

Article

Performance of Wild Non-Conventional Yeasts in Fermentation of Wort Based on Different Malt Extracts to Select Novel Starters for Low-Alcohol Beers

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Featured Application: A mixed promising starter for the production of low-alcohol beers with pleasant organoleptic characteristics was individuated.

Abstract: Nowadays, the increasing interest in new market demand for alcoholic beverages has stimulated the research on useful strategies to reduce the ethanol content in beer. In this context, the use of non-*Saccharomyces* yeasts to produce low-alcohol or alcohol-free beer may provide an innovative approach for the beer market. In our study, four wild non-*Saccharomyces* yeasts, belonging to *Torulaspora delbrueckii*, *Candida zemplinina* and *Zygosaccharomyces bailii* species, were tested in mixed fermentation with a wild selected *Saccharomyces cerevisiae* strain as starters for fermentation of different commercial substrates used for production of different beer styles (Pilsner, Weizen and Amber) to evaluate the influence of the fermentative medium on starter behaviour. The results obtained showed the influence of non-*Saccharomyces* strains on the ethanol content and organoleptic quality of the final beers and a significant wort–starter interaction. In particular, each starter showed a different sugar utilization rate in each substrate, in consequence of uptake efficiency correlated to the strain-specific metabolic pathway and substrate composition. The most suitable mixed starter was P4-CZ3 (*S. cerevisiae*–*C. zemplinina*), which is a promising starter for the production of low-alcohol beers with pleasant organoleptic characteristics in all the tested fermentation media.

Keywords: non-*Saccharomyces* yeasts; *Candida zemplinina* (*Starmerella bacillaris*); *Zygosaccharomyces bailii*; *Torulaspora delbrueckii*; low-alcohol beer; mixed cultures; wort–starter interaction



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1. Introduction

In order to satisfy the new market demand for alcoholic beverages with low alcohol content, research has devoted attention toward useful strategies to reduce the ethanol content in alcoholic beverages such as beer. These approaches can be divided into two main categories, physical and biological methods, which operate at two different points of the production process. The different physical methods actually available are applied to finished beer by selective removal of ethanol without modification of the quality characteristics of the products. Biological methods, conversely, act during fermentative process and are addressed to limit the ethanol production by yeasts during fermentation.

In this context, the use of yeast strains different from *Saccharomyces* yeasts, traditionally inoculated as starter cultures for brewing, is attracting increasing interest. The production of low-alcohol beers by the use of non-*Saccharomyces* yeasts is related to the limited ability of these yeasts to ferment wort sugars. Furthermore, these yeasts are characterised by metabolic activities different from *Saccharomyces*, which can potentially introduce new

flavours in these beers. In fact, the fermentative process plays a key role in determining the characteristics of the final product, as yeast metabolism affects not only the ethanol yield from the sugar substrate but also aroma composition. The yeast strains chosen for wort fermentation and beer conditioning play a fundamental role in beer quality, as the production of numerous aromatic compounds is strain-dependent [1,2]. Pyruvate produced by yeasts during glycolysis provides carbon skeletons for the synthesis of several aroma compounds, such as esters and higher alcohols [3]. Furthermore, yeast's enzymatic activities can modify the phenolic composition of wort, determining the release of volatile organic compounds. As a consequence, the use of non-conventional yeasts might allow the production of low-alcohol beers with peculiar aromatic traits. Therefore, management of fermentation by using novel yeast starters represents the widest space in which the brewer can easily operate for beer diversification. This is very attractive for the current beer market, which is seeking to extend their product portfolio to satisfy consumer demand, always looking for new styles of beer. Furthermore, this approach might be very useful for small and craft breweries. In these production plants, changing the yeast is a feasible modification compared with the substantial equipment investment necessary for the application of physical methods for dealcoholisation.

In recent years, the selection of non-conventional yeasts for the production of low-alcohol beers has expanded. Although these yeasts have been conventionally considered detrimental for alcoholic beverages, as they can negatively affect sensorial characteristics of the final products [4–8], the set-up of a suitable selection protocol and the accurate management of fermentative process can contribute to the production of fermented beverages with innovative aromatic characteristics that fulfil the modern consumer's expectations of products with novel aromatic tastes obtained without the use of chemical additives.

Numerous studies report the use of non-*Saccharomyces* yeasts as bio-flavouring agents in wine production [9–11] but, in recent years, studies describing the use of these yeasts for brewing processes have become available [7,12,13]. As non-conventional yeasts generally are characterised by lower ethanol yield than *Saccharomyces*, they are frequently used in co-fermentation or in sequential fermentation with classical *Saccharomyces* brewing yeasts, but some studies report the use of these yeasts as pure starter cultures for producing low-alcohol (0.5–1.2% *v/v*) or even alcohol-free (<0.5% *v/v*) beers, which are increasingly demanded beverages [14]. Strains belonging to *Brettanomyces* or *Dekkera* genera are among the most investigated non-conventional yeasts for beer fermentation [15]. Most of these yeasts are able to ferment the main sugars present in wort; furthermore, these yeasts hydrolyse glucoside-bound monoterpenes present in the hops, liberating monoterpenes such as linalool that are the key aroma substances from hops [16]. However, other yeasts have recently been used in the brewing process. For instance, *Saccharomyces ludwigii* [17] and *Pichia kluyveri* [18], characterised by an inefficient fermentation of maltose and maltotriose, were successfully used to produce alcohol-free beers with rich flavour. *Torulospira delbrueckii* has been proposed for a long time as a mixed culture in winemaking for its positive influence on wine aroma, whereas in the last few years, this species has also been used for ethanol reduction both in wine and beer [19–21].

The studies investigating the contributions of *T. delbrueckii* in beer have demonstrated that the use of this yeast, both in pure and mixed fermentations with *S. cerevisiae*, produces low-alcohol beers with a distinctive analytical and aromatic profile [19,21–23]. Similar results were reported for *Zygosaccharomyces rouxii*, which consumed ethanol under aerobic conditions and produced desirable flavour compounds, obtaining low-ethanol and flavourful beers [24]. *Lachancea thermotolerans* was proposed by Domizio et al. [25] as a pure culture for the production of sour beers without the use of lactic acid bacteria, as this yeast lowered the pH better than *S. cerevisiae*.

In this study, four wild non-*Saccharomyces* strains, namely *T. delbrueckii* LC2-1, *Candida zemplinina* TSF and CZ3, *Zygosaccharomyces bailii* CR1, were investigated in mixed fermentation with *S. cerevisiae* for their application in the production of low-alcohol beer. The mixed starters were tested in fermentation media obtained from different malt extracts in

order to evaluate the influence of the medium on the fermentative performance and aroma production of each yeast culture.

