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Unlocking the potential of *Zygosaccharomyces bailii*: A novel non-*Saccharomyces* yeast in brewing

Beer has long been an important part of culture, with the brewing industry combining both traditional methods and modern innovation, which has helped beer's evolution over time. Today, the increasing prominence of microbreweries and their expanding market influence have led to a growing interest in identifying elements that can distinguish one beer from another. One such factor is the use of non-*Saccharomyces* yeast strains for fermentation. Among these, *Zygosaccharomyces bailii* (*Z. bailii*), typically known as a spoilage microorganism, presents promising potential as an unconventional yeast for brewing. This study aimed to evaluate the fermentation characteristics of *Z. bailii* and its potential to produce beers with desirable features. To assess its fermentative capacity, *Z. bailii* was tested both as a monoculture and in mixed fermentations with *Saccharomyces cerevisiae* (*S.cerevisiae*) at ratios of 1:1, 1:10, and 1:20, across two original gravities (16 °P and 12 °P) at 20 °C. After confirming that *Z. bailii* could successfully initiate and complete the fermentations, the final beer characteristics were analyzed. The results demonstrated that *Z. bailii* can produce an alcoholic beverage with unique attributes, including a significant decrease in the pH of the final beer. Furthermore, no issues arose during fermentation at high original gravities, and all fermentations achieved the desired ethanol levels. Notably, a strong phenolic character was detected in the final beers, though it was not considered particularly off-flavor. In conclusion, the use of *Z. bailii* offers the potential to create a complex and innovative beer profile that may appeal to consumers. While *Z. bailii* is not yet suitable for large-scale industrial applications, its distinct traits suggest that it could become a valuable yeast strain for future beer production.

Descriptors: *Zygosaccharomyces bailii*, beer, fermentation, volatile compounds, co-fermentation

1 Introduction

Non-*Saccharomyces* yeasts, once viewed as spoilage microorganisms, are now recognized for their potential to enhance complexity and flavor diversity in beer. Species like *Brettanomyces*, *Torulaspora* and *Lachancea* are widely used today to introduce unique flavors, such as fruity esters and phenolic notes. Modern brewing incorporates these yeasts to produce a variety of craft beers, including sour and wild ales [1–4]. Many breweries are also experimenting with these yeasts to produce low-alcohol or non-alcoholic beers, leveraging their unique metabolic pathways for fermentation without high ethanol production.

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Zygosaccharomyces bailii (*Z. bailii*) shows remarkable resilience, growing even at low pH levels and in high concentrations of weak acids. Its acetic acid tolerance and ability to thrive with low acetic and lactic acid levels enable its potential as a starter culture in fermentation processes. Traditionally a spoilage yeast, *Z. bailii* has recently been explored in co-cultures with *Saccharomyces cerevisiae* (*S. cerevisiae*), enhancing wine aroma via ethyl esters that add fruity and floral notes [5]. Its robust adaptation to acidic conditions, as demonstrated by [6], shows potential for lactic acid production, with strains able to grow in media containing 10 g/L acetic acid or 60 g/L lactic acid at pH 3.

Although *Z. bailii* is well-known for its fermentative strength in various foods and beverages, significant gaps in knowledge about its metabolism in diverse culture conditions and ester production capabilities still exist [7]. Certain strains produce esters like 2-phenylethyl acetate, isoamyl acetate, ethyl acetate, and other ethyl esters [3] that enhance flavor profiles in wines and other fermented products, and simultaneously increase polysaccharide levels, improving wine body and taste [8].

Additionally, *Z. bailii* has been used to remove residual sugars in sluggish wine fermentations, specifically in Cabernet Sauvignon and Syrah [9]. In brewing, it has been tested in Pilsner beer for ethanol reduction when co-cultured with *S. cerevisiae* [3, 10]. Studies on *Z. bailii* also highlight its importance in spirit flavors like Maotai

liquor, where its production of higher alcohols, esters, and acids contributes to the aroma profile [11–13].

Although various non-*Saccharomyces* yeasts have been investigated for their use in beer production [4, 14, 15], *Z. bailii*, a promising alternative yeast, has yet to be tested. Thus, the objective of this study was to evaluate the capacity of *Z. bailii*, typically a spoilage wine yeast, to metabolize wort sugars and produce beer with desirable characteristics. After confirming the yeast's ability to ferment key wort sugars, two different initial worts (12 and 16 °P) were prepared. Fermentations were conducted at 20 °C, both in pure cultures and in mixed fermentations. Malt extract was used for wort production, and the fermentation process was monitored via specific gravity measurements. At the end of fermentation, the products were analyzed for their aromatic profile, ethanol content, bitterness and color.

2 Materials and methods

2.1 Yeast strains

A strain of *Zygosaccharomyces bailii* (Zb-K29Y2), previously isolated from the Greek terroir (Macedonia, Greece) [16] and characterized at strain level was used in this study [17]. The isolate was stored at − 20 °C in Nutrient Broth supplemented with 30 % glycerol (Serva, Heidelberg, Germany). One commercial strain of *Saccharomyces cerevisiae*, SafAle™ S.c. US-05 (Fermentis by Lesaffre, Marcq-en-Baroeul, France) was also used. Prior to experimental use, the *Z. bailii* isolate was purified in duplicate by streaking onto YPD agar plates [(g/L): Yeast extract 10, Bacteriological peptone 20, Dextrose (D-Glucose) 20, Agar 20], followed by incubation at 28 °C for 48 h. Precultures were subsequently subcultured in YM broth (1 % glucose, 0.5 % peptone, 0.3 % yeast extract, 0.3 % malt extract) at 25 °C for 48 h, so as to increase their fermentation capability. The purity of the *Z. bailii* (Zb-K29Y2) inoculum was confirmed by Repetitive Polymerase Chain Reaction (rep-PCR) using the GTG5 (5′-GTG GTG GTG GTG GTG-3′) primer as described in detail previously [17]. The genomic fingerprint of the DNA template from Zb-K29Y2 and the extracted DNA from the inoculum is presented in Supplementary data (S1).

2.2 Chemicals

Diethyl ether (95 %), pentane (95 %), acetonitrile (HPLC grade), water (HPLC grade), octanol standard solution, and anhydrous sodium sulfate were purchased from Chem. Lab (Athens, Greece). The rest constituents (yeast and malt extract, peptone, glucose, $(NH_4)_2SO_4$, $MgSO_4$, $ZnSO_4$, KH_2PO_4, and $KHPO_4$) were purchased from Merck KGaA (Darmstadt, Germany).

