research Centre, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic.

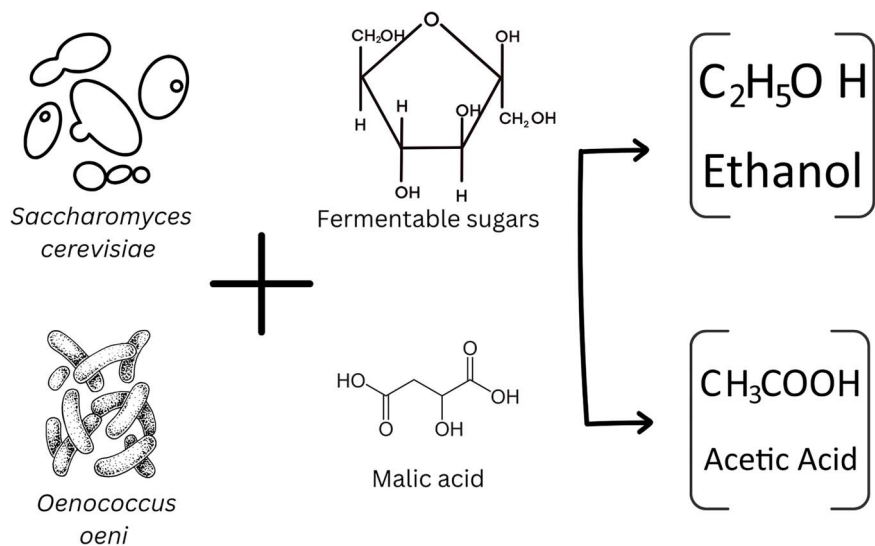
Email: lucia.benesova@uniag.sk

Abstract

The experiment dealt with the different procedures for malic acid degradation in red wine using the malolactic fermentation (MLF) process, its influence on qualitative parameters, and the formation of undesirable acetic acid in wine in the vintages of 2020 and 2021. We used the "Cabernet Sauvignon" variety, active dry *Saccharomyces cerevisiae* yeast and pure culture of the *Oenococcus oeni* malolactic bacteria. We found that MLF by noble, selected cultures can take place simultaneously when fermentable carbohydrates are present without significantly increasing acetic acid levels. All inoculation variations reached final acetic acid values of 0.807–0.820 g/L in 2020 and 0.753–0.780 g/L in 2021. The control variations without inoculation had the highest acetic acid values, which were 0.897 g/L (2020) and 0.803 g/L (2021) at the end of fermentation. In 2021, the control variant proved inadequate, leaving 0.53 g/L of malic acid in the wine. The duration of fermentation was shortened by simultaneous co-inoculation of yeast and malolactic bacteria without affecting unwanted acetic acid. During this process, it is, in addition to monitoring acetic acid, extremely important to control the pH value, which must be lower than 3.5. The alternative of using MLF as a technology involving the co-inoculation of yeast and malolactic bacteria starter culture without a negative impact on the wine's quality proved to be suitable and effective.

Keywords: red wine, malolactic fermentation, malic acid, acetic acid

Graphical abstract



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Introduction

The majority of red wines are produced using malolactic fermentation (MLF), a process involving the biological degradation of L-malic acid into L-lactic acid through the metabolic activity of lactic acid bacteria (Payan et al., 2023). This transformation reduces the wine's acidity and contributes to its microbiological stability and sensory refinement (Burns & Osborne, 2013; Vasyagin et al., 2024; Vion et al., 2024). Malic acid, a dicarboxylic acid responsible for the harsh taste of unripe grapes, is enzymatically decarboxylated to the milder L-lactic acid, with the release of carbon dioxide, resulting in a rounder and smoother flavour profile (Bauer & Dicks, 2017). The MLF process removes malic and citric acid, which are key carbon sources for bacterial metabolism, and thus limits the potential for unwanted degradation reactions post-bottling. The bacteria involved in MLF are generally classified into homofermentative (e.g., *Pediococcus*) and heterofermentative (e.g., *Lactobacillus*, *Oenococcus*) genera. Among these, *Oenococcus oeni* is the most widely used due to its high tolerance to wine's harsh conditions, including low pH, elevated ethanol content, and the presence of sulphur dioxide (Bartle et al., 2019; Muñoz et al., 2014). Despite its benefits, MLF is not without risks. When spontaneous, or carried out by non-noble or non-selected bacterial strains, the fermentation of residual sugars can lead to the formation of undesirable compounds, such as acetic acid and mannitol, resulting in sensory and quality defects in the final product (Bartle, 2020; Bartle et al., 2019). Therefore, controlling MLF through inoculation with noble, selected strains of *O. oeni* is increasingly recommended to ensure predictable results and reduced risk of spoilage (Ailer & Pintér, 2023; Paramithiotis et al., 2022).

Commercially available strains of *O. oeni* are developed to promote clean MLF with minimal by-product formation. These strains are capable of fermenting malic acid even in the presence of high concentrations of fermentable sugars, under conditions such as pH < 3.5 and SO₂ levels below 40 mg/L (Mendes Ferreira & Mendes-Faia, 2020; Filimon et al., 2022; Mangas et al., 2023). The strategy of co-inoculating yeast (*Saccharomyces cerevisiae*) and MLF starter cultures at the beginning of alcoholic fermentation, referred to as early co-inoculation, has been introduced as a technological innovation to streamline vinification and ensure microbiological safety (Costello et al., 2023; Semon et al., 2001). This co-inoculation approach can shorten the duration of fermentation, stabilise wine more efficiently, and allow earlier addition of protective sulphur dioxide. However, the risk of excessive volatile acidity—mainly acetic acid production—remains a concern, particularly in sugar-rich environments. Noble strains of *O. oeni*, selected for their aromatic neutrality and low volatile acid production, have been engineered to mitigate this issue, but practical validation under diverse vinification conditions is still limited (Bartle et al., 2019; Romero et al., 2018).