2. Materials and Methods

2.1. Yeast Strains

Five indigenous yeast strains from the UNIBAS Yeast Collection (UBYC), University of Basilicata (Potenza, Italy), were tested in this study. The strains used were *Candida zemplinina* (now reclassified as *Starmerella bacillaris*) CZ3 and TSF, *Zygosaccharomyces bailii* CR1, *Torulasporea delbrueckii* LC2-1 and *Saccharomyces cerevisiae* P4 [26]. The strains were previously isolated from grape must or fruit juice and identified by restriction analysis of the amplified ITS region [27] and analysis of the variable D1/D2 domain of the large-subunit (26S) rDNA gene [28]. In addition, three commercial *S. cerevisiae* strains, Fermentis SAFALE WB-06 (code SW), Fermentis SafAle US-05 (code SP) and Wyeast London Ale No. 1028 (code SA), were used.

The strains were maintained at 4 °C on YPD agar medium (1% (*w/v*) yeast extract; 2% (*w/v*) peptone, 2% (*w/v*) glucose; Oxoid, Hampshire, UK) for short-term storage and in YPD broth supplemented with 50% of glycerol (Sigma, St. Louis, MO 63304, USA) at −20 °C until further analysis.

2.2. Wort Preparation

The wort was prepared by using three different malt extracts (MEs) provided by Mr. Malt Company, which were sealed by the company for the production of Pilsner, Weizen and Amber beer styles. The characteristics of the MEs were the following:

- ME for Pilsner beer: original specific gravity (SG) 1036, pH 5.92;
- ME for Weizen beer: SG 1036, pH 5.94;
- ME for Amber beer: SG1038, pH 5.91.

The wort used for the microfermentation trials was prepared by firstly reconstituting dry malt extract (1.5 kg) in sterilised pure drinking water (12 L). The mixture was stirred until complete homogenization. The final wort was stored at 4 °C and, before inoculation, the absence of viable cells was checked by plate counting on Wallerstein Laboratory Nutrient Agar medium (WL; Oxoid, Hampshire, UK). The worts obtained by reconstituting the three different MEs were coded as following: PW (ME for Pilsner beer), WW (ME for Weiss beer) and AW (ME for Amber beer).

2.3. Microfermentation Trials

The 4 *S. cerevisiae* strains (3 commercial and 1 indigenous) were tested in a pure culture fermentation at lab scale in order to test the fermentative performance of the wild *S. cerevisiae* strain (P4). The 4 non-*Saccharomyces* strains were tested in a mixed fermentation at lab scale with the P4 *S. cerevisiae* strain. The inoculation levels of each strain, both as a single and mixed starter, are reported in Table 1.

Table 1. Inoculum level of pure and mixed starters used in the different microfermentation trials.

STARTER	INOCULUM LEVEL
P4-TSF	1×10^3 (P4) + 1×10^7 (TSF)
P4-CZ3	1×10^3 (P4) + 1×10^7 (CZ3)
P4-CR1	1×10^6 (P4) + 9×10^6 (CR1)
P4-LC2-1	1×10^6 (P4) + 9×10^6 (LC2-1)
P4; SP; SW; SA	1×10^7

All the fermentations were carried out at 20 °C ± 1 °C in flasks containing 100 mL of wort under sterile conditions. Pre-cultures of strains were grown in 10% Malt Extract Broth (Oxoid, Hampshire, UK) at 20 °C ± 1 °C for 48 h for *S. cerevisiae* strains and for 72 h for non-*Saccharomyces* strains. The fermentation trials were performed in duplicate under

static conditions. The fermentation kinetics were monitored by measuring total soluble solids (TSS) with a refractometer and also by measuring the weight loss of the flasks due to the CO₂ evolution until the end of fermentation, indicated by a constant weight and TSS for 3 consecutive days.

At the end of the primary fermentation, each beer, containing the remaining yeast (counts ranging between 1×10^5 and 1×10^6 cells/mL), was transferred into 100-mL sterile bottles and supplemented with sucrose (5 g/L; Oxoid, Hampshire, UK). The secondary fermentation was performed in the bottle at 19 ± 1 °C for 2 weeks. Experiments were performed in duplicate.

The growth kinetics during the fermentative process were monitored by colony forming unit (CFU) counts on WL Nutrient Agar (Oxoid, Hampshire, UK). At different fermentation times, 1 mL sample from each flask, serially diluted with 0.1% (*w/v*) peptone water, was spread by plating on WL medium; the plates were incubated at 26 °C for 5 days. Plates containing a statistically representative number of colonies were counted and around 20 colonies, randomly selected and representative of different morphologies, were purified on YPD plates for identification. The selected colonies were identified by 5.8S ITS-RFLP analysis.

2.4. Analytical Determination

The specific gravity of experimental beers was measured using a density meter and was used for calculation of apparent and real attenuation, according to Vidgren et al. [29], whereas the volatile acidity and ethanol content were determined according to the official methods, following the procedure reported by Canonico et al. [19].

Apparent attenuation was calculated by the equation:

$$\text{Apparent Attenuation \%} = 100 \left(1 - \frac{\text{Final apparent extract}}{\text{Original extract}} \right)$$

Real attenuation was calculated by the equation:

$$\text{Real Attenuation \%} = 100 \left(1 - \frac{\text{Final real extract}}{\text{Original extract}} \right)$$

The content of secondary compounds, such as acetaldehyde, ethyl acetate, *n*-propanol, isobutanol, *n*-butanol, acetoin, volatile acidity, active-amyl and isoamyl alcohols, were determined by direct injection of the sample using gas chromatography. Beer samples (1 µL) were directly injected into a chromatograph (Agilent 7890 A) with a 180 cm × 2 mm glass column with Carbowax 20% (Supelco, Sigma-Aldrich, Milan, Italy). The inlet temperature was 200 °C and the oven temperature was set to rise from 70 °C to 120 °C at a rate of 5 °C/min at 120 °C, 2 °C/min at 130 °C and 7 °C/min at 180 °C. The duration of the running was 22 min. The mobile phase gas was helium (He) at a flow rate of 23 mL/min. The quantitative analysis was performed by the internal standard calibration curve of the compounds of interest.

2.5. Statistical Analysis

Analysis of variance (ANOVA) was applied to the experimental data for the main characteristics of the 3 different beers used in this study, which was done after the verification of variance homogeneity (Levene's test, $p < 0.05$). Tukey's test was used to compare the mean values between mixed and pure fermentations at laboratory scale of each type of wort used.

The analytical data of the experimental beers were analysed by a heatmap, a method used to reduce the dimensionality of the data and to find the best differentiation between yeast strains and the 3 different worts used (PW, WW and AW). The data obtained were converted to Z-scores, calculated as follows: $Z\text{-score} = (X - \mu) / \sigma$, where, for each parameter in each wort, X is the concentration, μ is the mean value and σ is the standard deviation

among all the starters [30]. The software used for all the statistical analyses was PAST version 3.26 [31].