2.3 Wort production and fermentation

Dry malt extract was used for wort production, with two batches prepared at specific gravities of 12 and 16 °P. To achieve the desired specific gravity for each batch, water was heated to 65 °C, and the appropriate amount of dry malt extract was added. Hop extract containing 6 % iso-alpha acids, isomerized (Brouwland, Beverlo, Belgium), was also added in order to achieve an initial 30 IBU. The initial characteristics of the wort were: specific gravity 12.1 ± 0.1 °P and 16.1 ± 0.1 °P; pH 5.1 ± 0.1. The wort was separated in batches of 20 L, each was inoculated with the appropriate yeast or mixture, and fermented at 20 °C. The fermentations included pure cultures of *S. cerevisiae* and *Z. bailii*, as well as mixed cultures with *S. cerevisiae* to *Z. bailii* ratios of 1:1, 1:10, and 1:20. The fermentations were carried out in containers equipped with a spigot and an airlock. The spigot was sanitized before and after each sampling, and the containers remained sealed throughout the fermentation process. Duplicate fermentations were also utilized, with samples collected for volatile and sensory analysis. The results from these samples were within the statistical deviation. The initial inoculation rate was 6 x 10^6 cells/mL either in pure or mix cultures fermentations. Fermentation data were collected over approximately 1300 hours, allowing for a detailed comparison of fermentation dynamics across yeast strains and initial sugar levels. The end of the fermentation was determined by three daily successive equal specific gravity values. Subsequently, the product was transferred into bottles after racking.

2.3 Yeast Cell Counting

The cell counting was performed by microscopy, using a microscope CX60 (Olympus Corporation, Center Valley, USA) and a Thomas type hemocytometer and their viability was evaluated by the methylene blue method, according to Lange et al. (1993) [18].

2.4 Determination of the main beer characteristics

2.4.1 Wort specific gravity, pH and Free Amino Nitrogen (FAN)

Specific gravity was determined (DMA 35, Anton-Paar) according to the official ASBC method Beer-2B, rev. 2014, pH (ASBC method Beer-9) and FAN was determined in samples withdrawn during beer fermentation, using ninhydrin, according to the official method of the American Society of Brewing Chemists (ASBC) Wort-12A, rev. 2010 [19].

2.4.2 Ethanol content, bitterness and colour determination

The ethanol content of the produced beers was determined by distillation of decarbonated samples, according to the official ASBC method Beer- 4A, rev. 2018.

The bitterness (expressed in IBU) and the color (expressed in SRM) of the final products were determined according to the official ASBC methods Beer-23A, rev. 2018, and Beer-10A, respectively [20].

2.4.3 Sensory screening

The beers produced were assessed by a team of five beer experts in two consecutive sessions of five beers each as follows: In the initial session, the beers of 12 °P specific gravity were evaluated, then a break of 1 hour took place, followed by the second session in which all beers of 16 °P specific gravity were assessed. All assessments took place in individual off-white booths under a mix of natural and artificial light, of odourless 20 ± 2 °C air-conditioned room (ISO 8589:2007). The beers were presented in a randomized order for each assessor following a partially balanced design and

samples were labeled with unique three-digit codes. Beer samples, (25 ml portions), were served at room temperature (18 – 21 °C) in ISO standard tasting glasses.

The experts that evaluated the samples were all with extensive experience in brewing beer either through working – collaborating in the brewing industry or as experienced home brewers, all having attended several seminars with specific beer flavors and off-flavors. The objective in sensory evaluation sessions was to assess foam stability, aroma (in the nose and in the mouth), basic tastes and mouthfeel of the beers, in order to screen out any samples with possible sensory defects. Data were collected in ballots and assessors were free to use their own vocabulary for both in nose and in mouth characteristics. The collected results were solely qualitative and therefore no statistical process was applied, instead the comments for each sample were summarized across the group and recorded in a table.

2.4.4 Extraction and analysis of volatile compounds

The extraction and analysis of the volatile products was performed according to Drosou et al. [2]. Briefly, beer (50 mL) was mixed with 1-pentanol and diethyl ether (25 mL each). The mixture was stirred for 10 min at room temperature, centrifuged at 1370 g for 10 min, and the aqueous phase was subjected to a second extraction under the same conditions. The organic layers were mixed, washed with distilled water in a separation funnel, and the excess water was removed by anhydrous sodium sulphate. The obtained solution was filtered (filter paper Whatman No 42, Maidstone, UK), 10 µL of 3-octanol solution in chloroform (2500 ppm) was added as internal standard, condensed in a Vigreux column, and adjusted to a final volume of 100 µL. Analysis was performed by Gas Chromatography/Mass Spectrometry (GC/MS), in a Shimadzu, Nexis GC-2030 with an autosampler/ injector (Shimadzu, AOC 20i Plus) and a mass spectrometer (Shimadzu, GCMS QP2020 NX) and equipped with a fused silica capillary column, 30 m × 0.32 mm i.d., 0.25 µm coating thickness (HP-5MS, Agilent Technologies, Santa Clara, CA,USA). One µL of sample was injected using a split ratio of 100:1. The injector temperature was set at 250 °C, the carrier gas was helium at a constant flow rate of 1.5 mL/min, and the oven temperature program was set as previously mentioned. The identification of the volatile compounds was based on the data system library (NIST 11).

2.4.5 Statistical analysis

All experiments were conducted in duplicate, and their mean values and standard deviations are presented. Experimental data were subjected to analysis of variance to detect significant differences among the investigated factors (yeasts, extract, fermentation temperature). Duncan's multiple-range test was applied in the cases of significant differences ($p < 0.05$). Analysis was performed using the software Statistica 14.0 (StatSoft, Tulsa, OK, USA).

3 Results and Discussion

3.1 Fermentation and Beer Characteristics

Figure 1 demonstrates the fermentation kinetics of the studied yeasts in pure and mixed cultures fermentations at 12 °P and 16 °P. The initial inoculation rate was 6×10^6 cells/mL either in pure or mixed culture fermentations. In all experiments, this standardized inoculation level was deliberately set lower than the concentrations commonly employed in industrial or high-gravity brewing. This approach was intended to assess the strains' tolerance under extreme conditions (i.e., high sugar concentrations) [21], which reasonably explains the prolonged lag phases observed in some cases.

In the 12 °P initial condition, *S. cerevisiae* displayed a rapid decline in gravity, reaching 2.9 ± 0.1 °P within 200 hours, indicating a highly efficient fermentation process. This finding is consistent with *S. cerevisiae*'s established reputation for fast and comprehensive sugar utilization in brewing. In contrast, *Z. bailii* showed a slower and less pronounced decrease in gravity, achieving a final reading of 4.0 ± 0.1 °P after 1000 hours, which was significantly higher ($p < 0.05$) than that of the conventional yeast. In the mixed culture fermentations, the rate of gravity reduction varied depending on the ratio of *S. cerevisiae* to *Z. bailii*. The 1:10 mix reached a final gravity of 4.0 ± 0.1 °P within approximately 600 hours, whereas the 1:1 ratio took longer (~ 1000 hours), to reach the same level. This delay observed in the 1:1 mix, may be attributed to species-specific interactions between *S. cerevisiae* and *Z. bailii*. According to Wang et al. (2016), the interactions between Saccharomyces and non-*Saccharomyces* species during alcoholic fermentation are species- and strain-specific, potentially leading to competition for

