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Original article

Application of *Metschnikowia Pulcherrima* in Sequential Fermentations for the Production of Pilsner-Style Craft Beer

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Abstract

Previous studies have demonstrated promising potential and interest in the application of *Metschnikowia pulcherrima* in sequential fermentations in co-culture with Saccharomyces cerevisiae for the production of ale-style craft beer. This technique has yielded favorable results for top-fermenting beers, while its application in lager-style fermentations remains more limited. For this purpose, the indigenous S. cerevisiae 31 and M. pulcherrima 62 strains, isolated respectively from the grape varieties Shesh i Zi and Kallmet, were used to ferment a Pilsner-style beer in sequential inoculation. As a reference, a fermentation was prepared using the commercial lager strain S. pastorianus S-23 as a single starter. Fermentation kinetics and key physicochemical parameters were evaluated. The results showed that co-inoculation with M. pulcherrima 62 facilitated the completion of fermentation of S. cerevisiae 31, unlike the fermentation carried out with S. cerevisiae 31 alone. Furthermore, the co-cultures exhibited similar final alcohol content and pH values to those observed with the commercial strain S-23. However, higher levels of acetaldehyde were detected in beer obtained by sequential inoculation, while, glycerol concentrations were lower compared to beer obtained with S. pastorianus S-23. These preliminary findings suggest that the use of local non-Saccharomyces yeasts in Pilsner-style bottom fermentations is technologically feasible. Although not yet equivalent to commercial strains in performance, these findings are promising and provide a basis for the development of novel beer profiles using locally isolated starter cultures.

Keywords: Saccharomyces Cerevisiae, Metschnikowia Pulcherrima, Fermentation Characteristics, Sequential Inoculation, Pilsner Style, Craft Beer

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INTRODUCTION

Beer is the most popular alcoholic beverage in the world and in recent years there has been a significant shift in consumer preferences towards craft beer, leading to an increase in research and interest in this segment. Over the past two decades, craft breweries and their consumers have significantly influenced global beer markets, contributing to the rise of beer's popularity around the world. This trend is particularly notable among Millennials, who show a growing preference for non-alcoholic and low-alcohol beer styles, reflecting a broader societal shift toward health-conscious consumption (Chorbadzhiev, P. et, al., 2025; Withers, E.T. et, al., 2017; Garavaglia, C. et, al.,2017; Aquilani, B. et, al., 2015 ; Bellut, K. et, al., 2019). The assertion that most commercially available low and non-alcoholic beverages have a poor flavor profile, leading to consumer rejection, is generally accurate but becoming less so as the market evolves (Staub. et, al., 2022) In recent years, brewing industries have been adopting biotechnological strategies that involve the use of non-Saccharomyces yeasts, alone or in combination with Saccharomyces species, to increase sensory complexity and improve the acceptability by consumers of beers with reduced alcohol content (Catarino, M. et, al., 2011; Vrînceanu, C.R. et, al., 2025; Klimczak, K. et, al., 2024). Species such as M. pulcherrima have been extensively studied and applied in beer fermentation due to their effectiveness in producing low-alcohol beers while simultaneously enhancing the aromatic profile (Postigo, V. et, al., 2022 ; Karaulli, J. et, al., 2024). Due to its production of pulcherriminic acid, M. pulcherrima is widely used in the wine industry as a biocontrol agent, primarily for its ability to inhibit spoilage microorganisms but is not presently employed as a biocontrol agent in the brewing industry (Kregiel, D. et, al,. 2024 ; Canonico, L. et, al., 2023). In brewing, this yeast species is primarily used to enhance aroma-active compounds in beer, a function directly linked to the yeast's enzymatic activities such as β-glucosidase, β-lyase, and protease enzymes. β-glucosidase enzyme can hydrolyze glycoconjugate precursors and promote the release of active aromatic compounds from hop such as terpenes (Einfalt, D. et, al., 2021 ; Han, X. et, al., 2023 ; Karaulli, J. et, al., 2024) . Additionally, β-lyase activity produced by M. pulcherrima strains results in the release of volatile flavor-active thiols (Michel, M. et, al., 2019). M. pulcherrima, typically found in vineyards, is primarily used in brewing, especially in top-fermenting Ale styles where fermentation temperatures are similar to winemaking (Drosou, F. et, al., 2022). Additionally, M. pulcherrima is commonly used in co-culture with Saccharomyces yeast strains because of its low ethanol tolerance, which limits its metabolic activity during later stages of fermentation (Morata, A. et, al., 2019) Previous studies have also confirmed that M. pulcherrima, despite its antimicrobial properties, does not negatively impact the viability or metabolic performance of S. cerevisiae (Karaulli, J. et, al., 2024 ; Morata, A. et, al., 2019 ; Oro, L. et, al., 2014 ; Comitini, F. et, al., 2021). The aim of this study was to evaluate different fermentation trials using M. pulcherrima 62 in co-culture with S.cerevisiae 31 isolated from vineyard of Albania for the production of Pilsner-style beer.