3. Results and Discussion

3.1. Mixed Fermentation Trials in Three Worts

In the preliminary step, the indigenous *S. cerevisiae* strain P4, used in a previous work [26], was tested in a laboratory fermentation of different worts, obtained by the three MEs, in comparison with the three commercial *S. cerevisiae* starters routinely used for Pilsen (SP), Weizen (SW) and Amber (SA) beer production, supplied by Mr. Malt Company. Figure 1 shows the apparent (AA) and real (RA) degree of attenuation of the beers produced with P4, SP, SW and SA as single cultures for each tested wort. In all the trials, P4 exhibited the highest value both for the AA and RA, except for the fermentation performed in Amber wort (AW), where P4 and SA showed similar AA and RA levels. Considering that the attenuation level is an important physico-chemical parameter of the beer, being correlated to the sugar content in the final product, the indigenous *S. cerevisiae* strain P4 demonstrated good performance in all beers, compared with each reference commercial starter, and therefore was used in mixed culture for the fermentation of the three different worts.

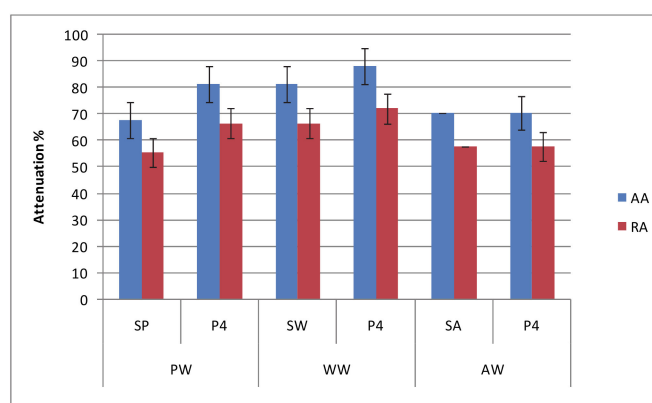


Figure 1. The apparent (AA) and real (RA) degree of attenuation (%) of the beers obtained by P4 and the three commercial starters (Pilsen starter (SP), Weizen starter (SW) and Amber starter (SA)) in wort obtained from malt extract for Pilsen (PW), Weizen (WW) and Amber (AW) beers. Data are the mean of two independent experiments.

This strain (P4) was isolated from sourdough, indicating that this substrate might represent a powerful source for isolation of brewing starter yeasts. In both brewing and baking, fermentation of cereal-derived sugars occurs; consequently, it is not surprising that yeasts isolated from these two sources are characterised by high phenotypic similarities, such as the ability to utilise maltose and often maltotriose [32]. Different studies reporting baking as a useful source of new brewing yeast strains are available. Thus, Rossi et al. [33], analysing yeasts isolated from different fermented foods and beverages (wine, cider and baking), found that baking yeasts yielded the best results during brewing trials. Other authors [34–36] compared strains isolated from sourdoughs with commercial brewing starters during pilot-scale fermentations; these experiments produced beers similar or better to those obtained by using the commercial brewing starter, with regard to yield and flavour profile.

3.2. Cell Growth Dynamics of the Strains and Evolution of Total Soluble Solids during Mixed Fermentation

The P4 *S. cerevisiae* strain was tested in pure and mixed fermentations with four non-*Saccharomyces* strains, which were CR1 (*Z. bailii*), LC2-1 (*T. delbrueckii*), TSF and CZ3 (*C. zemplinina*). These four non-*Saccharomyces* strains were chosen for their inability to ferment maltose (data not shown), resulting in suitable candidates for production of low-

alcohol beers. Among the non-*Saccharomyces* yeasts used in this work, *T. delbrueckii* was first suggested as a potential brewing yeast by King and Dickinson [37]. After that, different studies evaluated the use of this yeast species for brewing [19,22], whereas it should be emphasised that, currently, few data have been reported on the use of *Z. bailii* and *C. zemplinina* strains for brewing [38]. The fermentation trials were performed in duplicate and monitored by evaluation of the cell growth dynamics of each strain and evolution of total soluble solids (TSS). The results are reported in Figure 2.