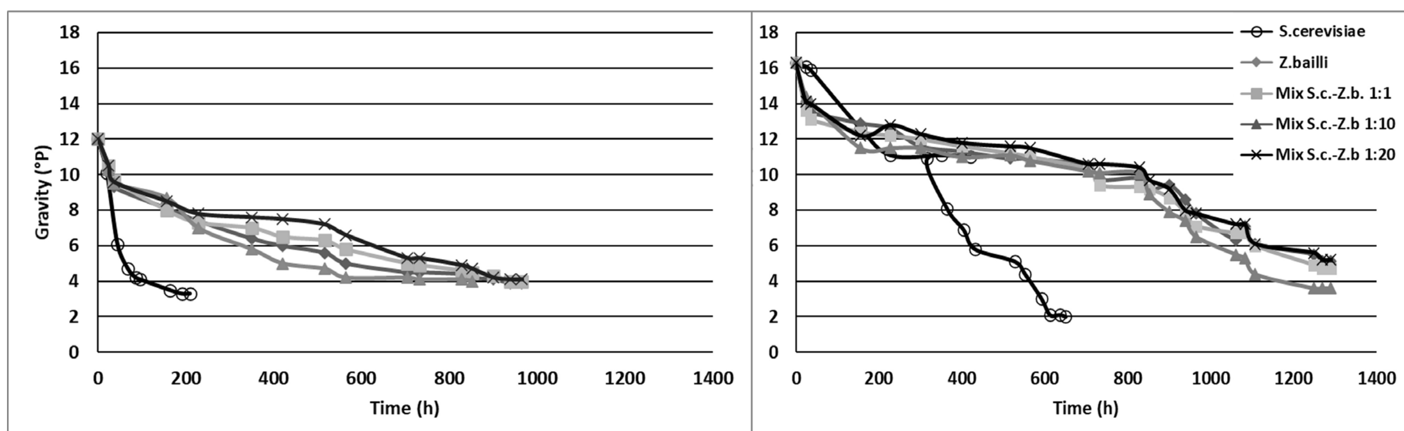

Fig. 1 Fermentation kinetics of pure and mixed cultures of *S. cerevisiae* and *Z. bailii* with initial gravity of 12 °P (left) and 16 °P (right) at 20 °C. The standard deviation in Plato measurements ranges between 0.0 and 0.1

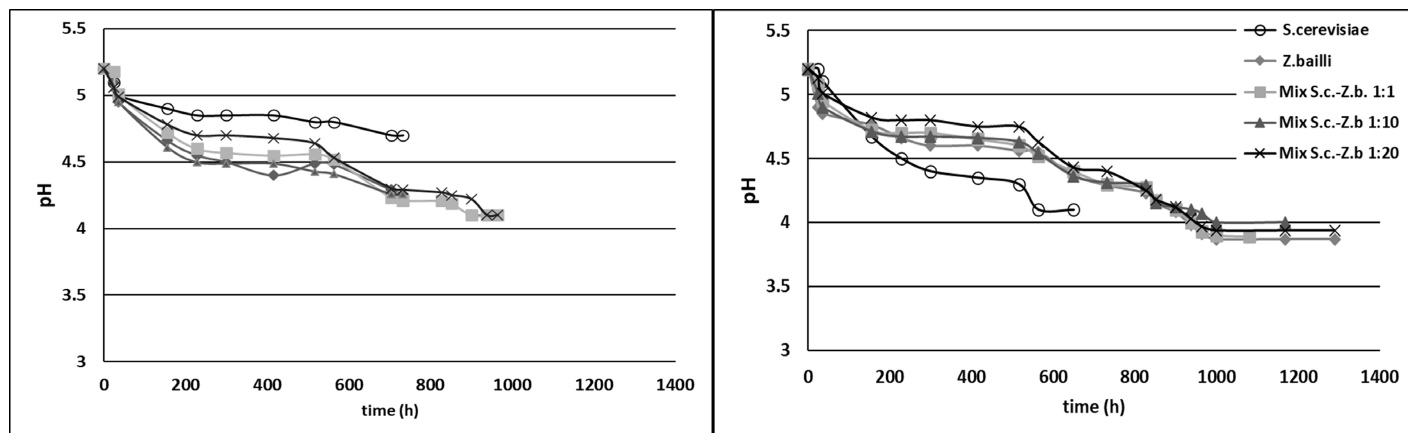

Fig. 2 pH change during pure and mixed cultures fermentations of 12 °P and 16 °P over time

nutrients, metabolic rate differences, or the production of inhibitory compounds [22]. Such strain-specific interactions might explain the prolonged fermentation time in the 1:1 ratio. Notably, all fermentations, except for the conventional yeast, concluded with a final gravity of 4.0 ± 0.1 °P, suggesting a limitation in the extent of fermentation achievable by *Z. bailii* and mixed cultures.

In the 16 °P initial gravity (Fig. 1), additionally, fermentations displayed the same behavioral pattern as those conducted at 12 °P, supporting the interpretation that the prolonged lag phase is due to high sugar content and strain stress. *S. cerevisiae* maintained a steady and consistent reduction, requiring approximately 650 hours to reach completion, due to high sugar concentration. *Z. bailii* again exhibited a much slower fermentation rate, with a substantial amount of residual sugar remaining at the end of the fermentation (5.1 ± 0.1 °P), indicating that *Z. bailii* may be less effective in high-sugar environments where high-efficiency fermentation is desired. The mixed cultures in this condition mirrored the pattern observed in the 12 °P condition, with fermentation rates and outcomes similarly influenced by the interaction between the two yeast species.

Yeast autolysis could have also contributed to the observed drop in extract after 800 hours. Under prolonged fermentation and high stress conditions, some yeast cells may lyse, releasing intracellular components into the medium [23]. These released nutrients could be utilized by the remaining viable yeast, potentially altering fermentation dynamics and accelerating the reduction of extract. Notably, none of the mixed cultures surpassed a final gravity of 5.0 °P, regardless of the *S. cerevisiae* to *Z. bailii* ratio, suggesting a consistent limitation in their ability to reduce gravity beyond this point under high initial sugar concentrations.

Figure 2 shows the change in pH over time during fermentation at two different initial gravities: 12 °P (left) and 16 °P (right). Each graph depicts the pH reduction across all fermentations with samples withdrawn in several time intervals. In both graphs, the pH decreases progressively over time. Acetic acid is a key byproduct of *Z. bailii*, and its production can contribute to the lowering of the pH in the fermentation environment, such as in wine [24]. This yeast can produce acetic acid even under conditions where other yeasts might not survive, such as in high alcohol and acidic environments.

Table 1 The main beer characteristics including ethanol, FAN, color and bitterness