The chemical and sensory impact of MLF is broad, including changes in pH, reductions in bitterness and astringency, and the production or modification of key aroma compounds, such as diacetyl, acetoin, and esters (Plavša et al., 2021; Bartowsky et al., 2015; Sumbly et al., 2014; Swiegers et al., 2005). Some bacterial strains, especially from *Lactobacillus plantarum*, have been shown to enhance wine aroma through the production of enzymes, such as β -glucosidase, esterases, and decarboxylases, thereby further enriching the organoleptic profile (Du Toit et al., 2011). The complexity of MLF is compounded by the influence of multiple variables, such as pH, ethanol concentration, temperature, SO₂ levels, yeast–bacteria interactions, and nutrient availability (Cinquanta et al., 2018; Guzzon et al., 2016; Liu et al., 2017). These factors affect not only the kinetics of malic acid degradation but

also the risk of undesirable compound formation. Of particular importance is the level of free SO₂ (Bevardi et al., 2018). At 15 mg/L, most *O. oeni* cells die within hours, while 5 mg/L allows MLF to proceed over several weeks (Bartle et al., 2019). The second most abundant acid in grapes, L-malic acid, is known for its sensitivity to environmental factors, such as temperature, vineyard location, and cultivation practices, all of which influence its final concentration in must (Gerós et al., 2012; Zhang et al., 2023). In contrast to tartaric acid, which is chemically stable, malic acid degrades easily during fermentation. This variability further underscores the importance of controlled MLF, particularly in red wines intended for ageing or undergoing complex post-fermentation processes, such as sur lie maturation or micro-oxygenation (Pollon et al., 2023). Given the growing interest in MLF optimisation, particularly via co-inoculation strategies, further research is needed to assess their efficacy under practical winemaking conditions. The development of new bacterial strains capable of completing MLF even at higher sugar contents without raising acetic acid levels presents an opportunity to improve both process efficiency and product quality (Azzolini et al., 2010; Izquierdo-Cañas et al., 2016; Russo et al., 2020).

The aim of this study was to evaluate different procedures for the degradation of malic acid in red wines using MLF and to monitor their effects on key quality parameters, especially the production of undesirable acetic acid. A particular focus was placed on testing whether the selected heterofermentative lactic acid bacterium *O. oeni* produces excessive acetic acid in the presence of fermentable sugars when co-inoculated with yeast.

Materials and methods

Experimental design

In this study, the “Cabernet Sauvignon” variety was grown in the Nitra wine-growing area of Slovakia. Geographic coordinates of the vineyard: 48.301220° N, 18.100772° E. Brown soils predominate in this locality, and the skeletal content is minimal. The average rainfall in the area is 580 mm, and the average temperature is 11.3 °C. During the growing season, the average temperature was 18 °C. The site is characterised by mild winters. The active CaCO₃ content is 7%. The vineyard is managed on the Rhine-Hesse vine shape. The vines were grafted on rootstock SO₄. The vineyard was planted in 2007. The grape harvest took place over 2 years on 28 October 2020 and 5 October 2021. For each vintage, we used 220 kg of hand-picked grapes. After destemming and crushing the grapes, we adjusted the refraction of the must with sucrose to 20.5% in both vintages to obtain the desired ethanol content in the product above a 12% vol. Conditions for successful malolactic fermentation by noble malolactic bacteria strains are summarised in Table 1. As malolactic bacteria is extremely sensitive to the SO₂ content, we did not use any sulphurisation until the lactic acid value of the wine was below 0.1 g/L. The mash was inoculated on the same day with a pure culture of the noble yeast *S. cerevisiae* ZYMAFLORE RX60 (Laffort, France). Three variations in the timing of malolactic bacteria inoculation relative to alcoholic fermentation and one variation without the use of noble malolactic bacteria were generated. After inoculation with yeast, we divided each variant into three repetitions, each of 15 L of mash. Alcoholic and MLF was carried out at a controlled temperature of 19–22 °C. The mash was fermented in 30 L stainless steel containers. During fermentation, we manually mixed it three times a day. After the final measurement of physicochemical parameters (23 November 2020 and 5 November 2021), we added SO₂ to all variants in the amount of 40 mg/L in the form of potassium pyrosulphite.

Table 1. Conditions for successful malolactic fermentation by noble malolactic bacteria strains.

| Factor | Value |
|--|--|
| Number of starting strains of <i>Oenococcus oeni</i> | Minimum 106/ml |
| Alcohol concentration | Maximum 15% (vol/vol) |
| Temperature | Optimum 22–25 °C |
| pH value | Minimum 3.3 |
| Total SO ₂ | Lower than 40 mg/L |
| Yeast sludge and nutrients | Leave the yeast sludge, the addition of nutrients is also possible |
| Oxygen content | Microaerophilic environment |
| Interactions between malolactic bacteria and yeast | Use only mutually compatible strains |
| Exclusive production of lactic acid | No formation of diacetyl, acetic acid, or mannitol |

Note. Source: (Liu et al., 2017; Mozzi et al., 2010; Steidl, 2002; Technical Sheet Laffort, 2020).

Table 2. Experimental application of *Oenococcus oeni* at different times after initiation of alcoholic fermentation.

| Variant | Application date of <i>O. oeni</i> (2020) | Time after initiation of AF (30 Oct 2020) | Variant | Application date of <i>O. oeni</i> (2021) | Time after initiation of AF (5 Oct 2021) |
|---------|---|---|---------|---|--|
| 7001 | 30 Oct | 0 days | 8001 | 5 Oct | 0 days |
| 7002 | 5 Nov | 6 days | 8002 | 10 Oct | 5 days |
| 7003 | 10 Nov | 11 days | 8003 | 19 Oct | 14 days |
| 7004 | Control variant (without <i>O. oeni</i> addition) | - | 8004 | Control variant (without <i>O. oeni</i> addition) | - |

Note. AF = alcoholic fermentation of saccharides; 7001 = co-inoculation malolactic bacteria (on the same day as the *S. cerevisiae* starter); 7002 = malolactic bacteria added 6 days after *S. cerevisiae* fermentation; 7003 = malolactic bacteria added 11 days after *S. cerevisiae* fermentation; 7004 = control variant without inoculation with a noble malolactic bacteria culture; 8001 = co-inoculation malolactic bacteria (on the same day as the *S. cerevisiae* starter); 8002 = malolactic bacteria added 5 days after *S. cerevisiae* fermentation; 8003 = malolactic bacteria added 14 days after *S. cerevisiae* fermentation; 8004 = control variant without inoculation with a noble malolactic bacteria culture.