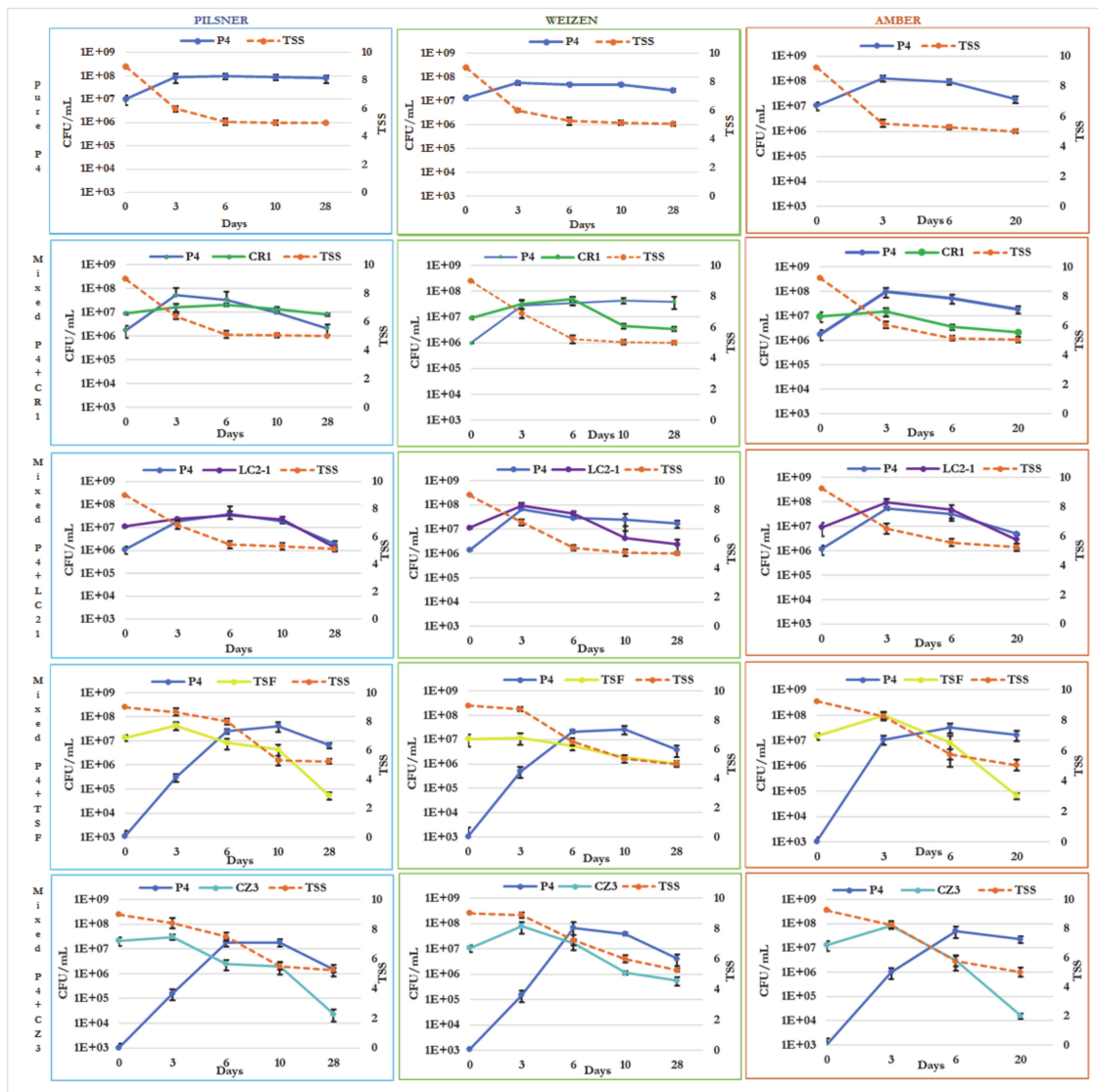


Figure 2. Fermentation kinetics of single and mixed starter cultures composed of *S. cerevisiae* (P4) and non-*Saccharomyces* strains (*Z. bailii* CR1, *T. delbrueckii* LC2-1, *C. zemplinina* TSF and CZ3) during fermentation in three different worts (PW, WW, AW), reported as cell counts on the left y-axis and reduction of total soluble solids (TSS) on the right y-axis. Continuous lines, viable cells; dotted lines, TSS. Data are the mean values \pm standard deviations of two independent experiments.

The overall duration of the fermentations was different, depending on the wort and the starter culture used. In fact, for Pilsner and Weizen beers, the primary fermentation performed with P4, P4-CR1 and P4-LC2-1 finished in 10 days, whereas more days were necessary to complete the primary fermentation by P4-TSF and P4-CZ3. The primary fermentation in AW was faster than in the other two worts, although differences were found among the starter cultures. As already reported for PW and WW fermentations, P4, P4-CR1 and P4-LC2-1 starter cultures consumed the sugars before P4-TSF and P4-CZ3.

The duration of the refermentation in bottle was 14 days for all beers, independently of the wort used. The initial amount of TSS was 9 degrees Plato ($^{\circ}\text{P}$) in PW and WW, and 9.5°P in AW, whereas the final content of TSS was around 5°P in all the beers produced. As regards the reduction of TSS during the time, the mixed cultures including *Z. bailii* and *T. delbrueckii* (P4-CR1 and P4-LC2-1, respectively) showed a similar trend to the pure P4 culture in PW and WW, with a progressive decrease from 9 to 5°P until the sixth day of fermentation and successive stability until the end of the process.

Conversely, in AW, these two mixed starter cultures showed a slower sugar reduction than the P4 strain. As regards *T. delbrueckii*, most previous studies reporting the use of this yeast in beer production found that the overall speed of fermentation seemed to be slower than that of the usual *S. cerevisiae* brewing strains. The results obtained in our study were different, as in PW and WW fermentation, the rate of TSS reduction in mixed fermentation including the *T. delbrueckii* strain was similar to those exhibited by a pure culture of *S. cerevisiae*, whereas a slightly slower reduction was observed in AW fermentation, confirming that the fermentation performance of *T. delbrueckii* strongly depends on the strain [39]. The reduction of TSS in the fermentations performed by the two mixed cultures P4-TSF and P4-CZ3, both consisting of *S. cerevisiae* and *C. zemplinina*, occurred very slowly; in fact, the reduction of TSS until 5°P required 10 days in PW fermentation, whereas more time was necessary in the WW and AW fermentations.

The cell dynamics during the process are an important parameter in fermentation. In brewing practice, the yeast biomass collected after fermentation is used as the inoculum for subsequent batch fermentations. Therefore, keeping yeast cell viability as high as possible is a desired trait for the brewing process. Moreover, cell viability has an impact on the fermentation kinetics and beer quality [40].

Usually, the number of viable cells diminishes substantially during beer fermentation inoculated with *S. cerevisiae* [41]. In our study, the *S. cerevisiae* P4 strain in single fermentation showed similar pattern in all the worts during the first 6–10 days of the process, with an increase in cell numbers in the first 3 days. Subsequently, the number of viable cells remained constant in Pilsner fermentation, whereas a decrease was observed in the other two worts, particularly in Amber production, where the cell number significantly decreased at the end of the process.

In the mixed fermentations, different trends were recorded in the function of the non-*Saccharomyces* strain included in the starters. In the fermentation by mixed culture P4-CR1, the non-*Saccharomyces* strain (*Z. bailii*) maintained similar cell viability during Pilsner production, whereas a high decrease in cell viability during the process was observed for the P4 strain. For the fermentations performed in WW and AW, the CR1 strain underwent a decrease (from 9.35×10^6 to 3.50×10^6 and from 9.50×10^6 to 2.15×10^6 , respectively), whereas the P4 strain showed similar cell viability to single fermentation. These results might indicate a competition between *S. cerevisiae* and non-*Saccharomyces* strains, as the prevalence of the non-*Saccharomyces* strain over *S. cerevisiae* was observed when the cell numbers of the *S. cerevisiae* strain were lower than non-*Saccharomyces* cells, such as in Pilsner fermentation.

A different result was detected in the fermentation by the mixed culture P4-LC2-1, where the two strains (*S. cerevisiae* and non-*Saccharomyces*) exhibited similar trends in Pilsner and Amber productions, with the viable cells of the non-*Saccharomyces* strain (*T. delbrueckii*) were very similar to the cell numbers of P4, i.e., 1.30×10^6 and 1.85×10^6 , respectively, in Pilsner, and 2.85×10^6 (LC2-1) and 4.95×10^6 (P4) in Amber fermentation. In the WW, a similar trend between the two strains was observed in the first few days, while after the sixth day, the cell numbers of the *S. cerevisiae* strain (P4) were higher (1.70×10^7) than those of LC2-1 (2.40×10^6). This result was very similar to data obtained in WW fermentation with the P4-CR1 mixed starter.

The two mixed cultures with *C. zemplinina* as a non-*Saccharomyces* yeast (P4-TSF and P4-CZ3) showed a similar trend of cell numbers in each wort, whereas differences in the evolution of cell number were found as a function of the wort used. In PW, both TSF

and CZ3 showed a decrease in cell numbers after the third fermentation day, with high reduction during bottle refermentation (after the 10th day), reaching 5.40×10^4 (TSF) and 2.35×10^4 (CZ3) cells/mL. In WW fermentation, similar trends for the evolution of *C. zemplinina* cells were found in the first step, after primary fermentation (until the 10th day), for both the strains, whereas in the last step, the number of viable cells was higher than the levels detected in PW fermentation (1.05×10^6 for TSF and 5.55×10^5 for CZ3). For brewing performed in AW, a rapid decrease in *C. zemplinina* cells was observed after the third day, with the final number of viable cells being very similar to those found in PW fermentation, which was 6.60×10^4 (TSF) and 1.60×10^4 (CZ3). In all the mixed fermentations, the CZ3 strain showed a lower ability to survive during the process than TSF, as at the end of the process, the number of viable cells of CZ3 was lower than that of TSF. As regards *S. cerevisiae*, the evolution of cell number of the P4 strain exhibited a similar trend in all the mixed fermentations with *C. zemplinina* strains, with a high increase in the first part of the processes (until the sixth day), reaching values between 1.85×10^7 and 6.80×10^7 cells/mL, followed by a reduction in viable cells at the end of the fermentation, with the lowest reduction in AW fermentation (1.50×10^6 and 2.25×10^7 cells/mL for P4-CZ3 in PW and P4-TSF in AW, respectively).