Specific Gravity	Yeast strains	% ABV	FAN (mg/L)	COLOR (SRM)	BITTERNESS (IBU)
	Initials	**0.00 ± 0.00**	**200.00 ± 1.00**	**5.00 ± 0.30**	**29.00 ± 1.00**
12 °P	***S. cerevisiae***	5.80 ± 0.10^{a}	40.76 ± 0.24^{d}	4.80 ± 0.30^{a}	26.70 ± 0.00^{a}
	Z. bailii	4.90 ± 0.10^{ab}	38.00 ± 0.11^{e}	3.00 ± 0.10^{c}	22.70 ± 0.01^{c}
	Mix S.c.-Z.b. 1:1	4.40 ± 0.00^{b}	42.10 ± 0.00^{c}	3.50 ± 0.03^{b}	26.05 ± 0.01^{a}
	Mix S.c.-Z.b 1:10	5.00 ± 0.50^{ab}	44.20 ± 0.20^{b}	3.60 ± 0.03^{b}	24.35 ± 0.01^{b}
	Mix S.c.-Z.b 1:20	5.20 ± 0.20^{ab}	49.00 ± 0.50^{a}	3.00 ± 0.06^{c}	24.10 ± 0.02^{b}
16 °P	**Initials**	**0.00 ± 0.00**	**272.00 ± 2.00**	**4.00 ± 0.50**	**29.00 ± 1.00**
	S. cerevisiae	9.30 ± 0.20^{a}	48.30 ± 0.00^{a}	3.80 ± 0.06^{a}	26.70 ± 0.04^{a}
	Z. bailii	7.70 ± 0.30^{c}	63.70 ± 0.31^{b}	3.50 ± 0.20^{ac}	23.25 ± 0.01^{b}
	Mix S.c.-Z.b 1:1	8.20 ± 0.10^{bc}	111.00 ± 0.10^{c}	3.70 ± 0.25^{a}	19.05 ± 0.01^{c}
	Mix S.c.-Z.b 1:10	8.70 ± 0.10^{ab}	109.70 ± 1.05^{c}	3.75 ± 0.10^{a}	19.30 ± 0.05^{c}
	Mix S.c.-Z.b 1:20	8.50 ± 0.10^{b}	113.00 ± 0.50^{c}	3.30 ± 0.03^{c}	22.20 ± 0.04^{b}

*superscript letters in the same column present significant differences ($p < 0.05$) between yeast strains at the same initial gravity (a>b>c>d>e)

Table 2 Summary of sensory comments given by the experts to the beer samples and inclusion/exclusion from the next step of analysis

Specific Gravity	Beers produced	Summary comments related to smell	Summary comments related to tastes and mouthfeel	Choice for GC-MS analysis (YES/NO)
12 °P	***S. cerevisiae***	Not intense, No off-flavour	Typical American Pale Ale style	YES
	Z. bailii	Very phenolic, smell of 4-vinyl guaiacol	High sweetness	YES
	Mix S.c.-Z.b. 1:1	Very phenolic, smell of 4-vinyl guaiacol	Less sweet compared to *Z. bailii* monoculture	NO
	Mix S.c.-Z.b. 1:10	Phenolic but also fruity	Well-structured mouthfeel, good balance between sweet and sour, good foam stability	YES
	Mix S.c.-Z.b. 1:20	Off-flavour, spontaneous sour beer type	too sour	NO
16 °P	***S. cerevisiae***	Slight solvent -paint	Typical American Pale Ale style	YES
	Z. bailii	Strong phenolic character, 4-vinyl guaiacol	Medium sweetness	YES
	Mix S.c.-Z.b. 1:1	Off-flavour, acetic acid	too sour	NO
	Mix S.c.-Z.b. 1:10	Off-flavour, acetic acid, smell of oxidised wine	Average sweetness and sourness	NO
	Mix S.c.-Z.b. 1:20	Phenolic but also floral and fruity character, aroma complexity	Full structure - body. Balanced taste and aftertaste	YES

However, to maintain pH homeostasis and survive the acid stress, *Z. bailii* employs mechanisms like pumping out protons (H^+ ions) using energy from ATP [24–26]. The rate of pH decreases, and final pH values vary slightly between samples fermented either with a lower proportion of *Z. bailii* in the mixed fermentations (1:1, 1:10 and 1:20) or with pure *Z. bailii*, suggesting that both the strain and concentration of the yeast impact the pH reduction pattern, with similar trends observed across both initial concentrations. In both cases the conventional yeast, ended with the highest pH values (4.1 and 4.6, respectively). In general, these results do not suggest any evidence of bacterial contamination, as the typical pH range for beer is between 3.8 and 4.7 [27].

The main characteristics of the obtained beers are shown in table 1. All yeast strains produced higher amounts of ethanol at 16 °P than at 12 °P, with significant variations depending on the yeast strain used. *S. cerevisiae* yielded the highest ethanol concentrations at both initial gravities ($p < 0.05$), followed closely by the mixed fermentation. Specifically, in mixed-culture wine fermentations, *Z. bailii* is often paired with *S. cerevisiae* to potentially lower the overall ethanol level. Studies from the wine industry indicate that mixed fermentations of these two yeasts typically produce 1 – 2 % vol. less ethanol than *S. cerevisiae* monocultures, due to *Z. bailii*'s tendency to redirect sugar metabolism toward other byproducts [8, 28]. In monoculture, *Z. bailii* produced a lower ethanol concentration overall, yielding 4.90 ± 0.10 % ABV at 12 °P and 7.70 ± 0.30 % ABV at 16 °P (Table 1), which is still sufficient for a Pale Ale beer [29].

The final concentration of free amino nitrogen (FAN) is also depicted in table 1. Though optimal FAN levels may vary depending on the yeast strain and specific fermentation conditions, a target range of 100 – 140 mg/L has been recommended for worts with an original gravity of 10 – 12 °P [30, 31]. The initial levels of FAN were measured at 200.00 ± 1.00 mg/L and 272.00 ± 2.00 mg/L, respectively. The highest demand for FAN was observed during the monoculture fermentation of *Z. bailii*, followed by the conventional yeast. In contrast, the mixed fermentations utilized less nitrogen, with the 1:20 ratio showing the highest residual FAN of 49.00 ± 0.50 mg/L, indicating a lower FAN uptake. This indicates that mixed cultures can be more efficient in nitrogen utilization, which may impact the overall fermentation process and flavor profile of the resulting beer [30]. The results for higher gravity fermentations exhibit similar trends (Table 1).

The final color and bitterness of the produced beers, as detailed in table 1, indicate that the monoculture of *Z. bailii* exhibited the lowest color intensity at 12 °P, with a value of 3.00 SRM. In the mixed culture fermentations, particularly at the highest ratio of non-*Saccharomyces* yeast, similar color results were observed. In high gravity fermentations, the mixed cultures achieved a slightly higher color intensity, reaching 3.30 SRM. Last but not least, the bitterness associated with the monoculture fermentation of *Z. bailii* has the minimum value at 12 °P, a pattern that is also observed in mixed fermentations, where the bitterness decreases as the proportion of *Z. bailii* increases, which can be attributed to the absorbance of a small portion of hop acids by the yeast cells or to biotransformation reactions that take place [32]. On the other hand, at 16 °P, the lowest bitterness units were observed in the mixed fermentations at ratios of 1:1 and 1:10. In all conditions, the beers of *S. cerevisiae* exhibited a bitterness level of 26.7 IBU.