Table 3. Total duration and timetable of analysis of the content profile at different times after the addition of the *Oenococcus oeni* culture in 2020.

| Date of analysis | Variant | | | | |
|------------------|------------|------------|------------|------|---|
| | 7001 | 7002 | 7003 | 7004 | |
| 28 Oct | | | | | Control variant (without <i>O. oeni</i> addition) |
| 2 Nov | 4-day MLF | | | | |
| 6 Nov | 8-day MLF | 2-day MLF | | | |
| 10 Nov | 12-day MLF | 6-day MLF | 1-day MLF | | |
| 23 Nov | 25-day MLF | 19-day MLF | 14-day MLF | | |

Note. MLF = malolactic fermentation; 7001 = co-inoculation malolactic bacteria (on the same day as the *S. cerevisiae* starter); 7002 = malolactic bacteria added 6 days after *S. cerevisiae* fermentation; 7003 = malolactic bacteria added 11 days after *S. cerevisiae* fermentation; 7004 = control variant without inoculation with a noble malolactic bacteria culture.

Identification of variants

The designation of the experimental variants is presented in Table 2. The control was the variant without malolactic bacteria inoculation, which is still common among many winemakers. A commercial malolactic bacteria culture of *O. oeni* (Lactoenos SB3 Direct, Laffort, France) was used to inoculate the fermenting must according to the manufacturer's instructions. The bacterial strain used is intended for the direct inoculation of red wines (wine-centrum.sk) in a controlled MLF process. It is classified for the processing of products intended for direct human consumption in the wine industry and in accordance with Commission Delegated Regulation (EU) 2019/934 (Commission Delegated Regulation (EU) 2019/934 of 12 March 2019 Supplementing Regulation (EU) No. 1308/2013 of the European Parliament and of the Council as Regards Wine-Growing Areas Where the Alcoholic Strength May Be Increased, Authorised Oenological Practices and Restrictions Applicable to the Production and Conservation of Grapevine Products, the Minimum Percentage of Alcohol for by-Products and Their Disposal, and Publication of OIV Files, C/2019/1869, OJ L 149, 7.6.2019, p. 1–52., n.d.).

The characteristics of the *O. oeni* strain used according to the manufacturer (Lactoenos SB3 Direct, Laffort, France) were as

follows: low volatile acidity production, aromatic neutrality, and no biogenic amine production. We rehydrated the freeze-dried bacteria to 20 times their weight in non-chlorinated water at 20 °C for 15 min maximum and added them to the mash. We used the following inoculation methods: early co-inoculation (bacteria inoculation started simultaneous with alcoholic fermentation), co-inoculation 6 days after starting alcoholic fermentation, and co-inoculation 11 days after starting alcoholic fermentation. The specific timepoints were chosen based on the actual dynamics of alcoholic fermentation, aiming to reflect realistic winemaking conditions and identify phases during which co-inoculation might influence acetic acid formation. These intervals were selected operationally rather than arbitrarily, to capture different fermentation stages and their impact on MLF performance. The MLF time in the inoculated variants ranged from 14 to 30 days (Tables 3 and 4).

Methods for must and wine analysis

An ALPHA analyser (Bruker Optik GmbH, Ettlingen, Germany) was utilised to determine the chemical parameters in the wine samples. The measuring technique used was Fourier transform infrared (FT-IR) spectroscopy with attenuated total

Table 4. Total duration and timetable of analysis of the content profile at different times after the addition of the *Oenococcus oeni* culture in 2021.

| Date of analysis | Variant | | | |
|------------------|------------|------------|------------|---|
| | 8001 | 8002 | 8003 | 8004 |
| 5 Oct | | | | Control variant (without <i>O. oeni</i> addition) |
| 8 Oct | 4-day MLF | | | |
| 13 Oct | 9-day MLF | 4-day MLF | | |
| 20 Oct | 16-day MLF | 11-day MLF | 2-day MLF | |
| 5 Nov | 30-day MLF | 25-day MLF | 17-day MLF | |

Note. MLF = malolactic fermentation; 8001 = co-inoculation malolactic bacteria (on the same day as the *S. cerevisiae* starter); 8002 = malolactic bacteria added 5 days after *S. cerevisiae* fermentation; 8003 = malolactic bacteria added 14 days after *S. cerevisiae* fermentation; 8004 = control variant without inoculation with a noble malolactic bacteria culture.

Table 5. Analytical evaluation of primary grape must.

| Parameter | Year 2020 (28 Oct 2020) | Year 2021 (5 Oct 2021) |
|------------------------------|-------------------------|------------------------|
| Fructose (g/L) | 97.32 ± 0.055 | 99.85 ± 0.120 |
| Glucose (g/L) | 92.35 ± 0.116 | 94.14 ± 0.240 |
| Refraction (%) | 19.04 ± 0.012 | 19.97 ± 0.095 |
| Density (g/cm ³) | 1.08 ± 0.0001 | 1.08 ± 0.0008 |
| Malic acid (g/L) | 3.22 ± 0.012 | 4.88 ± 0.0536 |
| pH | 3.21 ± 0.012 | 3.09 ± 0.021 |
| Tartaric acid (g/L) | 4.17 ± 0.088 | 4.17 ± 0.033 |
| Total acid content (g/L) | 8.37 ± 0.088 | 8.85 ± 0.058 |
| Total sugar content (g/L) | 182.07 ± 3.067 | 197.13 ± 0.260 |

The values are given as the arithmetic mean of three separate repetition measurements ± SE of the mean. Note. To obtain the desired ethanol content in the product above a 12% vol before alcoholic fermentation, we adjusted the refraction of the must with sucrose to 20.5% (205 g/L total sugar content) in both vintages.