These results indicate that the strains exhibited different fermentation activity and growth kinetics, depending on the medium used.

3.3. Analytical Parameters of the Experimental Beers

At the end of the fermentation process, the experimental beers produced by single and mixed starters in the three different worts (PW, WW and AW) were analysed for analytical parameters and by-products related to organoleptic quality. The results regarding the main analytical parameters are reported in Table 2. The data of each beer produced by mixed cultures were compared by one-way ANOVA with the data from the beer obtained from the single starter P4 in the corresponding wort. No significant differences were detected for the final real extract (FRE), expressed as Plato degree, which ranged between 3.0 and 4.0 in Pilsner wort. The lowest value was recorded by P4 beer in WW (2.5) and the same value (4.0) was found in all the beers produced in AW (data not shown).

Table 2. Main analytical characteristics of the experimental beers produced by pure (P4) and mixed cultures (P4-CR1, P4-LC2-1, P4-TSF, P4-CZ3) in the three different worts (PW, WW and AW). For each parameter and each wort, data with different superscript letters mean significant differences (Tukey's test; $p \leq 0.05$) between beers produced by the single and mixed starters.

		P4	P4-CR1	P4-LC2-1	P4-TSF	P4-CZ3
PW	AA	81.24 ± 6.78	81.30 ± 6.70	67.70 ± 5.8	81.54 ± 4.31	81.52 ± 4.8
	RA	66.65 ± 3.90	66.70 ± 4.33	55.52 ± 3.98	66.90 ± 3.54	66.88 ± 3.55
	VA	0.13 ± 0.01 ^a	0.23 ± 0.009 ^b	0.30 ± 0.013 ^c	0.31 ± 0.015 ^c	0.41 ± 0.007 ^d
	AC	3.09 ± 0.02 ^a	3.03 ± 0.05 ^{abc}	2.89 ± 0.01 ^b	2.87 ± 0.06 ^b	2.80 ± 0.03 ^b
WW	AA	88.10 ± 4.8	74.60 ± 4.78	81.22 ± 4.75	81.54 ± 4.31	81.30 ± 4.88
	RA	72.20 ± 3.96	61.10 ± 3.92	66.64 ± 3.90	66.90 ± 3.54	66.69 ± 3.55
	VA	0.24 ± 0.013 ^a	0.35 ± 0.012 ^b	0.39 ± 0.01 ^b	0.35 ± 0.006 ^b	0.60 ± 0.008 ^c
	AC	3.10 ± 0.02 ^a	3.02 ± 0.06 ^a	2.86 ± 0.02 ^b	2.92 ± 0.01 ^{ab}	2.63 ± 0.05 ^c
AW	AA	70.60 ± 6.4	70.58 ± 0.08	70.27 ± 0.11	70.38 ± 0.31	70.38 ± 0.13
	RA	57.90 ± 3.73	57.88 ± 0.02	57.63 ± 0.37	57.72 ± 0.74	57.68 ± 0.32
	VA	0.51 ± 0.018 ^a	0.44 ± 0.016 ^{bc}	0.56 ± 0.004 ^a	0.55 ± 0.015 ^a	0.41 ± 0.020 ^c
	AC	3.19 ± 0.04	3.25 ± 0.03	3.13 ± 0.07	3.12 ± 0.08	3.08 ± 0.02

AA = apparent attenuation (%); RA = real attenuation (%); VA = volatile acidity (g/L); AC = alcohol content (% v/v).

As regards the apparent and real attenuation (AA and RA), high values were found in beers produced both by the mixed cultures and the pure culture (P4) in PW and WW, whereas the Amber beers exhibited the lowest values for both parameters. Statistical differences were found in all the beers for volatile acidity (calculated as acetic acid) and

for alcohol content (AC) in all the samples, except for those produced in AW. In beers obtained from PW and WW, the single starter fermentations produced beers containing a significantly lower level of volatile acidity than samples from mixed starters, whereas in AW beers, the lowest level of volatile acidity was detected in beer fermented with the mixed starter containing the *Z. bailii* strain (P4-CR1). Our results are in contrast to the traditional features assigned to *Z. bailii* species, as this yeast is frequently associated with wine spoilage in consequence of high production of volatile acidity [42,43], confirming the key role of biodiversity at strain level. On average, the beers obtained by AW fermentation contained more volatile acidity than other samples, although the highest level was found in samples obtained with the P4-CZ3 mixed starter in WW. This starter also yielded the highest level of volatile acidity among PW samples, whereas in AW fermentation, the highest volatile acidity was detected in samples fermented with the mixed starter containing the *T. delbrueckii* strain (P4-LC2-1).

The alcohol content of all the beers ranged from 2.63 to 3.25% (*v/v*), with significant differences in the function of starters and beers. In order to verify if the differences in ethanol content were due to significant differences in residual sugar, ANOVA was carried out on the final real extract, expressed as °P. The results showed that the residual sugar in all the beers was not significantly different (data not shown). These results indicate that the differences in the ethanol content of final experimental beers were mainly related to the starter cultures performing the fermentation processes.

All the beers produced by mixed fermentation exhibited a lower alcohol content than the beers obtained by pure fermentation with *S. cerevisiae* P4, with the exception of the starter P4-CR1 in Amber beer. In particular, the beers produced by the mixed culture P4-CZ3 exhibited the lowest values of AC in all the tested worts (2.80% *v/v*, 2.63% *v/v* and 3.08% *v/v* in PW, WW and AW, respectively).

Figure 3 summarises the ethanol reduction obtained across all of the mixed fermentation trials, calculated as difference between the alcohol content in beers obtained from each mixed starter and the ethanol content of beers obtained with the P4 starter. The highest reduction was observed in WW fermentations for almost all the mixed cultures, except P4-TSF. The mixed starter P4-CZ3 had the highest reduction in all the beers produced (ethanol reduction of 0.29 in PW, 0.47 in WW and 0.11 in AW).