In terms of the sensory evaluation, the first session in which all 12 °P samples were profiled, yielded the following outcomes: To begin with, the sample of the pure *Z. bailii*, gave a product with a

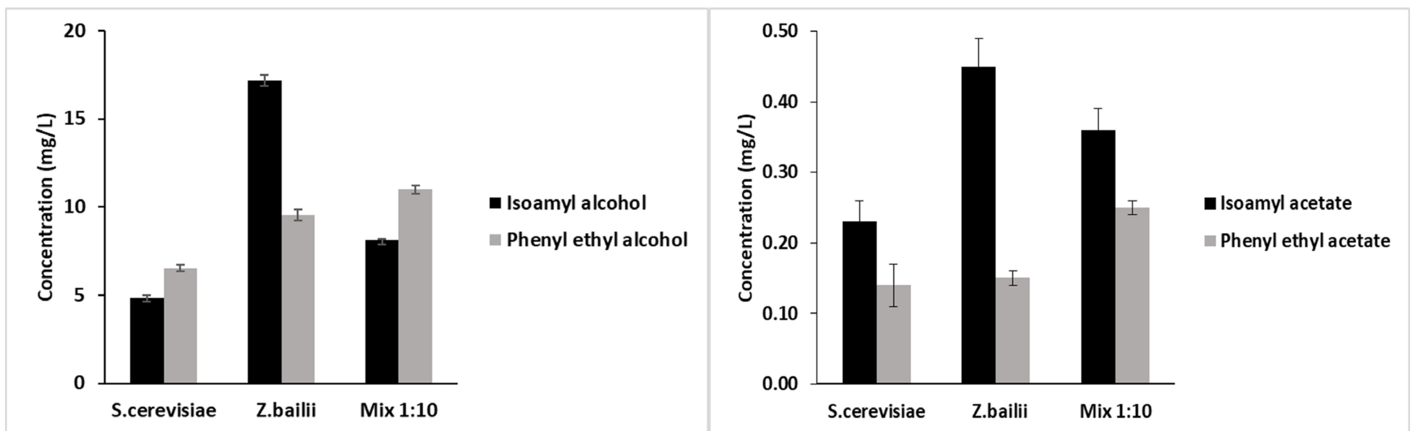

Fig. 3 The main higher alcohols and their corresponding esters in 12 °P initial gravity in mono- and mixed culture fermentation of *Z. bailii* in 1:10 ratio

distinct phenolic note on the nose, reminiscent of "Weissen" type of beers, but without strong fruitiness and was perceived as the sweetest of all samples in that specific gravity range. The mix fermentation of *Z. bailii* and *S. cerevisiae* at 1:1 ratio was very similar to the pure *Z. bailii* sample in terms of aroma but less sweet in taste. The beer fermented by *Z. bailii* and *S. cerevisiae* at 1:10 ratio, had the most well-structured body, good foam stability, and aromas characterized by both phenolic and fruity notes (Table 2). As far as it concerns the beer fermented by *Z. bailii* and *S. cerevisiae* at 1:20 ratio exhibited several off-flavours reminiscent of certain spontaneously produced sour beers [33], and was subsequently eliminated from further analysis (Table 2). Finally, the sample produced from the conventional yeast was free of off-flavours but also relatively neutral without any profound fruitiness.

In the next session, the beers with 16 °P initial specific gravity were evaluated as follows: First, the beer fermented by the mix fermentation of *Z. Bailii* and *S. cerevisiae* at 1:1 ratio was perceived as having an overly strong off-flavour of acetic acid. Similarly, the mix of 1:10 ratio was also evaluated as a sample with strong acetic notes, reminiscent of the aroma profile of an oxidized wine (Table 2). Consequently, the above samples were excluded from further analysis. The beer produced from the monoculture of *Z. bailii*, again presented a pronounced phenolic smell (both ortho and retronasally), similar to the 12 °P beers, and was kept as the sample fully expressing the potential of the yeast. Last but not least, the beer produced by the mixed culture fermentation of *Z. bailii* and *S. cerevisiae* at 1:20 ratio, had phenolic as well as floral and fruity notes, which were perceived as balanced, with good structure (mouthfeel) and flavor complexity, and was retained for further GC-MS analysis (Table 2). The control sample, produced by *S. cerevisiae*, did not exhibit any particularly strong qualities to make it stand out, but it was analysed further as the control condition.

4 Production of Volatile Compounds

The primary advantage that brewers seek when using non-*Saccharomyces* yeasts is the enhanced aromatic complexity they provide to the final product. As shown in figure 3, variations in the original gravity of the fermentation significantly affect the volatile compounds produced. In addition to gravity, the formation of active flavor compounds is closely linked to nitrogen availability, particularly the residual free amino nitrogen (FAN) at the end of fermentation. This parameter is crucial for influencing the production of various flavor compounds. Additionally, the impact on volatile production is not only determined by the specific yeast strain used but also by the fermentation's yeast composition. Mixed fermentations, in which various yeast strains are combined in different ratios, can further enhance the diversity of aromatic profiles, as each strain contributes unique metabolic byproducts. The combination of strains, especially when used in precise ratios, can result in a more complex and varied flavor profile, influencing both the intensity and the character of the aromas in the final product. Therefore, many brewers experiment with mixed fermentations, as they allow for more control over the aromatic outcome [10, 15, 34].

In the fermentation trials, for the 12 °P wort, the 1:10 yeast culture ratio emerged as the most promising combination based on sensory evaluation, whereas for the 16 °P wort, the optimal ratio was found to be 1:20. These selected ratios demonstrated the most desirable sensory qualities, making them ideal candidates for further analysis. To gain insight into the specific volatile compounds contributing to these sensory profiles, the chosen samples were analyzed using gas chromatography-mass spectrometry (GC-MS).

In figure 3, the results of higher alcohols and their corresponding esters at 12 °P are presented in pure and mixed culture fermentations of *S. cerevisiae* and *Z. bailii* in a ratio of 1:10. In the monoculture fermentation, *Z. bailii* produces a significantly higher concentration (17.20 ± 0.29 mg/L) of isoamyl alcohol, whereas in the mixed fermentation, the higher values were detected in phenyl ethyl alcohol (11.00 ± 0.25 mg/L). *S. cerevisiae* produced significantly lower concentrations of the two higher alcohols ($p < 0.05$). As far as ester production is concerned, *S. cerevisiae* produced the least amount of isoamyl acetate, whereas in *Z. bailii*'s fermentation the production reached the highest concentration (0.45 ± 0.03 mg/L). The concentration of phenyl ethyl acetate produced by conventional yeast was comparable to the amount of the pure fermentation of *Z. bailii*. The mixed culture produced 0.25 ± 0.01 mg/L of phenyl ethyl acetate, significantly higher between the examined fermentations. Contrary to other oenological studies [35] conducted in red wines that report exceptionally high amounts of acetate esters (ranging from 1.77 – 4.9 mg/L for isoamyl acetate and 2.59 to 121 mg/L for

phenylethyl acetate) the concentrations observed in this study were much lower. Similarly, the concentration of isoamyl acetate in mixed wine fermentations conducted by Capece et al. (2022) was detected at 9.12 ± 1.07 mg/L, concentration 9 times higher than detected [28]. This disparity may stem from differences in the fermentation medium, conditions, as well as the different yeast strain used.

Figure 4 presents the production of medium-chain fatty acids (MCFAs) and their corresponding esters produced during fermentation at an initial gravity of 12 °P, in pure culture fermentations and a mixed of 1:10 co-culture of *S. cerevisiae* and *Z. bailii*. In the left, concentrations of several MCFAs are displayed, including hexanoic acid, octanoic acid, n-decanoic acid, dodecanoic acid, and hexadecanoic acid. The mixed culture exhibited significantly higher ($p < 0.05$) concentrations of these acids (over 4 mg/L of octanoic and hexadecanoic acid) compared to the pure cultures, with the exception of hexadecanoic acid, for which the final concentration was similar to *Z. bailii*'s fermentation.