Table 6. Acetic acid content during and after malolactic fermentation in individual variants.

| Acetic acid (g/L) | | | Acetic acid (g/L) | | |
|-------------------|-------------|---------------|-------------------|-------------|---------------|
| Variant | Date (2020) | Mean ± SD | Sample | Date (2021) | Mean ± SD |
| 7001 | 2 Nov | 0.243 ± 0.006 | 8001 | 8 Oct | 0.470 ± 0.020 |
| | 6 Nov | 0.557 ± 0.015 | | 13 Oct | 0.503 ± 0.021 |
| | 10 Nov | 0.790 ± 0.020 | | 20 Oct | 0.510 ± 0.010 |
| | 23 Nov | 0.807 ± 0.006 | | 5 Nov | 0.753 ± 0.015 |
| 7002 | 2 Nov | 0.287 ± 0.006 | 8002 | 8 Oct | 0.507 ± 0.015 |
| | 6 Nov | 0.683 ± 0.012 | | 13 Oct | 0.607 ± 0.015 |
| | 10 Nov | 0.863 ± 0.012 | | 20 Oct | 0.650 ± 0.010 |
| | 23 Nov | 0.807 ± 0.006 | | 5 Nov | 0.763 ± 0.021 |
| 7003 | 2 Nov | 0.277 ± 0.006 | 8003 | 8 Oct | 0.570 ± 0.026 |
| | 6 Nov | 0.767 ± 0.176 | | 13 Oct | 0.720 ± 0.030 |
| | 10 Nov | 0.737 ± 0.006 | | 20 Oct | 0.717 ± 0.021 |
| | 23 Nov | 0.820 ± 0.001 | | 5 Nov | 0.780 ± 0.010 |
| 7004 | 2 Nov | 0.327 ± 0.015 | 8004 | 8 Oct | 0.590 ± 0.020 |
| | 6 Nov | 0.717 ± 0.006 | | 13 Oct | 0.647 ± 0.023 |
| | 10 Nov | 0.873 ± 0.012 | | 20 Oct | 0.723 ± 0.015 |
| | 23 Nov | 0.897 ± 0.015 | | 5 Nov | 0.803 ± 0.012 |

Note. SD = standard deviation of three repetitions.

reflection. This technique enables the simultaneous study of multiple parameters with a single measurement. The settings of the analyser were calibrated for wine and must sample. The accredited DAKS Institute Heidger (Osann Monzel, Germany) measured the wine calibration spectra and calibrated the settings based on reference values. Over 2,000 red and white wines comprised the calibration data. It was discovered what was the root mean square error of prediction (RMSEP) was. After filtration and centrifugation, wine samples were examined without further tampering. The flow-through cell was injected with about 10 ml of wine sample. The material was heated to 40 °C before the spectrum was capture in the range of 0.78–1,000 nm. Within 70–100 s, the equipment examined the sample,

and the results were recorded. By comparing the reference values of each parameter to the standard wine for analysis (Bruker Optic GmbH, Germany), the accuracy of the process was verified.

Statistical analysis

The data were analysed using the statistical software XLSTAT v.2021.4.1 (Addinsoft, Paris, France). The data distribution was tested using the Shapiro–Wilk test at the 0.05 level of statistical significance. The statistical significance of the difference between the samples was evaluated using the Kruskal–Wallis test (post-hoc Dunn test) at a significance level of $p = .05$.

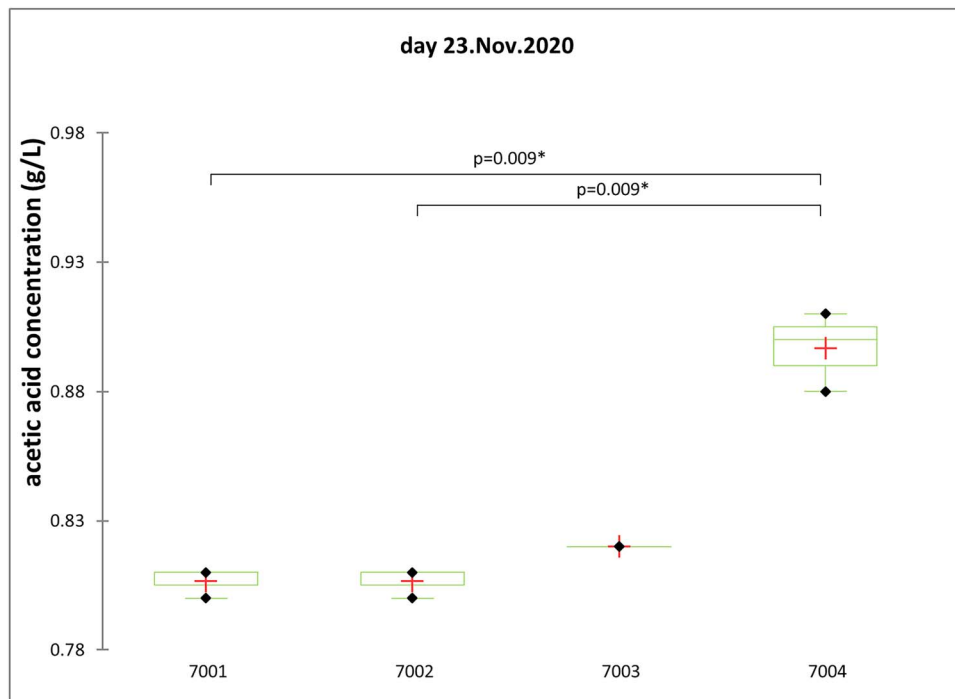


Figure 1. Statistical evaluation of acetic acid concentrations (g/L) at the end of malolactic fermentation in individual variants (vintage 2020).

Results and discussion

Physicochemical parameters of grape must

The results of the analysis of the initial must prior to the initiation of alcoholic fermentation and MLF are shown in Table 5. The total sugar content in the must was higher in 2021 than in 2020. The total acid and tartaric acid contents in must were similar in the two compared years, while the malic acid content was 1.66 g/L higher in 2021 than in 2020. The pH value was 0.12 lower in 2021 than in 2020, which was related to the higher malic acid content. Other monitored parameters were comparable in both years.