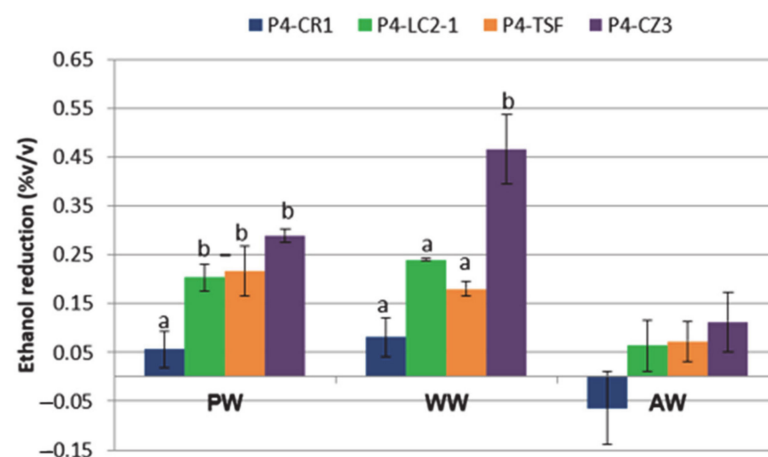


Figure 3. Alcohol reduction in the beers produced by the mixed cultures (P4-CR1, P4-LC2-1, P4-TSF, P4-CZ3) in the three different worts (PW, WW, AW). These values were calculated as difference between the alcohol content in beers obtained from each mixed starter and the ethanol content of beer obtained with the pure starter (P4). Data with different superscript letters (a, b) within each trial are different according to Tukey's test ($p \leq 0.05$).

These results indicate that the *C. zemplinina* strains tested in this study, mainly the CZ3 strain, might be promising yeast starters for the production of low-alcohol beers.

This species has been extensively tested in wine fermentation owing to its fermentative behaviour, both for the production of low-alcohol wines and for its positive contribution to the overall sensory quality of wine, whereas very few studies have been available until now on the use of this yeast species in brewing.

Estela-Escalante et al. [38,44] investigated the application of a *C. zemplinina* strain, isolated from overripe grapes for craft beer production in different wort extracts. When tested in malt wort alone, this strain of *C. zemplinina* produced a low-alcohol beer (1.5% v/v), confirming the inability of this species to ferment maltose. Instead, the use of different adjuncts had a different effect on ethanol production, with a high increase in ethanol production when apple juice was used as an adjunct. This study demonstrated that *C. zemplinina* could be suitable for the production of craft beer with new sensory characteristics using innovative adjuncts, and for production of low-alcohol beers by using wort without the use of adjuncts.

Conversely to *C. zemplinina* strains, the mixed starter containing the *Z. bailii* strain (P4-CR1) was less efficient for ethanol reduction, as it yielded the lowest reduction in the PW and WW fermentations.

In AW fermentation, this mixed starter produced beer containing a higher level of alcohol than beer obtained by using the single starter P4. *Z. bailii* is a yeast species widely present in various food fermentations, although it is considered a spoilage yeast due to its high resistance to preservatives and high tolerance to various stresses. However, some applications of this yeast in the food industry have also been proposed [9,45], such as the use of *Z. bailii* in a mixed starter with *S. cerevisiae* to improve the production of ethyl esters [46]. While *Z. rouxii* is considered a suitable yeast for production of beers with a low alcohol content [14,47] in consequence of its total or partial inability to ferment maltose, only one study [48] reported the use of *Z. bailii* for the production of alcohol-free beers. However, when unfermented maltose could be present in the final beers, it is necessary to use non-*Saccharomyces* yeasts able to produce flavours that can mask the wort-like off-flavours created by residual wort sugars.

3.4. Main Volatile Compounds in the Different Experimental Beers

To evaluate the influence of the mixed starters used on the analytical profiles, the different beers produced from PW, WW and AW worts were analysed for the main by-products, such as acetaldehyde, ethyl acetate, acetoin and higher alcohols (*n*-propanol, isobutanol, active amyl alcohols). In Table 3, the levels of these compounds, detected after bottle refermentation, are reported.

The variance analysis (ANOVA) of these data showed significant differences ($p < 0.05$) among the different experimental beers for almost all the compounds, except ethyl acetate in WW and AW fermentations, and isoamyl alcohol in Weizen beers, demonstrating the influence of non-*Saccharomyces* strains on the aromatic characteristics of the experimental beers.

Regarding Pilsner beers, the main differences between the single and mixed starters were related to the lowest content of *n*-propanol, isobutanol, *n*-butanol and acetoin found in beers fermented with the *S. cerevisiae* P4 strain. Regarding acetaldehyde, the P4, P4-CR1 and P4-LC2-1 beers contained similar amounts, significantly higher than the acetaldehyde content of the experimental beers obtained with the mixed starters including *C. zemplinina* strains (P4-TSF and P4-CZ3). The level of ethyl acetate, which is responsible for a fruity aroma [2], was significantly higher in beers produced by mixed fermentation with the P4-LC2-1 and P4-TSF starters (15.42 and 16.94 mg/L respectively). The lowest amount was found in P4-CZ3 beer (7.55 mg/L). Active amyl alcohol levels were very similar in all the samples, with the exception of beer produced by P4-CZ3, which contained a significantly higher level of this compound. The highest level of active amyl alcohol was detected in experimental beer obtained with the mixed starter including the *T. delbrueckii* strain (P4-LC2-1).

Table 3. Main by-products determined in the experimental beers produced by pure (P4) and mixed cultures (P4-CR1, P4-LC2-1, P4-TSF, P4-CZ3) in the three different worts (PW, WW and AW).