The results indicate a consistent pattern in which octanoic acid and its ethyl ester along with hexadecanoic acid are the most abundant compounds produced in both fermentations. However, the substantially lower concentrations of the esters relative to their precursor acids suggest limited esterification under these specific fermentation conditions. Octanoic acid ethyl ester was detected at the highest concentration among the esters in both *Z. bailii* monoculture (0.12 ± 0.02 mg/L) and mixed culture samples (0.19 ± 0.03 mg/L), though its levels remained significantly lower than those of the acids. Notably, the concentration of octanoic acid ethyl ester was higher in the mixed culture than in the monoculture. Despite this increase, the detected levels were significantly below its reported flavor threshold of 0.9 mg/L [36, 37]. Other esters, such as hexanoic acid ethyl ester and decanoic acid ethyl ester, were present at comparatively lower concentrations. Previous studies, such as Garavaglia et al. (2014), reported high concentration of these esters in synthetic medium fermentations produced by different strains of *Z. bailii* originated from red wine oak barrel, which varied from 1 mg/L to 80 mg/L depending on the strain used [35]. Last but not least, while ale yeasts are typically associated with higher ester production compared to lager yeasts, the particular *S. cerevisiae* strain used in our experiments exhibited limited ester formation, as outlined in the producer's specifications. Consequently, *S. cerevisiae* produced the lowest levels of hexanoic acid ethyl ester and a concentration of octanoic acid ethyl ester comparable to that detected in the monoculture fermentation of *Z. bailii*.

The production of higher alcohols and their acetate esters during 16 °P fermentation was compared between *S. cerevisiae* and *Z. bailii* pure cultures and mixed-culture fermentations in a 1:20 ratio (Fig. 5). For higher alcohols (left graph), isoamyl alcohol emerged

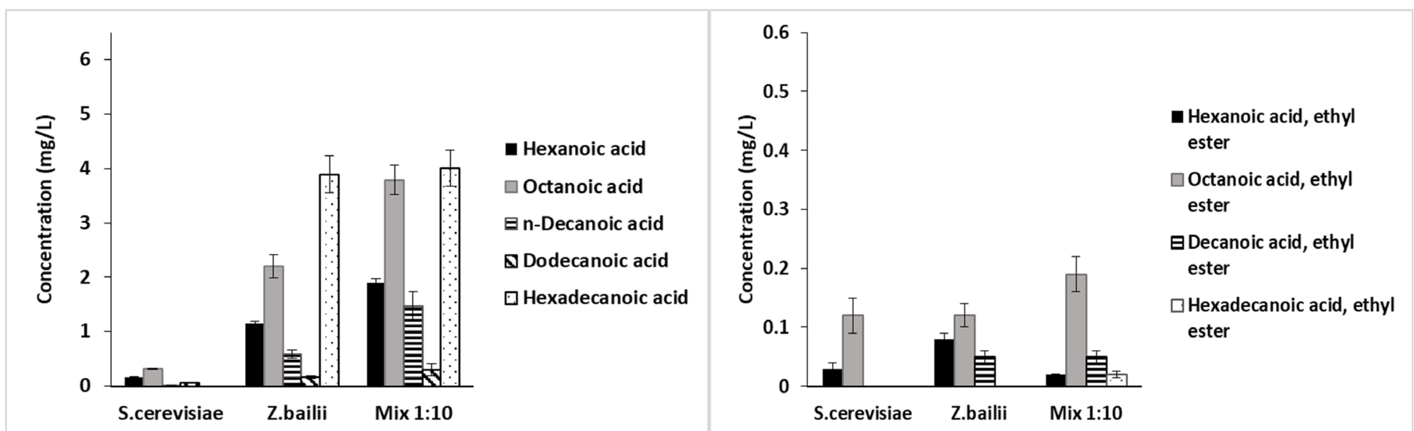

Fig. 4 The produced Medium Chain Fatty Acids and their corresponding esters in 12 °P initial gravity in in mono- and mixed culture fermentation of *Z. bailii* in 1:10 ratio

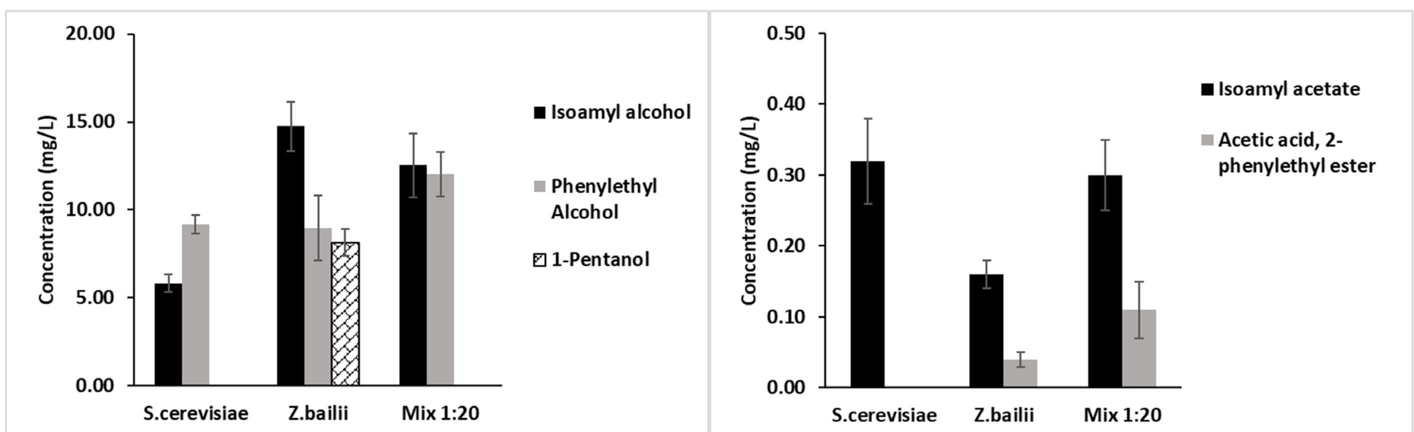

Fig. 5 The main higher alcohols and their corresponding esters in 16 °P initial gravity in mono- and mixed culture fermentation of *Z. bailii* in 1:10 ratio