Acetic, malic, and lactic acid concentrations and pH of wine

Samples of each experimental variant were subjected to an analysis of parameters that could potentially be affected by the MLF process and the malolactic bacteria inoculation date. The following conditions are sufficient for the formation of acetic acid by spontaneous flora malolactic bacteria: residual sugar content above 4 g/L, aerobic conditions, temperature over 25 °C, and microbially damaged grape. While performing MLF, it is important to prevent excessive increases in the acetic acid concentration. The legislative limit for the volatile acid content in wine within the EU is above 1 g/L, depending on the wine category. A volatile acid content over 1.4 g/L is already considered a fault (Ailer et al., 2021). The main goal of MLF is to metabolise malic acid, which has a harsh sensory effect with tannins, without the formation of unwanted by-products, especially acetic acid. During MLF, the pH increases, while astringency and bitterness perception decrease (Ailer et al., 2020). The production of acetic acid was the lowest in variants co-inoculated with *O. oeni* (7001 and 8001) during both years. The highest acetic acid concentrations were observed in the control samples without *O. oeni* inoculation (variants 7004 and 8004) in both years, namely 0.897 g/L in 2020 and 0.803 g/L in 2021. In both monitored years, the acetic acid content in the control variants was significantly higher ($p \leq .05$) compared to

the inoculated variants (Table 6, Figures 1 and 2). Lonvaud-Funel (2022) states that the best energy substrates for *O. oeni* are sugars: glucose, fructose, and pentoses. If the alcoholic fermentation was properly completed, any increase in volatile acidity caused by heterofermentative lactic acid bacteria is limited, and the acetic acid value does not exceed 1.0 g/L. The noble bacteria strain *O. oeni* used by us, even when used simultaneously with yeast, did not produce an increased concentration of acetic acid even in a sugar environment, and this was the main goal of the research.

We found a statistically significant influence ($p \leq .05$) of vintage on the malic acid content in the must (2020—3.22 g/L; 2021—4.88 g/L; Table 5). The lactic acid content in the experimental variants at the end of MLF correlated with the malic acid content, and the difference between years was statistically significant ($p \leq .05$; 2020—1.2 g/L; 2021—2.2 g/L; Figures 3 and 6). The malic acid content in the basic must did not affect the total duration of MLF. In samples co-inoculated with *O. oeni* and yeast (variants 7001 and 8001), malic acid concentrations decreased almost linearly, with a corresponding increase in lactic acid concentrations during MLF. All three experimental variants had less than 0.1 g/L malic acid values at the end of MLF. The control variants at the same time of analysis had malic acid values less than 0.1 g/L in 2020 but up to 0.53 g/L in 2021, which confirmed the risks and uncertainties of spontaneous MLF (Figures 4 and 7). The effect of using malolactic bacteria was more pronounced in 2021 from the point of view of the controlled formation of malic acid and acetic acid. For an even more accurate comparison of the effect of individual inoculation, it is necessary to carry out a physicochemical analysis of malic and lactic acid contents between the 12th and 21st days of fermentation, at least every 3 days. In our research, we primarily focused on monitoring the formation of unwanted acetic acid using individual malolactic bacteria inoculation methods.

A positive result is that the early date of *O. oeni* inoculation resulted in lower acetic acid levels at earlier measurement dates compared to the other variants. These results agree with those of Cañas et al. (2015). However, there are also studies in which

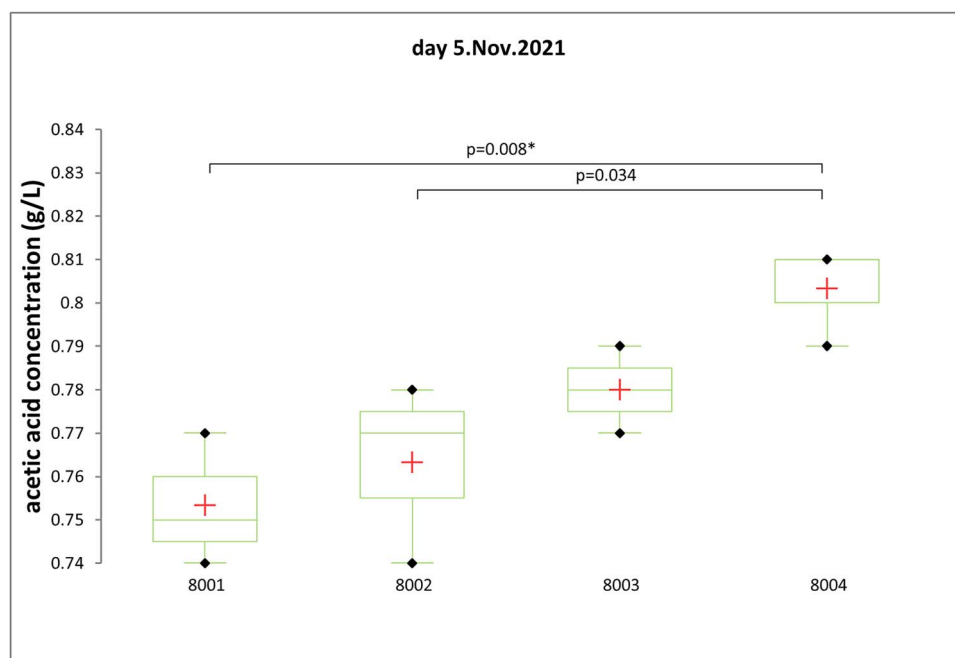


Figure 2. Statistical evaluation of acetic acid concentrations (g/L) at the end of malolactic fermentation in individual variants (vintage 2021).

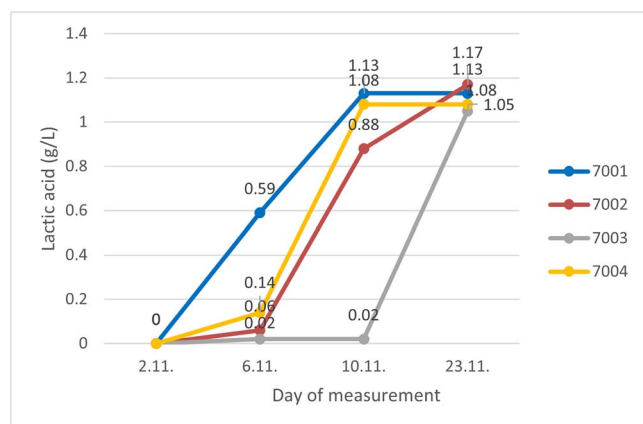


Figure 3. Lactic acid concentration (g/L) during and after malolactic fermentation in individual variants (vintage 2020).