		Main By-Products (mg/L)				
		P4	P4-CR1	P4-LC2-1	P4-TSF	P4-CZ3
PW	<i>n</i> -Propanol	8.47 ± 0.37 ^a	9.19 ± 0.41 ^{ac}	9.04 ± 0.36 ^{ac}	11.58 ± 0.01 ^b	9.80 ± 0.14 ^c
	Isobutanol	86.50 ± 1.63 ^a	98.19 ± 4.06 ^{ab}	110.65 ± 12.22 ^{bc}	127.16 ± 3.31 ^c	115.59 ± 3.35 ^c
	<i>n</i> -Butanol	132.61 ± 5.04 ^a	147.39 ± 0.18 ^b	160.75 ± 5.60 ^{bc}	157.97 ± 0.14 ^{bc}	173.43 ± 4.80 ^c
	Active amyl alcohol	21.13 ± 2.69 ^a	21.82 ± 0.81 ^a	21.87 ± 0.61 ^a	20.35 ± 2.31 ^a	33.09 ± 1.14 ^b
	Isoamyl alcohol	59.17 ± 1.02 ^a	57.82 ± 3.42 ^{ab}	70.07 ± 0.37 ^{ac}	61.90 ± 4.61 ^a	63.27 ± 2.65 ^a
	Ethyl acetate	9.45 ± 0.52 ^a	10.42 ± 4.44 ^a	15.42 ± 1.72 ^b	16.94 ± 0.37 ^b	7.55 ± 0.35 ^a
	Acetaldehyde	52.46 ± 1.91 ^a	57.00 ± 2.12 ^a	58.81 ± 0.39 ^a	45.74 ± 3.46 ^{ab}	38.2 ± 5.44 ^b
	Acetoin	12.86 ± 2.42 ^a	12.82 ± 1.46 ^a	15.11 ± 2.28 ^a	14.95 ± 1.41 ^a	26.31 ± 0.10 ^b
WW	<i>n</i> -Propanol	14.43 ± 0.47 ^a	12.32 ± 0.67 ^b	8.54 ± 0.11 ^c	10.08 ± 0.16 ^c	9.80 ± 0.55 ^c
	Isobutanol	53.68 ± 2.55 ^a	69.73 ± 2.94 ^b	78.51 ± 3.83 ^b	76.63 ± 1.56 ^b	115.79 ± 3.14 ^c
	<i>n</i> -Butanol	65.87 ± 3.44 ^a	112.44 ± 2.19 ^b	123.21 ± 4.91 ^b	115.55 ± 5.29 ^b	173.42 ± 4.64 ^c
	Active amyl alcohol	20.50 ± 1.07 ^a	22.95 ± 1.09 ^{ab}	20.03 ± 1.14 ^a	20.04 ± 0.38 ^a	28.10 ± 3.30 ^b
	Isoamyl alcohol	58.25 ± 2.13	62.05 ± 2.48	62.87 ± 2.66	56.54 ± 1.26	63.33 ± 3.86
	Ethyl acetate	6.28 ± 0.08	7.87 ± 0.18	7.67 ± 0.27	7.90 ± 0.18	7.69 ± 0.082
	Acetaldehyde	49.41 ± 6.91 ^a	60.23 ± 4.20 ^{ab}	52.35 ± 5.50 ^a	52.97 ± 3.65 ^a	38.29 ± 2.29 ^{ac}
	Acetoin	13.22 ± 2.07 ^a	13.75 ± 1.17 ^a	14.01 ± 0.06 ^a	14.28 ± 1.10 ^a	33.83 ± 2.79 ^b
AW	<i>n</i> -Propanol	13.68 ± 1.14 ^a	13.51 ± 0.79 ^a	9.71 ± 0.36 ^{ab}	15.01 ± 1.91 ^{ac}	17.34 ± 0.44 ^{ac}
	Isobutanol	64.85 ± 1.71 ^a	89.63 ± 3.49 ^b	93.60 ± 1.94 ^{bc}	101.88 ± 1.74 ^c	96.84 ± 4.22 ^{bc}
	<i>n</i> -Butanol	117.55 ± 9.07 ^a	131.63 ± 8.26 ^{ab}	146.78 ± 0.97 ^b	140.28 ± 7.70 ^{ab}	121.33 ± 5.51 ^{ab}
	Active amyl alcohol	32.90 ± 0.17 ^a	23.75 ± 2.06 ^b	22.75 ± 1.01 ^b	26.06 ± 0.38 ^{ab}	23.76 ± 1.22 ^b
	Isoamyl alcohol	89.07 ± 3.16 ^a	63.95 ± 3.49 ^b	64.98 ± 3.72 ^b	59.99 ± 0.91 ^b	62.06 ± 2.34 ^b
	Ethyl acetate	8.58 ± 0.95	8.89 ± 0.85	7.57 ± 0.65	8.42 ± 0.49	8.88 ± 0.68
	Acetaldehyde	39.19 ± 1.76 ^a	33.41 ± 0.16 ^b	35.68 ± 0.46 ^{ab}	51.66 ± 2.23 ^c	41.76 ± 0.66 ^{ad}
	Acetoin	8.04 ± 1.66 ^a	11.61 ± 1.29 ^{ac}	20.53 ± 0.07 ^{bc}	16.10 ± 1.96 ^c	10.62 ± 0.77 ^a

Data are means ± standard deviation. Data with different superscript letters (a, b, c, d) within each row and each wort are significantly different (Tukey's test, $p \leq 0.05$).

In WW, the beer produced by the pure P4 starter was different from beers produced by mixed starters in the content of all secondary compounds, except for ethyl acetate and isoamyl alcohol. The lowest amount of acetaldehyde was found in beer produced by P4-CZ3 (38.29 mg/L), similar to the data reported for PW fermentation. Fermentation with the pure P4 culture resulted in the highest amount of *n*-propanol and the lowest amounts of isobutanol, *n*-butanol and acetoin. These results, except for *n*-propanol levels, were also in agreement with data obtained from the analysis of Pilsner beers. As regards amyl alcohols, similar levels were found among all the samples, except for the experimental beer produced by P4-CZ3, which was characterised by the highest level of active amyl alcohol.

This mixed starter showed a similar metabolic behaviour both in PW and WW fermentations, as the beers produced by P4-CZ3 were characterised by the highest amounts of numerous compounds, such as isobutanol, *n*-butanol, acetoin and active amyl alcohol.

In Amber beers produced by the different starters, significant differences in the level of all secondary compounds were found, except for ethyl acetate. *n*-Propanol was produced at varying amounts; the lowest value was in P4-LC2-1 beer and the highest amount was in P4-CZ3 beer.

Regarding isobutanol, *n*-butanol and acetoin, differences were recorded between beers produced by the P4 single starter and those by mixed starters. The lowest values of these compounds were produced by the single *S. cerevisiae* P4 starter, as already reported for PW and WW fermentation, whereas the highest values were found in beer produced by the mixed starter P4-TSF for isobutanol and in beer produced by the mixed starter P4-LC2-1 for *n*-butanol and acetoin. The beer produced by P4 differed from those produced by mixed starters for the highest content of active amyl and isoamyl alcohols.

The analysis of the data relating to the secondary compounds showed a non-univocal behaviour of the starter cultures in the three tested beers, even if some results were confirmed in all the fermentations, such as the low production of isobutanol, *n*-butanol and acetoin by the single starter P4. The low production of acetoin has already been described in strains isolated from the wine environment, where this feature was found to be the more common pattern in *S. cerevisiae*. The high frequency of the low production phenotype corroborates the genetic evidence of a dominant trait in the *S. cerevisiae* species [49].

In order to evaluate the weight of the different factors on the content of main secondary compounds, these data were further analysed by two-way ANOVA (analysis of variance), considering two fixed factors (A and B) and their interaction. In our case, Factor A was the starter used and Factor B was the type of wort used for the production of the beers. The results of the two-way ANOVA are reported in Table 4. As regards the influence of the starter, this factor mainly affected the contents of isobutanol and *n*-butanol and volatile acidity; the wide variability between the results can be linked to the strain-specific metabolic pathways that lead to the production of these compounds.

Table 4. Two-way ANOVA of secondary compounds determined in laboratory-scale beers, using five different starters and three different worts.