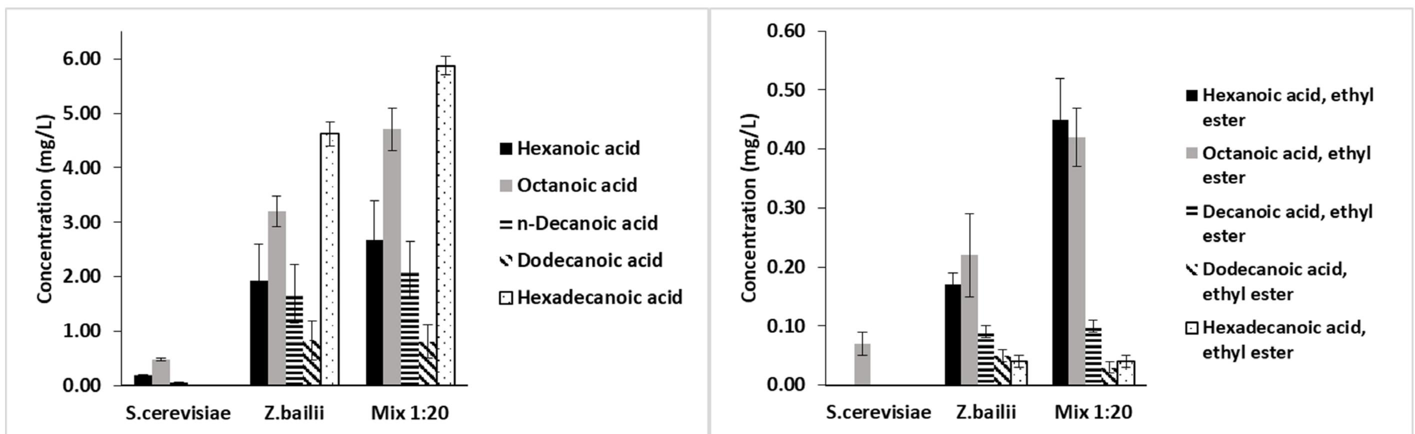

Fig. 6 The produced Medium Chain Fatty Acids and their corresponding esters in 16 °P initial gravity in mono- and mixed culture fermentation of *Z. bailii* in 1:10 ratio

as the most abundant compound in the monoculture of *Z. bailii*. *Z. bailii* is recognized for its robust isoamyl alcohol production under stress conditions, such as high sugar concentrations (16 °P), reflecting its efficient conversion of branched-chain amino acids into fusel alcohols [11]. Notably, 1-pentanol was exclusively detected in the fermentation of *Z. bailli* and only under 16 °P fermentation conditions, with no traces in the mixed fermentation. In the mixed fermentation, isoamyl alcohol and phenylethyl alcohol concentrations showed no statistically significant differences. In the mixed fermentation, concentrations of isoamyl alcohol and phenylethyl alcohol showed no statistically significant differences. Under these conditions, *S. cerevisiae* and *Z. bailii* produced comparable amounts of phenylethyl alcohol, measuring 9.19 ± 0.54 mg/L and 8.98 ± 1.83 mg/L, respectively.

For the acetate esters (right graph), isoamyl acetate was the predominant ester in all fermentations, with significantly higher concentrations ($p < 0.05$) in the conventional and mixed culture compared to *Z. bailii*. A similar trend was observed for phenylethyl acetate, although its increase in the mixed culture was less pronounced. Studies report that *Z. bailii* is particularly efficient at producing acetate esters like isoamyl acetate and phenylethyl acetate in mixed fermentations with *S. cerevisiae*, enhancing the overall aroma complexity of fermented products [3, 5, 28]

When considering the aforementioned results alongside the 12 °P fermentations, a consistent trend was observed: *Z. bailii* produced the highest isoamyl alcohol concentrations in monoculture, while phenylethyl alcohol levels dominated in the mixed fermentation. This shift in dominance could be attributed to the higher original gravity at 16 °P, which provides a richer nutrient base, and the metabolic efficiency of *Z. bailii* under nutrient-rich conditions. The observation that lower pH enhances ester production aligns with findings from fermentation studies involving *Z. bailii*. Specifically, Garavaglia et al. (2014) demonstrated that a pH of 3.5 facilitates higher ester production by *Z. bailii* BCV 08 in static cultures using grape must as a substrate, indicating a clear dependence between pH levels and ester biosynthesis [5]. This phenomenon is also evident in these experiments, where higher gravity fermentations lead to lower final pH values due to the accumulation of organic

Table 3 Other important volatile compounds detected in the fermentations of 12 and 16 °P

Compounds		Concentration at 12 °P (mg/L)			Concentration at 16 °P (mg/L)		
		S.cerevisiae	*Z.bailii*	Mix 1:10	*S.cerevisiae*	*Z.bailii*	Mix 1:20
Acids	Acetic acid	0.00 ± 0.00^a	0.03 ± 0.00^b	0.24 ± 0.06^c	0.00 ± 0.00^a	0.67 ± 0.28^c	0.26 ± 0.03^b
	9-Decenoic acid	0.00 ± 0.00^a	0.29 ± 0.05^b	0.56 ± 0.10^c	0.00 ± 0.00^a	0.29 ± 0.09^b	0.41 ± 0.13^b
Alcohols	1-Hexanol, 2-ethyl-	0.00 ± 0.00^a	0.07 ± 0.00^b	0.08 ± 0.01^b	0.00 ± 0.00^a	0.15 ± 0.03^b	0.10 ± 0.02^b
	3-Furfuryl alcohol	0.00 ± 0.00^a	0.04 ± 0.00^b	0.07 ± 0.01^b	0.00 ± 0.00^a	0.07 ± 0.03^b	0.07 ± 0.03^b
	Tyrosol	0.25 ± 0.06^a	0.12 ± 0.03^a	0.36 ± 0.03^b	0.39 ± 0.07^b	0.21 ± 0.11^{ab}	0.41 ± 0.06^a
	Tryptophol	2.66 ± 0.17^b	0.55 ± 0.03^a	0.66 ± 0.03^a	2.40 ± 0.19^b	0.34 ± 0.02^a	0.43 ± 0.07^a
	Methionol	0.00 ± 0.00^a	0.22 ± 0.03^b	0.37 ± 0.03^c	0.00 ± 0.00^a	0.28 ± 0.11^b	0.22 ± 0.13^b
Esters	Ethyl hydrogen succinate	0.00 ± 0.00^a	0.11 ± 0.03^b	0.08 ± 0.03^b	0.00 ± 0.00^a	0.16 ± 0.07^b	0.19 ± 0.10^b
Phenols	2-Methoxy-4-vinylphenol	0.05 ± 0.01^a	0.99 ± 0.09^c	0.32 ± 0.06^b	0.06 ± 0.01^a	0.61 ± 0.08^b	0.08 ± 0.03^a
	2,4-Di-tert-butylphenol	0.02 ± 0.00^a	0.43 ± 0.01^b	1.79 ± 0.30^c	0.18 ± 0.01^a	1.83 ± 0.58^b	2.40 ± 0.70^b
Lactones	γ-Nonalactone	0.00 ± 0.00^a	0.04 ± 0.00^b	0.05 ± 0.01^b	0.00 ± 0.00^a	0.07 ± 0.02^b	0.12 ± 0.03^b

* superscript letters in the same specific gravity present significant differences ($p < 0.05$) between different fermentations

acids and yeast metabolism. These factors collectively contribute to the enhanced synthesis of certain higher alcohols and esters, reflecting the metabolic interplay between the yeast strains.

Figure 6 illustrates the concentrations of medium-chain fatty acids (MCFAs) and their esters during 16 °P fermentations conducted by *S. cerevisiae* and *Z. bailii* in monoculture and mixed-culture fermentations of 1:20 ratio. For MCFAs (left graph), hexadecanoic acid displayed the highest concentrations in both fermentation conditions involving *Z. bailii*, with mixed fermentation producing significantly higher levels compared to monoculture ($p < 0.05$). Research highlights that *Z. bailii* has a robust lipid metabolism, allowing it to tolerate and adapt to stress conditions. This adaptation involves significant remodeling of lipid biosynthesis, including fatty acid production, as a survival mechanism. For example, lipidomic studies have shown that these yeasts alter their lipid composition, including increasing the production of long-chain saturated fatty acids like hexadecanoic acid, in response to stressors such as acetic acid and ethanol [25, 38]. Notably, hexadecanoic acid was found in 5.87 ± 0.17 mg/L in the mixed fermentation, while its concentration in the monoculture did not exceed the 4.62 ± 0.22 mg/L. Regarding hexanoic acid, n-decanoic acid, and dodecanoic acid, their concentrations remained relatively consistent across both fermentation conditions. In the case of *S. cerevisiae*, octanoic acid was the most abundant medium-chain fatty acid, though its levels did not exceed 1 mg/L, followed by hexanoic acid, while n-decanoic acid was only detected in trace amounts.