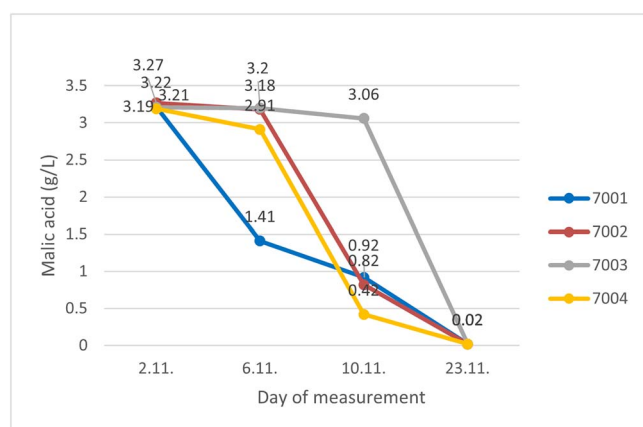


Figure 4. Malic acid concentration (g/L) during and after malolactic fermentation in individual variants (vintage 2020).

the authors observed reduced (Tristezza et al., 2016) or increased acetic acid concentration after yeast and malolactic bacteria co-inoculation compared to sequential inoculation (Garofalo et al., 2015; Knoll et al., 2012; Liu, 2002). The variability in the results of different studies may be due to different must compositions, vinification conditions and malolactic bacteria strains. Our results showed that the acetic acid concentration at the end of MLF in experimental variants was not dependent on the timing of *O. oeni* inoculation relative to alcoholic fermentation and the values were not statistically significantly different ($p \leq .05$). The measured final acetic acid concentration in our study slightly exceeded the threshold concentration of its perception (0.7 g/L) but was within the range of the current legislation for red dry wines (1.2 g/L) and did not have a negative impact on the quality of the final product. According to Miranda et al. (2017) and Zhang et al. (2023), the olfactory perception of acetic acid and ethyl acetate in wines depends on their age and degree of sweetness. The higher the sugar content, the higher the perception threshold. In sweet Madeira, they indicate a threshold of up to 1.4 g/L. Since red wines are generally produced dry, it is necessary to control the undesirable formation of volatile acids. Parra et al. (2023) noted that in the formation of acetic acid in wine, contamination with *Acetobacter aceti* bacteria from the operating premises and the vineyard is also of great importance. We eliminated this by processing healthy grapes and ensuring the hygiene of the research premises. Our results suggest that MLF by *O. oeni* culture can take place even in the presence of fermentable sugars without a significant increase in acetic acid levels, which can cause wine defects. Overall, spontaneous MLF is more difficult to control, and the outcome is more difficult to predict compared to controlled malic acid degradation via pure malolactic bacteria cultures. Yilmaz and Gökmen (2021) and Paramithiotis et al. (2022) compared spontaneous and controlled MLF. Only 28% of malic acid was metabolised during 15 days of natural MLF, whereas all malic acid in red wine fermented with *O. oeni* was consumed for 7 days. While successful spontaneous MLF can enhance the wine's aromatic profile and reflect its terroir, uncontrolled processes

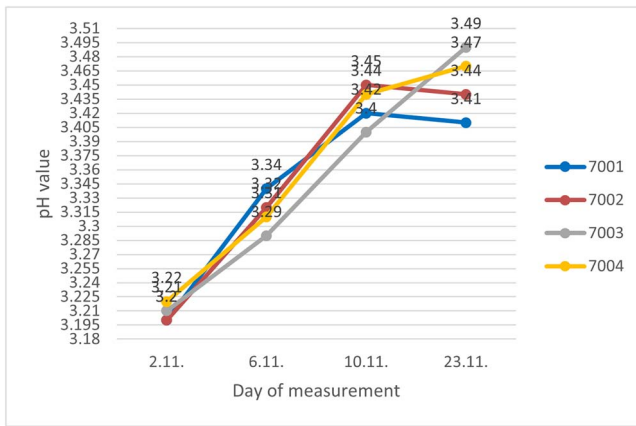


Figure 5. pH value during and after malolactic fermentation in individual variants (vintage 2020).

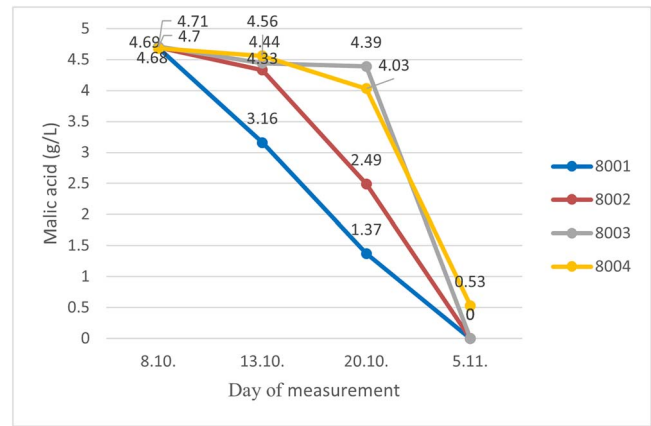


Figure 7. Malic acid concentration (g/L) during and after malolactic fermentation in individual variants (2021).

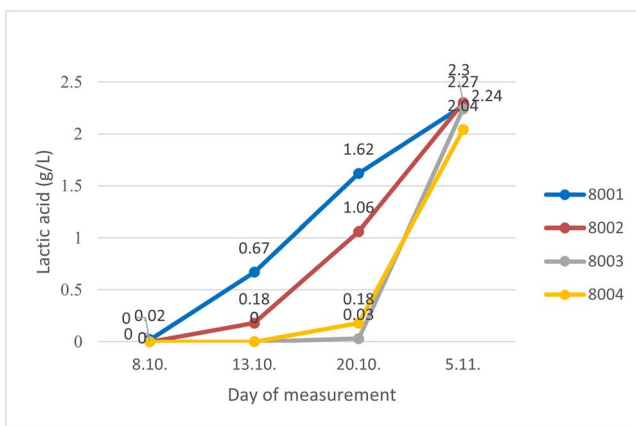


Figure 6. Lactic acid concentration (g/L) during and after malolactic fermentation in individual variants (vintage 2021).