A previous study showed that the use of M. pulcherrima 62 and S. cerevisiae 31 in sequential inoculations contributed to obtaining a better organoleptic and compositional quality of English Ale and American Wheat beers, compared to the same beers obtained when using S. cerevisiae 31 as a single starter (Karaulli, J. et, al. 2024).

MATERIAL and METHOD

Yeast Strains and Growth Condition

For this study *M. pulcherrima* 62 and *S. cerevisiae* 31 strains, belonging to the culture collection of the Agri-Food Research Centre of the Faculty of Biotechnology and Food of Agriculture University of Tirana, were used. These strains were previously isolated from autochthonous Albanian red grapes (Karaulli, J *et, al*., 2023) As a reference, the commercial yeast strain *S. pastorianus* SafLager™ S-23 (Fermentis, Lesaffre, Maisons-Alfort Cedex, France), was used. The yeasts were cultured aerobically at 28 °C in YEPD broth (Merck Millipore, Darmstadt, Germany) and after 48 h the broth cultures were centrifuged at 8,000 rpm for 10 min at 4 °C. Finally, the cell pellet was washed twice with saline solution (0.9% w/v NaCl) and used as inoculum. Cell density of inoculum was assessed using Thoma Counting Chamber (Thermo-Fisher Scientific) ((Karaulli, J. *et, al*. 2024)

Wort Production and Fermentation Tests

Beer wort was prepared using a Grainfather G Series brewing system (Bevie Handcraft, Nelson NZ Ltd) at the Department of Agricultural, Environmental and Food Sciences, University of Molise (Campobasso, Italy). For the production of pilsner wort, 100% Pilsner malt (Château Pale Ale, Castle Malting, Lambermont, Belgium) was utilized. Saaz hops (Barth-Haas, Nürnberg, Germany) were added during both the boiling and dry hopping stages. The main analytical characteristics of the beer wort are presented in Table 1.

Taste Panelists: The study was approved by the Ethical Committee, the University of Jordan: EMNMH 2219136 T: 3.1 A: 74173/2023. Human testing was conducted after oral consent was taken from each participant. Fifty volunteer panelists (16 males, 34 females, aged between 24 and 75 years old) were trained on sensory evaluation of pocket bread in November / 2023. They received two, 3-hour training sessions on the general aspects of organoleptic analyses and in-depth sensory evaluation of Arabic flat bread (Amr,1988;Elía, 2011). The training sessions were held by the Jordanian Society of Sensory Evaluation for Food. Analysis of Variance followed by Least significant Difference (LSD) test was performed on the responses of the volunteers to the overall acceptance of the bread by the end of the training sessions, and those with significantly different responses (four of them) to this attribute were excluded from the taste panel, thus only 46 panelists remained ranging in age between 24 and over 70 years (Table 1).

Table 1. Main chemical parameters of Pilsner beer wort

Parameters	Values
Density (g/cm^3)	1.043 ± 0.003
pH	5.62 ± 0.42
*FAN (mg/L)	222.3 ± 2.1
° Brix	11.4 ± 0.2
Maltose (g/L)	60.2 ± 0.5
Glucose (g/L)	6.1 ± 0.4
Parameters	Values

All values are expressed as the mean of three technical replicates ± standard deviation (n = 3). *FAN: Free Amino Nitrogen.

