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Potential for Lager Beer Production from *Saccharomyces cerevisiae* Strains Isolated from the Vineyard Environment

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Abstract: *Saccharomyces pastorianus*, genetic hybrids of *Saccharomyces cerevisiae* and the *Saccharomyces eubayanus*, is one of the most widely used lager yeasts in the brewing industry. In recent years, new strategies have been adopted and new lines of research have been outlined to create and expand the pool of lager brewing starters. The vineyard microbiome has received significant attention in the past few years due to many opportunities in terms of biotechnological applications in the winemaking processes. However, the characterization of *S. cerevisiae* strains isolated from winery environments as an approach to selecting starters for beer production has not been fully investigated, and little is currently available. Four wild cryotolerant *S. cerevisiae* strains isolated from vineyard environments were evaluated as potential starters for lager beer production at laboratory scale using a model beer wort (MBW). In all tests, the industrial lager brewing *S. pastorianus* Weihenstephan 34/70 was used as a reference strain. The results obtained, although preliminary, showed some good properties of these strains, such as antioxidant activity, flocculation capacity, efficient fermentation at 15 °C and low diacetyl production. Further studies will be carried out using these *S. cerevisiae* strains as starters for lager beer production on a pilot scale in order to verify the chemical and sensory characteristics of the beers produced.

Keywords: *S. cerevisiae*; vineyard environment; starter; lager beer production

Citation: Iorizzo, M.; Letizia, F.; Albanese, G.; Coppola, F.; Gambuti, A.; Testa, B.; Aversano, R.; Forino, M.; Coppola, R. Potential for Lager Beer Production from *Saccharomyces cerevisiae* Strains Isolated from the Vineyard Environment. *Processes* **2021**, *9*, 1628. https://doi.org/10.3390/pr9091628

Academic Editor: Giovanna Ferrentino

Received: 18 July 2021
Accepted: 8 September 2021
Published: 9 September 2021

1. Introduction

Beer is a fermented alcoholic beverage produced from water, malted cereal grains (generally barley and wheat) and hops [1,2]. The yeast metabolism during the fermentation and maturation process is crucial, as the levels of aroma-active compounds in beer depend not only on the raw materials and the fermentation conditions but also on the yeast strain [3,4]. By using specific yeasts, fermentation efficiency can be increased, new beer characteristics can be developed and the sensory complexity of the final product can be enhanced [5]. The yeast species dominating the production of alcoholic beverages worldwide belong to the *Saccharomyces* genus. The *Saccharomyces* sensu stricto species complex [6] contains some of the most important species to the food industry, including *S. cerevisiae*, used for wine, bread, ale beers and sake productions [7]; *S. bayanus*, involved in the wine and cider productions [8] and *S. pastorianus*, which is employed in lager beer production [9]. *S. pastorianus* is an interspecies hybrid of *S. cerevisiae* and *S. eubayanus* [10]. *S. cerevisiae* has been selected for some properties crucial to beer production, such as

flocculation capacity and alcohol tolerance, but it requires an optimal fermentation temperature above 15 °C. *S. eubayanus* is able to ferment at lower temperatures but does not metabolize maltotriose and shows low flocculation capacity [10–12].

The natural hybrid *S. pastorianus* shows tolerance to cold temperatures and the ability to metabolize maltose and maltotriose. These traits were inherited from genetic interactions between the parent genomes [13,14]. In particular, the *S. eubayanus* genome conferred enhanced cold tolerance, while the *S. cerevisiae* subgenome assured a vigorous and complete fermentation [15–17]. In spite of the multiplicity of different beers, actually, a small number of yeast strains are available to the brewing industry [18]. The selection and domestication of brewer's yeasts have reduced genotypic and phenotypic variability [19].

In recent years, new strategies have been adopted and new lines of research have been outlined to create and expand the pool of lager brewing starters, including the creation in laboratory of new lager yeast strains generated by interspecific hybridization [10,20,21].

The microbiomes of terrestrial environments constitute vast, under-explored and under-exploited sources of biodiversity. Among these environments, the vineyard microbiome represents an important reserve of yeast strains, with a wide genotypic and phenotypic variability potentially suitable to fermented beverage productions.

However, *S. cerevisiae* strains—isolated from winery environments as an approach to select new starters for beer production—have not been fully investigated, and little is currently available [22,23]. In the frame of a scientific project (PRIN, ADAPT: influence of agro-climatic conditions on the microbiome and genetic expression of grapevines for the production of red wines: a multidisciplinary approach), some studies have been carried out on the microbial biodiversity in vineyard ecosystems.

Research on the grape yeast communities has led to the selection of four cryotolerant *S. cerevisiae* strains.

In order to evaluate some technological performances, in this study, we tested the suitability of these yeasts, found in non-brewing environments, as starters for lager beer production.

2. Materials and Methods

2.1. Yeasts Isolation

The used yeasts were isolated from red grapes, Aglianico and Cabernet Sauvignon varieties, harvested at a fully ripened stage from six vineyards located in three different regions of Southern Italy: Campania, Sicily and Molise. Grape samples were collected randomly from the vineyards and transferred to the laboratory within 12 h. The grapes were crushed by a homogenizer (bagMixer 400, Interscience, Saint Nom la Brétèche, France), and appropriate serial dilutions of each sample were made using a physiological saline solution (0.9% *w*/*v* NaCl). Subsequently, 0.1 mL of each dilution was distributed on Petri plates containing WL nutrient agar (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 0.01% *w*/*v* chloramphenicol (Merck KGaA, Darmstadt, Germany). The plates were incubated at 28 °C for 1–3 days. After the incubation, 5 colonies from each sample were picked on the basis of typical *Saccharomyces* morphology: cream/white in color and smooth non-powdery surface [24,25]. Purification of the strains was performed on the YEPD agar (Thermo Fisher Scientific, Waltham, MA, USA) by successive subculturing. Stock cultures were maintained in YEPD broth (Thermo Fisher Scientific, Waltham, MA, USA) for daily use and were preserved in YEPD broth with 30% (*v*/*v*) sterile glycerol (Merck KGaA, Darmstadt, Germany) for long-term storage at −80 °C until further characterization.