		F-Value									
	FD	Ethanol	Volatile Acidity	<i>n</i> -Propanol	Isobutanol	<i>n</i> -Butanol	Active amyl Alcohol	Isoamyl Alcohol	Ethyl Acetate	Acetaldehyde	Acetoin
S	4	43.30 **	180.70 ***	23.4 **	143.7 ***	77.97 ***	18.56 **	10.68 *	21.51 **	12.83 *	52.59 **
B	2	93.49 ***	743.10 ***	96.95 ***	189.40 ***	123.10 ***	12.99 *	19.01 **	111.40 ***	36.85 **	19.87 **
S × B	8	4.68 *	108.00 ***	18.66 **	20.08 **	28.50 **	14.21 *	16.79 **	23.44 **	12.61 *	26.55 **

S = Strains (P4, P4-CR1, P4-LC2-1, P4-TSF, P4-CZ3); B = Beers (Pilsner, Weizen, Amber); S × B = Strains × Beers interaction. FD = freedom degree. Significance codes: $p < ***$, 10^{-9} , ** 10^{-5} , * 10^{-3} .

The influence of the wort used for fermentations was mainly correlated to the amount of four compounds, which are ethyl acetate, *n*-propanol, isobutanol and *n*-butanol, in addition to ethanol and volatile acidity levels. Furthermore, volatile acidity was the parameter most influenced by the interaction between the starter culture and the wort used for fermentation. In general, the type of the wort used for the production of the beers at laboratory scale was the factor with the major impact on the by-product's differences. In fact, ethyl acetate, for example, was detected in very different ranges in the three beers. In particular, in the experimental beers produced by PW fermentation, this compound was found to have the highest levels, ranging from 7.55 until 15.42 mg/L, whereas in the other two beer styles, the content of ethyl acetate was lower (6.28–7.90 and 7.57–8.89 mg/L in the WW and AW fermentations, respectively). However, the production of ethyl acetate, generally considered as an off-flavour due to its “solvent-like” aroma, remained, for all the starter cultures, below the sensory threshold of 21–30 mg/L in beer [50].

Another compound present in highly variable concentrations among the beers obtained with the three different worts was the higher alcohol isobutanol, with the highest levels detected in beers from PW fermentation (values ranging between 86.5 and 127.16 mg/L), whereas lower values were detected in Weizen beers. Among the higher alcohols identified in beer, isobutanol, *n*-propanol, active amyl and isoamyl alcohols are the most important in terms of flavour, as they induce an “alcoholic” flavour and aroma [51]. Isoamyl alcohol (descriptors: “alcoholic”, “vinous”, “sweet”) is the most abundant higher alcohol and is typically found well above its flavour threshold in beer [52]. In addition, active amyl alcohol and isobutanol, which have very similar sensory properties, increase the impact of isoamyl alcohol significantly. In this study, the only starters that were able to produce isoamyl alcohol above its sensory threshold of 70 mg/L [53] were *S. cerevisiae* P4 in AW beer (89.07 mg/L) and P4-LC2-1 in PW (70.07 mg/L). Isobutanol was found as the higher alcohol present in the highest concentration in all experimental beers obtained in this study. Our results confirm that the final concentrations of higher alcohols in beer are influenced by wort composition, fermentation profile and yeast strains [1]. In fact, each

starter showed a different sugar utilization rate in each substrate, as reported in Figure 2, in consequence of the uptake efficiency correlated to the strain-specific metabolic pathway and the substrate composition.

Finally, in order to evaluate the influence of each starter, removing the media effect, Z-scores were calculated for all analytical parameters (ethanol, volatile acidity and volatile compounds) in each fermentation medium and represented in the heatmap reported in Figure 4. As already reported, the single *S. cerevisiae* P4 starter produced beers characterised by higher ethanol content and volatile acidity (except in Pilsner beer), whereas this starter produced less of almost all the volatile compounds detected in this study. Conversely, the mixed starter culture P4-CZ3 was characterised as producing a higher amount of the majority of volatile compounds and the lowest level of ethanol.

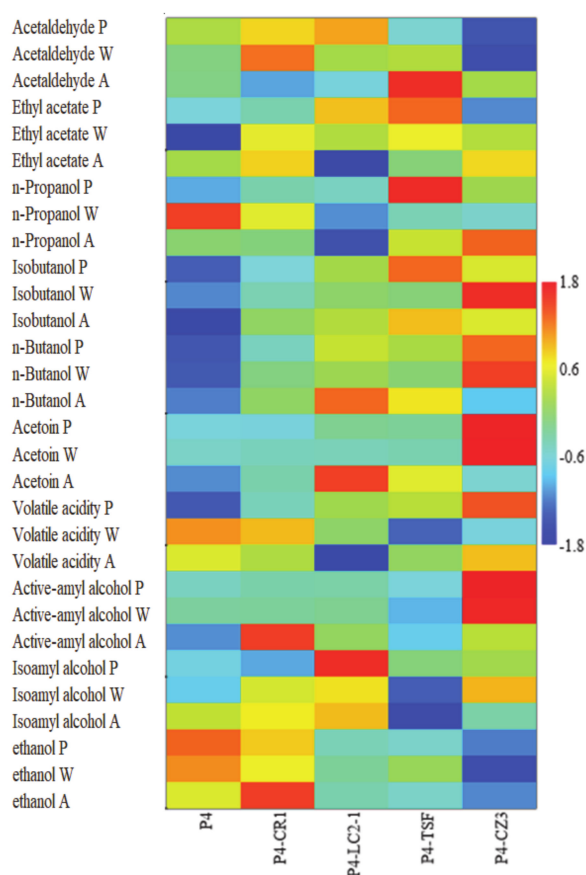


Figure 4. Heatmap based on all the analytical parameters detected in experimental beers obtained by mixed starters (P4-CR1; P4-LC2-1; P4-TSF and P4-CZ3) and the single strain (P4) in Pilsner (P), Weizen (W) and Amber (A) fermentations, together with their corresponding single-strain control (P4). Colours represent the range of calculated Z-scores (calculated over the rows and for each fermentation medium), with blue indicating lower than average production, light green indicating average production and red indicating higher than average production of each parameter.

4. Conclusions

This study presents, for the first time, a screening of non-conventional yeasts with evaluation of aroma production and fermentative properties in three substrates based on different commercial malt extracts used for the production of different beer styles. Our results confirm that the selection of indigenous non-*Saccharomyces* yeasts is a suitable strategy to produce beer with desirable characteristics, such as low ethanol and acceptable organoleptic quality. In particular, this study demonstrates that the metabolic behaviour of yeast strains is substrate-dependent, indicating that during the selection protocol of a starter culture, the beer styles in which the starter will be used have to be considered.

Among the mixed starters tested, almost all were able to produce beers with lower ethanol content than the control beer. The most suitable combination was P4-CZ3 (*S. cerevisiae*–*C. zemplinina*), which is a promising starter for the production of low-alcohol beers with pleasant organoleptic characteristics in all the tested fermentation media. Furthermore, the similar metabolic behaviour of P4-CZ3 in the PW and WW fermentations might indicate the suitability of this starter, mainly for the production of these beer styles. The next necessary step will be the evaluation of the selected starters' performance at large scale in order to validate the starters' behaviour at a real scale and evaluate the sensorial characteristics of the beers produced.

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