The flowchart shown in Figure 1 illustrates the main steps of the brewing process. Specifically, four independent fermentation tests were conducted under distinct experimental conditions. In Test A, fermentation was performed exclusively with the commercial yeast strain S. pastorianus S-23. Test B involved inoculation solely with the S. cerevisiae 31 strain. Test C employed a sequential inoculation strategy, with M. pulcherrima 62 strain introduced initially, followed by S. cerevisiae 31 inoculation after 48 h. Similarly, Test D utilized M. pulcherrima 62 as the primary inoculum, with subsequent inoculation of S. cerevisiae 31 after 96 h. Fermentation tests were carried out at 12°C ± 1°C using thermoregulated stainless steel tanks (capacity 30 L), containing 20 L of wort.

In all tests, the starter cultures were inoculated at an initial concentration around 106 CFU/ml. The fermentations were performed in triplicate. After 10 days of primary fermentation, 3.5 g/L of glucose was added as a primer for the secondary fermentation which took place in 330 mL dark brown glass bottles. After 40 days of maturation at 12 °C, the beers were subjected to chemical analysis. The progression of fermentation was assessed over time by monitoring pH and measuring density.

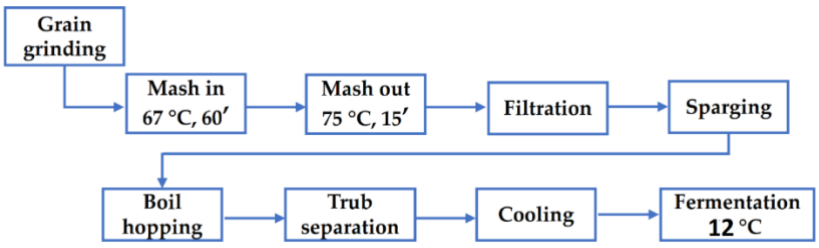

Figure 1. Flowchart of the brewing process

Chemical Analysis

Alcohol content (% *v/v*), density (g/cm³), and FAN (mg/L) were quantified following the analytical protocols outlined by the European Brewery Convention (EBC analytica *et, al.,* 2004). pH measurements were performed using a pH meter (Crison Basic 20, Barcelona, Spain). Volatile acidity was determined in accordance with the OIV MA AS313-02 method for wine, beer, and fermented beverages, employing an enochemical distillation unit (Gibertini model DEE, Milan, Italy). Glycerol (mg/L), total polyphenols (mg/L), and acetaldehyde (mg/L) concentrations were analyzed using

enzymatic assay kits (Steroglass, Perugia, Italy) following the manufacturer's instructions. Maltose, glucose, and sucrose contents were measured using Megazyme enzymatic kits (Bray Business Park, Ireland), also according to manufacturer protocols. Diacetyl concentration was determined based on analytical procedures described by the American Society of Brewing Chemists (Method 12 – Diacetyl), using a broad-spectrum spectrophotometric method (ASBC method), Merck KGaA, 64271 Darmstadt, Germany (Manual of Analysis Methods for the Brewery Industry , Method 12 – Diacetyl. *et, al*., 2014). All measurements were conducted in triplicate.

Statistical Analysis

Statistical analyses were performed using Statistics 10 (Analytical Software, Tallahassee, FL, USA). One-way analysis of variance (ANOVA) was applied to assess the effect of the physicochemical parameters in mixed fermentations in lager beers. When significant differences were found ($p < 0.05$), Tukey's Honest Significant Difference (HSD) test was used for post hoc comparisons among group means.

RESULTS and DISCUSSION

Fermentation Kinetics

The changes in density and pH monitored throughout alcoholic fermentation are shown in Figures 2 and 3.

In Test A, fermentation carried out using the commercial yeast strain S. pastorianus S-23 was considered complete after 9 days, as indicated by a reduction in density from 1.043 g/cm³ to 1.009 g/cm³. In Test B, the density dropped rapidly during the first days and stabilized after 9 days at 1.014 g/cm³ leading to an incompelte fermentation. As reported by Karaulli et al. (2024), this strain was previously shown to be incapable of fully fermenting maltose, likely due to weak maltase enzyme activity. The rapid initial decrease in density is likely attributed to glucose fermentation during the early stage (Dietvorst. J. et al., 2007). In Test C, initially inoculated with M. pulcherrima 62, the density decreased after 48 h of fermentation, from 1.043 to 1.039 g/cm³ with no significant changes in density, showning a low to moderate fermentative power compared with S-23 commercial yeast (Vincente, J. et, al., 2020). Following sequential inoculation with S. cerevisiae 31 after 48 h, the density reached 1.010 g/cm³ after 10 days of fermentation. This value is lower compared to Test B, which involved a single fermentation with S. cerevisiae 31. The improved attenuation may be attributed to the proteolytic activity of M. pulcherrima 62, which breaks down proteins into amino acids, thereby providing additional nutrients for S. cerevisiae 31 (Snyman, L. W. et, al., 2019; Fontana, M. et, al., 2009)