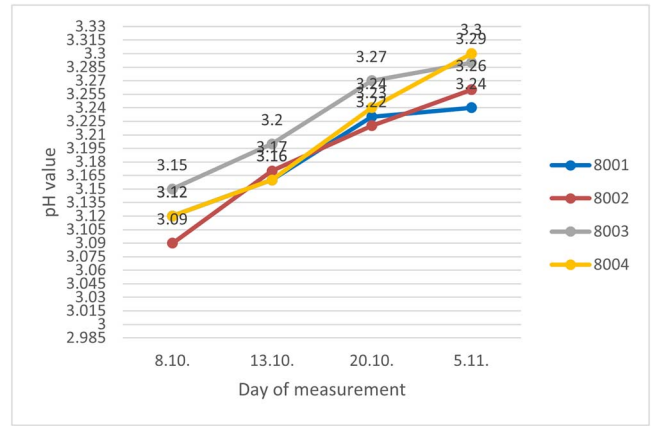


Figure 8. pH value during and after malolactic fermentation in individual variants (2021).

may negatively impact sensory quality (Sumby et al., 2019). In the future, innovative biotechnical methods for managing undesirable components of wine are also expected. Guo et al. (2024) investigated the metabolism of acetic acid in fruit wine using the yeast *Pichia barkeri* yeast can ferment acetic acid into alcohol. The acetic acid concentration in wines declined by 65%–73% compared to the pure fermentation of *S. cerevisiae*. Furthermore, the method boosted the variety and concentration of ester compounds, including ethyl decanoate, ethyl dodecanoate, and ethyl hexadecanoate, potentially improving the fruity flavour of the wine. The priority of technology for red wines intended for sur lie ageing and micro-oxidation is the complete malic acid degradation, which is microbially unstable. Microbial unstable components in long-term contact with yeast sludge can have a negative and undesirable effect, namely the production of sensorially undesirable volatile compounds (Pollon et al., 2023).

None of the samples had a pH exceeding 3.5, which is critical for increased acetic acid production in the presence of residual sugar (Budziak-Wieczorek et al., 2023; Vavřinik et al., 2022b). At the end of the study period, the resulting pH ranged from 3.40 to 3.45 in 2020 and from 3.24 to 3.30 in 2021 in all samples (Figures 5 and 8).

Total sugar and alcohol content

In both years, there was an almost complete overfermentation of total sugars, and a peak alcohol concentration was reached

within 2 weeks of alcoholic fermentation onset. The residual sugar content at the end of the observation period ranged from 1.80 (variant 7002) to 1.93 g/L (variant 7003) in 2020 (Figure 9). In 2021, the sugar content ranged from 1.83 (variant 8002) to 3.83 g/L (variant 8003) (Figure 10). The dynamics of the conversion of sugars to alcohol was almost the same for all inoculated and non-inoculated variants in both observation years.

Simultaneous inoculation of MLF starter cultures with yeast offers more favourable conditions for bacterial growth and metabolism, such as higher nutrient availability and lower ethanol levels (Russo et al., 2020). The availability of nutrients is greater in grape must than in wine, and there is less alcohol and other potential yeast-derived inhibitors (Bell & Henschke, 2005). These conditions are thought to allow lactic bacteria to acclimatise to ethanol as the concentration gradually increases, thereby improving MLF performance (Azzolini et al., 2010).

According to Zapparoli et al. (2016), wine with a high alcohol level may suppress lactic acid bacteria growth after inoculation. This indicates that, in the case of high-alcohol wines, early inoculation may be crucial to malic acid consumption.

Co-inoculation of yeast and malolactic bacteria represents an alternative method of application of microorganisms in commercial wine production, which is finding an increasing number of applications in common practice, especially in the production of red wines, due to the potential to reduce the overall duration of the vinification process and thus make the production process more efficient. The success of the co-inoculation process depends

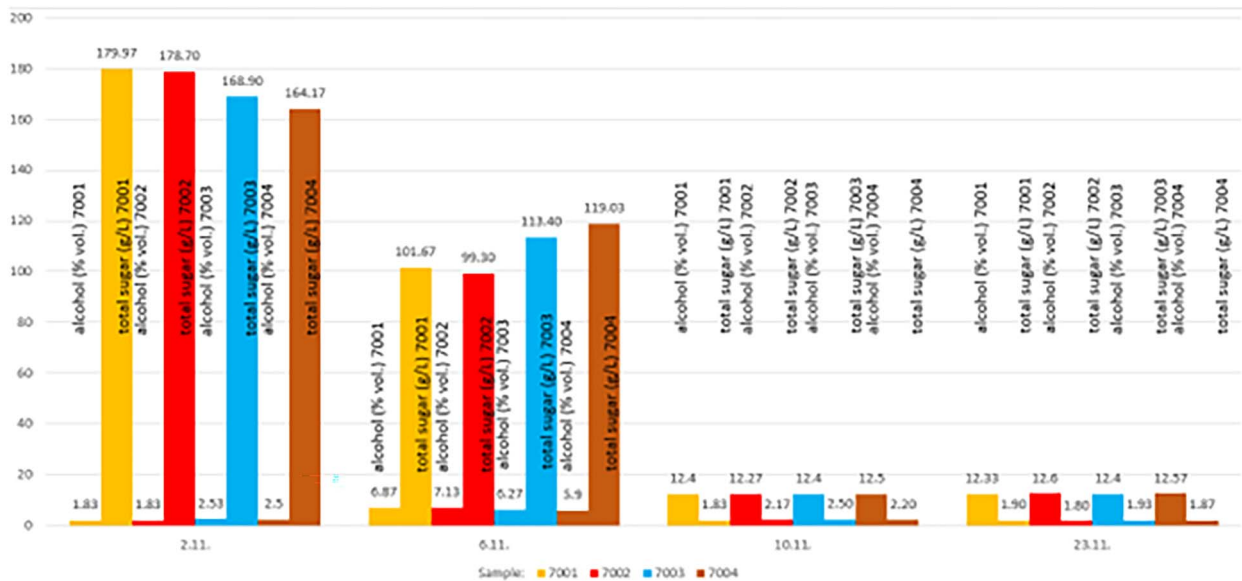


Figure 9. Alcohol content (% vol.) and total sugar concentration (g/L) during and after malolactic fermentation (vintage 2020).