On the other hand, for the MCFA esters (right graph), hexanoic acid ethyl ester and octanoic acid ethyl ester were the dominant compounds, with significantly higher concentrations ($p < 0.05$) observed in the mixed fermentation compared to the monocultures. Specifically, the mixed fermentation reached concentrations of 0.45 ± 0.07 mg/L and 0.42 ± 0.05 mg/L for hexanoic and octanoic acid ethyl esters, respectively, whereas *Z. bailii* produced lower levels at 0.17 ± 0.02 mg/L and 0.22 ± 0.07 mg/L. *S. cerevisiae*, on the other hand, produced only octanoic acid ethyl ester at a concentration of 0.07 ± 0.02 mg/L, with no other ethyl esters detected. This aligns with studies that highlight the enhanced ester production in mixed fermentations involving *Z. bailii* and *S. cerevisiae* [5, 8, 28]. The lower concentrations of these esters may be primarily due to the high concentrations of their corresponding acids, which not only indicate incomplete esterification but also suggest the potential for increased ester formation as the reaction progresses.

Table 3 presents the concentrations of other important volatile compounds produced during fermentation at 12 °P and 16 °P in pure cultures and mixed culture fermentations at 1:10 (12 °P) and 1:20 (16 °P) ratios. The presence of acetic acid at concentrations above its threshold (70 – 200 mg/L) degrades the organoleptic quality of the final product [36, 37, 39]. At 12 °P, the concentration of acetic acid is notably low in the *Z. bailii* monoculture (0.03 ± 0.0 mg/L) but increases substantially in the mixed culture (0.24 ± 0.06 mg/L). At 16 °P, *Z. bailii* produces significantly higher acetic acid levels (0.67 ± 0.28 mg/L) compared to the mixed culture (0.26 ± 0.03 mg/L). Acetic acid was not detected in *S. cerevisiae* under any of the conditions tested. The acetic acid levels observed do not indicate any microbial contamination that could lead to an increase in volatile acidity. This increase may be attributed to the inherent metabolic activity of *Z. bailii*, which is known to produce acetic acid as a stress response at higher sugar concentrations, rather than solely due to pH differences [24, 26, 38, 40, 41]. The concentration of 9-decenoic acid in mixed fermentations are higher than the monoculture at both gravities, suggesting that mixed cultures potentially enhance fatty acid metabolism. These results align with studies reporting that mixed cultures can increase acid production due to synergistic interactions between yeast strains, potentially improving sensory complexity [42].

Regarding alcohols, mixed cultures exhibit increased production of compounds such as tyrosol and tryptophol. More specifically, at 12 °P, tyrosol levels are three times higher in the mixed culture (0.36 ± 0.03 mg/L) than in *Z. bailii* (0.12 ± 0.03 mg/L). At 16 °P, tyrosol remains significantly elevated in mixed cultures (0.41 ± 0.06 mg/L) compared to monoculture (Table 3). Similarly, tryptophol concentrations are elevated in mixed fermentations at both gravities, with the conventional yeast producing levels nearly five times higher than those of the non-*Saccharomyces* yeast. This enhancement of aromatic alcohols suggests a possible increase in amino acid metabolism due to cross-feeding interactions in mixed cultures, corroborating previous findings by [43] on mixed fermentations' ability to increase aromatic profiles.

Malt contains various acids, such as ferulic and coumaric acids, which can be converted into phenolic compounds by certain yeast species, leading to the formation of phenolic off-flavors (POF). The most notable of these phenols is 2-methoxy 4-vinylphenol, commonly known as 4-vinylguaiacol (4VG). This compound imparts a characteristic clove-like aroma to beers, particularly in Belgian wit beers and German wheat beers when found in concentration above its flavour threshold (0.2 – 0.4 mg/L, [44]), by deriving from ferulic acid through thermal decarboxylation. Studies have shown that, in addition to *Brettanomyces* and *Dekkera* species, some non-conventional yeasts, like *Torulaspora delbrueckii*, can also produce beers enriched with these phenolic notes [15, 45]. Furthermore, [46] found that the concentration of 4VG is closely linked to the specific yeast strain used during fermentation. In this study, higher concentrations of 4VG were observed in pure fermentation of *Z.baillii*, suggesting a stronger phenolic profile under these conditions reaching almost 1 mg/L (Table 3). On the other hand, 2,4-di-tert-butylphenol, is substantially elevated in mixed fermentations. At 12 °P, mixed cultures produce nearly four times more 2,4-di-tert-butylphenol (1.79 ± 0.13 mg/L) than monocultures (0.43 ± 0.10 mg/L). A similar pattern occurs at 16 °P, where mixed fermentations generate 2.40 ± 0.70 mg/L compared to 1.33 ± 0.18 mg/L in *Z. bailii*. This result is supported by the observation that non-*Saccharomyces* yeasts, like *Metschnikowia pulcherrima* [47] and *Hanseniaspora* spp. [48], exhibit phenolic acid decarboxylase activity, converting phenolic acids into volatile phenols.

5 Conclusion

In general, the results confirmed that *Z. bailii* can complete fermentations, achieving desired alcohol levels, and significantly reducing the final beer pH. However, the prolonged fermentation time observed – 900 hours for normal gravity and 1100 hours for high gravity – pose a challenge for industrial applications. *Z. bailii*'s

slower fermentation, particularly at higher initial °P, suggests that while it may offer potential for flavor diversity or resilience, it is not ideal for high-efficiency fermentations when used alone. Mixed fermentations did not accelerate the process noticeably, especially under high gravity conditions. Notably, the phenolic character – a key feature of *Z. bailii* – was evident in all the beers produced, making this yeast particularly suitable for styles that traditionally embrace phenolic notes, such as Wheat beers. Given its ability to produce complex phenolic and fruity flavors, *Z. bailii* shows great promise to produce saison-style beers, which often feature these same distinctive characteristics, enhancing the complexity and profile of the final product. In the future, additional parameters such as aeration rate and temperature variation will be considered, as they are likely to influence the production of higher alcohols and esters. These aspects will be explored in further trials to enhance the robustness of the findings.

Author contributions

F. Drosou: Investigation, Data analysis, Writing original draft; A. Karagelis: Investigation, Data analysis; E. Koussissi: Sensory Data curation, Investigation; writing original draft; Th. Dourtoglou: Resources, Editing; Aik. Tzamourani: Yeast isolation, molecular typing, preculture preparation; M. Dimopoulou: Resources; P. Tataridis: Editing, Supervision.

Conflict of interest

The authors declare there are no conflicts of interest.

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