In Test D, following 96 h of fermentation with M. pulcherrima, the wort density decreased progressively to 1.030 g/cm³. This suggests that the yeast maintained fermentative activity throughout the period, although at a lower intensity compared to S. pastorianus S-23. Following sequential

inoculation with S. cerevisiae 31 at 96 h, the density decreased to 1.009 g/cm³ after 10 days of fermentation, comparable to the results observed in Test C (Figure 2).

Although S. cerevisiae 31 and M. pulcherrima 62 exhibited lower fermentative vigour compared to S. pastorianus S-23, our results show that inoculating initially with M. pulcherrima 62 did not negatively affect fermentation during the first 48 and 96 h. Furthermore, sequential inoculation of M. pulcherrima 62 followed by S. cerevisiae 31 improved fermentation performance of this strain, compared to using S. cerevisiae 31 alone, as reported by Karaulli et al. 2024 in a study on English Ale and American Wheat craft beers.

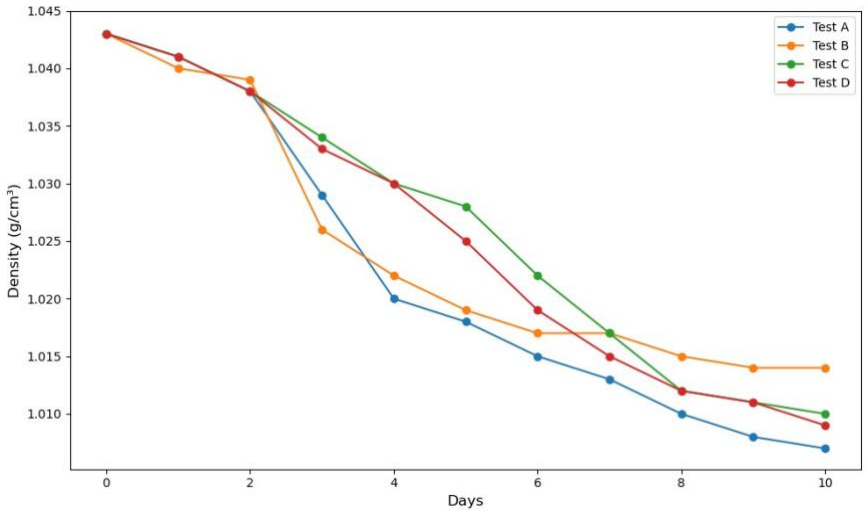

Figure 2. Density (g/cm³) trends during alcoholic fermentation of Pilsner beer

Test A: *S. pastorianus* S-23 as a single starter; Test B: *S. cerevisiae* 31 as a single starter; Test C: sequential inoculation with *M. pulcherrima* 62 followed by *S. cerevisiae* 31 after 48 h; Test D: sequential inoculation with *M. pulcherrima* 62 followed by *S. cerevisiae* 31 after 96 h

The initial wort had a pH value of 5.62. In Test A, the pH value declined progressively to reach a final value of 4.22 after eight days of fermentation and then remained stable. In Test B, the pH reached a final value of 4.18 despite incomplete fermentation. Following priming, the pH values of Tests A and B were comparable. In Tests C and D, the pH stabilised at 4.41 after 10 days of fermentation, with slight reductions observed after priming to 4.37 and 4.35, respectively.