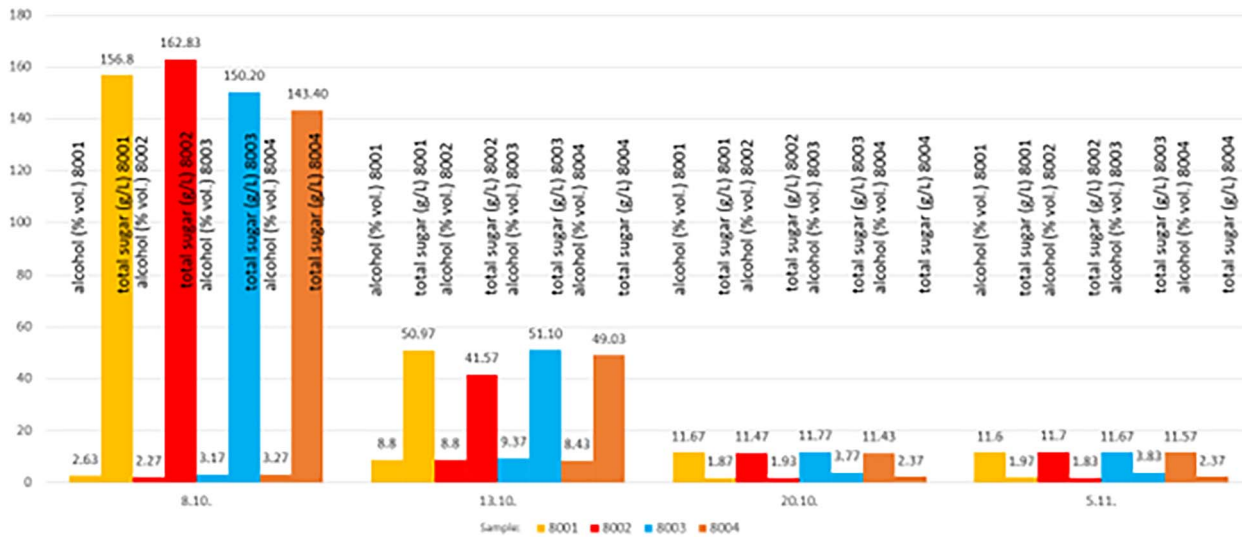


Figure 10. Alcohol content (% vol.) and total sugar concentration (g/L) during and after malolactic fermentation (vintage 2021).

largely on the mutual compatibility of the yeast and malolactic bacteria strains since yeast can stimulate or inhibit the MLF process, and malolactic bacteria in turn can stimulate or inhibit the alcoholic fermentation process (Versari et al., 2016). In our work, we used commercially available starter culture strains of yeast and *O. oeni* and did not observe any negative interactions between them. Sugar fermentation was smooth in all variants, without defects (Figures 9 and 10). In all inoculated samples, there was complete malic acid degradation (Figures 4–7).

The advantages of simultaneous inoculation with yeast and malolactic bacteria have led to its increasing validation in wine-making practices. Our acetic acid monitoring results followed those of previous studies (Guzzon et al., 2013; Lasik-Kurdyś et al., 2017), showing that shortening the amount of time between alcoholic fermentation and MLF lowers the danger of microbiological faults caused by the growth of unfavourable microorganisms. In our study, we monitored acetic acid content regularly to verify whether the selected heterofermentative malolactic bacteria *O. oeni* produced excessive amounts of acetic acid in the presence of

carbohydrates, as reported by Bartle et al. (2019). In the selected malolactic bacteria strains that we used, the process of excessive acetic acid production did not occur. A shorter time between alcoholic fermentation and MLF allows for accelerated vinification and the earlier addition of protective SO₂ levels (Beregal et al., 2017; Vavřinik et al., 2022a).

Abrahamse and Bartowsky (2012) evaluated the timing of malolactic bacteria inoculation under several regimes, including simultaneous yeast inoculation and inoculation at 4, 7 and 10 days after yeast inoculation, and found that all methods resulted in the effective completion of MLF; however, co-inoculation dramatically shortened the overall vinification time and changed the wine's volatile profile and pigments from versions that had malolactic bacteria inoculation later.

There were differences in fluency in the total duration of MLF between the different dates of *O. oeni* inoculation, and the earliest inoculated variants showed earlier malic acid degradation and thus a shorter vinification process. These results are consistent with those of other authors Tristezza et al. (2016) and Versari et al.

(2016), who compared co-inoculation and sequential inoculation methods for red wine production from the point of view of the dynamics of lactic acid formation and malic acid degradation. There are fewer mentions in scientific databases about sugar metabolism through malolactic bacteria in sequential MLF technology and its influence on acetic acid formation, which we focused on in our study.

The results of our study suggest that the early application of *O. oeni* culture is a comparable alternative to late inoculation in terms of the physicochemical parameters of the final product. We do not recommend the use of spontaneous MLF because the risks of defects and the creation of unwanted by-products in the future outweigh the benefits of specific local characteristics of the wine.

Conclusion

We found that MLF by noble, selected cultures of malolactic bacteria *O. oeni* (Lactoenos SB3 Direct, Laffort, France) took place simultaneously when fermentable carbohydrates were present, without significantly increasing acetic acid levels. The control variations without inoculation had the highest acetic acid values, which were 0.897 (2020) and 0.803 g/L (2021). In 2021, spontaneous degradation proved inadequate, leaving 0.53 g/L of malic acid in the wine. Earlier inoculation with *O. oeni* reduced the total vinification time. Co-inoculation of a compatible commercial *O. oeni* starter culture at the earliest date after alcoholic fermentation initiation proved to be the optimal date for MLF start-up in our conditions. The dynamics of acid degradation were more uniform and predictable with the early application method than with later application, while the resulting profile of the investigated contents was comparable. The duration of both fermentations (to the point when the concentration of malic acid dropped below 0.1 g/L and total sugar below 4 g/L) was shortened by up to 10 days in the variant with simultaneous inoculation of yeast and malolactic bacteria, which is a significant savings from the point of view of operational logistics. During this process, it is extremely important to control the pH value. In the case of applying the obtained results in practical oenology when using malolactic bacteria inoculations before pressing, it is extremely important to pay attention to hygiene and sanitation to avoid cross-contamination of musts and wines not intended for malolactic technology.

Data availability

The data that support the findings of this study are available upon request from the corresponding author.

Author contributions

Štefan Ailer (Conceptualisation, Methodology, Investigation, Writing—original draft, Supervision), Lucia Ailer (Visualisation, Data curation, Writing—review & editing), Zuzana Chlebová (Writing—review & editing, Software), Violeta Ivanova-Petropulos (Writing—review & editing, validation), and Dana Urminská (Project administration, Funding acquisition, Resources)

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Conflicts of interest

The authors declare that there is no conflict of interest, and all funding partners and collaborators have been acknowledged.

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