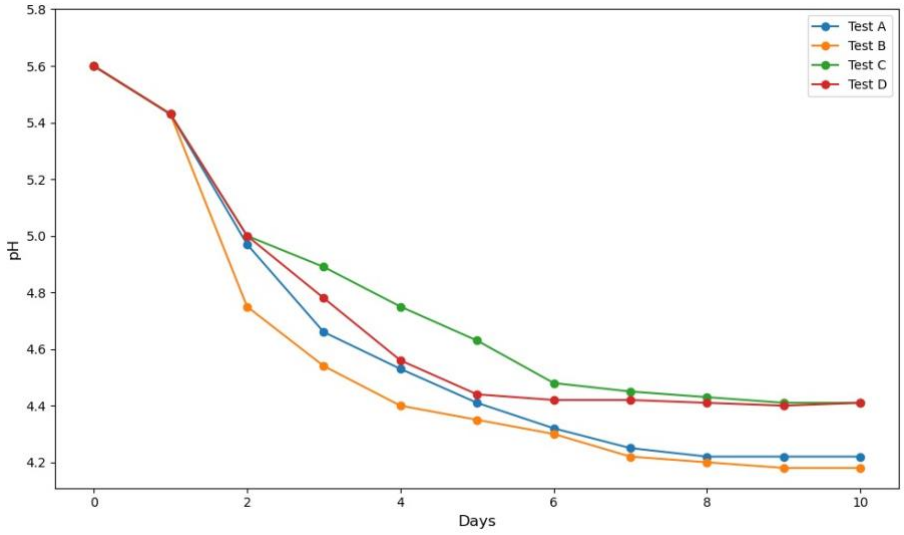

Figure 3. pH trends during alcoholic fermentation of Pilsner beer

Test A: *S. pastorianus* S-23 as a single starter; Test B: *S. cerevisiae* 31 as a single starter; Test C: sequential inoculation with *M. pulcherrima* 62 followed by *S. cerevisiae* 31 after 48 h; Test D: sequential inoculation with *M. pulcherrima* 62 followed by *S. cerevisiae* 31 after 96 h.

Main Chemical Parameters of Beers

The main physico-chemical parameters of the final beers are reported in Table 2. The highest alcohol contents were recorded in Test A (4.5% *v/v*), Test C (4.5% *v/v*), and Test D (4.4% *v/v*), with no significant differences among them. In contrast, Test B showed a significantly lower alcohol content (3.9% v/v), which can be attributed to incomplete fermentation.

Table 2. Main physical–chemical parameters of Pilsner beer style

Parameters	Test A	Test B	Test C	Test D
pH	4.22 ± 0.02^{c}	4.18 ± 0.03^{d}	4.37 ± 0.03^{a}	4.35 ± 0.02^{b}
Density (g/cm^3)	1.007 ± 0.001^{b}	1.014 ± 0.003^{a}	1.010 ± 0.002^{b}	1.009 ± 0.002^{b}
Alcohol (% v/v)	4.5 ± 0.2^{a}	3.9 ± 0.1^{b}	4.5 ± 0.3^{a}	4.4 ± 0.4^{a}
Volatile acidity (g/L)	0.42 ± 0.02^{b}	0.49 ± 0.03^{a}	0.48 ± 0.02^{a}	0.33 ± 0.02^{c}
Total polyphenols (mg/L)	108.5 ± 2.1^{c}	164.8 ± 6.6^{b}	252.0 ± 3.2^{a}	253.9 ± 2.8^{a}
Acetaldehyde (mg/L)	0.50 ± 0.03^{d}	3.60 ± 0.21^{b}	2.60 ± 0.12^{c}	8.80 ± 0.14^{a}
Diacetyl (mg/L)	0.16 ± 0.02^{a}	0.10 ± 0.01^{b}	0.06 ± 0.015^{c}	0.09 ± 0.01^{b}
Glycerol (g/L)	1.89 ± 0.07^{a}	1.41 ± 0.03^{b}	0.98 ± 0.01^{c}	0.98 ± 0.05^{c}
Maltose (g/L)	0.5 ± 0.1^{b}	6.23 ± 0.13^{a}	0.44 ± 0.04^{b}	0.62 ± 0.04^{b}
Glucose (g/L)	nd	nd	nd	nd

Test A: *S. pastorianus* S-23 as a single starter; Test B: *S. cerevisiae* 31 as a single starter; Test C: sequential inoculation with *M. pulcherrima* 62 followed by *S. cerevisiae* 31 after 48 h; Test D: sequential inoculation with *M. pulcherrima* 62 followed by *S. cerevisiae* 31 after 96 h.

All values are expressed as mean ± standard deviation (n = 3). Different superscript letters in each row indicate significant differences ($p < 0.05$). nd: not detected.

Beers obtained from Tests C and D exhibited higher total polyphenol content. This finding is consistent with a previous study by Karaulli *et al.* 2024. The increase is related probably to the β-

glucosidase enzymatic activity profile of *M. pulcherrima*, which can release polyphenols bound to glucosides, a mechanism well-documented in wine fermentation (Comitini, F. *et, al*., 2011). However, the extent of this enzymatic activity in beer fermentation remains limited.

Although some studies suggest that *M. pulcherrima* can produce elevated levels of acetic acid in the presence of oxygen, especially during wine fermentation, the amount of volatile acidity was very low in all fermentation tests (Comitini, F. *et, al*., 2011; Jolly, N. P. *et. al*., 2014). In line with previous research, *M. pulcherrima* 62 strain produced a low amount of acetic acid (Benito, S. *et, al*., 2015; Michel, M. *et, al*., 2016). Notably, Test D produced beer with significantly lower volatile acidity (0.33 mg/L), indicating better fermentation quality. These results emphasise the potential of *M. pulcherrima* 62 to control volatile acidity during fermentation, making it a valuable yeast for enhancing beer quality and optimising production processes.

Acetaldehyde is a key volatile compound generated during the fermentation and aging processes in the brewing industry. Where present at high concentration this compund is considered an off-flavor and couse an unpleasnt aroma in the final product (Moreira, M.T.G. *et, al*., 2024; Liu, C. *et, al*., 2018). Acetaldehyde in beer is primarily formed as an intermediate during yeast metabolism, especially during alcoholic fermentation under anaerobic conditions, pyruvate is decarboxylated by pyruvate decarboxylas, releasing CO_2 and forming acetaldehyde (Eram, M.S. *et, al*., 2013). The threshold values of acetaldehyde in beer have been reported to be 10–25 mg/L (MacGregor AW *et, al*., 1996). In our study, acetaldehyde concentrations were below the minimum detection threshold in all beer samples analyzed, with Test A exhibiting the lowest measurable value of 0.50 mg/L. In Test B, the acetaldehyde concentration was 3.60 mg/L, which was higher than in Test A and Test C. This increase is likely attributable to oxidation and suboptimal fermentation conditions, Test C expressed 2.60 mg/L value and Test D exibhit the hights value of 8.80 ± 0.14 mg/L. These differences should be considered preliminary and reflect the specific metabolic behavior of the yeast strains and the fermentation conditions applied in this study rather than definitive technological limitations. This observation can be attributed to the findings of Comitini. F. *et al*., 2011, which demonstrated that *M. pulcherrina* lacks strong alcohol dehydrogenase (ADH) activity and has limited capacity to reduce acetaldehyde to ethanol.

Glycerol is a polyalcohol with a slightly sweet taste, produced as a byproduct during the fermentation of wort by yeast. It is a colorless, odorless, and viscous liquid that contributes to the beer's body, fullness, and flavor intensity (Zhao, X. *et. al*., 2015; Li, H. *et. al*., 2015). Although recent studies have reported an increase in glycerol levels during fermentation in co-culture with *M. pulcherrima* (Mencher, A. *et. al*., 2021 ; Ivit, N.N. *et.al,* 2021 ; Karaulli, J *et, al*., 2023), our results indicate that Test A, exhibited the highest glycerol concentration, reaching 1.89 g/L. This value was higher compared to Test B (1.41 g/L), and Tests C and D, both showing the same glycerol concentration (0.98 g/L). These results suggest that the treatments used in beer production have different effects on glycerol content, which may influence the organoleptic properties and overall quality of the final product. This probably

because *S. pastorianus* are known to produce more glycerol than certain strains of *S. cerevisiae*, particularly under stress conditions such as low fermentation temperatures, or limited oxygen (Troianou, V. *et. al*., 2019). Under anaerobic conditions, *M. pulcherrima* could lead to increased production of glycerol and acetic acid in mixed fermentation with *S. cerevisiae* (Sadoudi, M. *et. al*., 2017 ; Lin, X. *et, al*., 2023 Karaulli, J. *et, al*., 2024) but as highlighted in previous study of Karaulli *et al*., 2024, it can be hypothesized that *M. pulcherrima* may consume part of the acetate to synthesize acetate esters during mixed fermentation with *S. cerevisiae*. These results should be interpreted as preliminary and indicative of the complex metabolic interactions occurring during sequential fermentations involving non-*Saccharomyces* yeasts and further investigations focusing on fermentation optimization and sensory evaluation are required to fully understand the impact of indigenous yeast strains on glycerol formation and overall beer quality

Diacetyl (butanedione or butane-2,3-dione) is a vicinal diketone generated by yeast as a by-product of amino acid metabolism during beer fermentation, imparting aroma characteristics described as butter, when detected above its flavor threshold > 0.1 mg/L (Stewart, G.G. *et, al*., 2017) In Test A, the concentration of diacetyl slightly exceeded the sensory detection threshold, reaching 0.16 mg/L. In Test B, the concentration was at the threshold level of 0.10 mg/L despite incomplete fermentation. In contrast, Tests C and D showed sub-threshold levels of 0.06 mg/L and 0.09 mg/L, respectively. These results suggest that different treatment conditions can significantly affect diacetyl concentrations in beer, which could impact the sensory profile of the final product.

Regarding glucose and maltose utilization, *S. pastorianus* S-23 in Test A was able to completely ferment both sugars. In contrast, in Test B, glucose was fully metabolized by *S. cerevisiae* 31, whereas maltose fermentation remained incomplete, with a residual concentration of 6.23 g/L. This incomplete utilization is likely attributable to low maltase activity, which has been associated with inefficient maltose fermentation in previous studies (Karaoglan, S.Y. *et. al.,* 2022 ; Karaulli *et al*., 2024).

In Tests C and D, the fermentation was fully completed reaching the value 0.44 g/L of maltose in Test C, and 0.62 g/L in Test D. Similar results were observed by Karaulli *et al*. (2024) during co-fermentation with *M. pulcherrima* 62 and *S. cerevisiae* 31 in the production of English Ale and American Wheat beers. This enhanced fermentation efficiency is likely due to the microaerobic environment created by *M. pulcherrima*, enabling more efficient sugar utilization and fermentation completion (Karaoglan, S.Y. *et. al.,* 2022; Karaulli *et al*., 2024).

The beer produced from Test B exhibited a notable residual maltose concentration following fermentation. This suggests either incomplete fermentation or insufficient treatment to ensure full sugar utilization. In contrast, Tests A, C and D demonstrated sugar profiles consistent with successful fermentation and are therefore considered more technologically appropriate. These results show that the

applied treatment can significantly affect sugars concentration in the final beer, which could impact its flavor and quality.

Conclusion

In recent years, M. pulcherrima, a non-Saccharomyces yeast species, has attracted increasing scientific interest due to its remarkable and versatile fermentative potential. This includes its ability to contribute to complex aroma profiles, influence microbial communities during fermentation and improve the quality of fermented products.

The use of M. pulcherrima 62, recently explored in the production of Ale-style beers, represents an innovative biotechnological approach to improving fermentation dynamics.

This study demonstrates that M. pulcherrima 62 effectively supports S. cerevisiae in sequential inoculation protocols for the fermentation of Pilsner-style beers. This improves fermentation efficiency and metabolic performance compared to using S. cerevisiae alone. The application of M. pulcherrima in the production of bottom fermented beers could contribute to improve the stability, flavor profile, and quality of the product, offering potential benefits for craft and industrial production. Further research is needed to validate these findings and to perform sensory analyses, including the characterization of volatile organic compounds, in order to fully assess the impact of these native yeasts on beer quality and their applicability at a broader industrial scale.

Additional Declaration

Author Contributions

In this study, the contribution of the authors was: Experiment and the laboratory analysis were carried out at the University of Molise under the supervison of professor Iorizzo and the assistance of Dr. Bruno Testa and Dr. Francesco Letizia. All the authors from Agricultural University of Tirana contributed equally to the development of the research idea, data analysis, writing and proofreading stages. The first author contributed to all stages.

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Responsible Artificial Intelligence Statement

No artificial intelligence support was received in any part of this study**.**

Ethics Approval

In all processes of this study, the principles of Pen Academic Publishing Research Ethics Policy were followed.

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