2.2. Molecular Identification of Yeasts

A total of 124 hypothetical *Saccharomyces* strains were analyzed. The isolated strains were aerobically cultured in 80 mL of YEPD broth at 28 °C for 18 h maintained under stirring using a digital orbital shaker (Heathrow Scientific, Illinois, USA) set at 200 rpm.

Each isolate (2 mL) was centrifuged at 7500 rpm for 5 min at 4 °C, and the pellet obtained was subjected to DNA extraction using a DNA extraction kit (Norgen Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. To improve the cell lysing and the DNA yield, a solution of 20 mg/mL of lyticase (Merck KGaA, Darmstadt, Germany) was added, and the mixture was incubated at 37 °C for 45 min.

The quantity and purity of the DNA were assessed using the NanoDrop spectrophotometer 2000 (Thermo Scientific, Wilmington, DE, USA).

Amplification of 28S rDNA region [26,27] was carried out using U1 and U2 primers. PCR was performed using a Mastercycler nexus gradient (Eppendorf, Hamburg, Germany).

The reaction mixture consisted of 10 µL TaqMan 2x PCR Master Mix (Norgen Biotek Corp., Thorold, ON, Canada), 2 µL of each primer (2.5 µM), 2 µL template DNA and the nuclease-free water was added to bring the final volume to 20 µL.

PCR products were separated in 1.5% (*w*/*v*) agarose gel by electrophoresis for 45 min at 80 V in 1X TAE buffer (Thermo Fisher Scientific, Waltham, MA, USA) and were subsequently visualized by UV transilluminator (Bio-Rad molecular image Gel Doc XR, Hercules, CA, USA) after ethidium bromide (50 µg/mL, Merck KGaA, Darmstadt, Germany) staining.

After purification (QIA-quick PCR Purification kit, QIAGEN GmbH, Hilden, Germany), the DNA products were sent to a commercial facility for sequencing (Eurofins MWG Biotech Company, Ebersberg, Germany). The Basic Local Alignment Search Tool Nucleotide (BLASTN) tool encoded in NCBI suite [28] was applied to the GenBank [29] database to identify the sequences. Sequence matches that showed high identity scores (98% and above) were considered acceptable for taxonomic placement at species level. Identification of *S. cerevisiae* species was validated via PCR using the ScHO-F/ScHO-R specific primers (Table 1) that amplify the HO/chromosome IV gene [30].

Table 1. List of primers used for yeasts identification and the respective PCR conditions.

PCR Primers	Target Region	Primer Sequences	PCR Conditions	References
U1	28S rDNA	5′-TGAAATTGTTGAAAGGGAA-3′	35 cycles 94 °C 1 min/55 °C 1 min/72 °C 2 min	[26]
U2		5′-GACTCCTTGGTCCGTGTT-3′		
ScHO-f	HO gene/ chromosome IV	5′-GTTAGATCCCAGGCGTAGAACAG-3′	35 cycles 94 °C 30 s/61 °C 30 s/72 °C 2 min	[30]
ScHO-r		5′-GCGAGTACTGGACCAAATCTTATG-3′		

2.3. Screening of S. cerevisiae Strains

2.3.1. Cryotolerance

The capacity to grow at 4 °C of all the 124 yeast isolates was evaluated by inoculating (10^6 cfu/mL) the yeast cultures into Erlenmeyer flasks (working volume of 100 mL) containing 80 mL of YEPD broth and maintained under stirring using a digital orbital shaker (Heathrow Scientific, IL, USA) set at 150 rpm. The growth was determined visually after 24 h.

The fermentation capacity at 15 °C was evaluated in a model beer wort (MBW) composed of: malt extract 70 g/L, glucose 30 g/L, peptone 1.5 g/L, K_2HPO_4 1.14 g/L, $MgSO_4.7H_2O$ 1.23 g/L, $CaCl_2.2H_2O$ 0.44 g/L, thiamine HCl 0.5 mg/L, $ZnCl_2$ 135 µg/L; pH was adjusted to 5.5 with HCl. The MBW was sterilized before use by 3 pasteurization

cycles of 5 min each at 80 °C. As for the inoculum, the yeast strains were cultured aerobically into Erlenmeyer flasks (working volume of 100 mL) containing 80 mL of YEPD broth, incubated at 20 °C for 24 h maintained under stirring using a digital orbital shaker (Heathrow Scientific, IL, USA) set at 150 rpm. After the incubation time, the broth cultures were centrifuged (5000 rpm for 10 min at 4 °C), the pellet obtained was washed with sterile physiological solution (0.9% *w*/*v* NaCl), and Erlenmeyer flasks (working volume of 250 mL) containing 150 mL of MBW were inoculated with 10^6 cfu/mL and incubated at 15 °C without shaking. The progress of the fermentation was monitored by the daily weight loss through CO_2 release.

All the chemical compounds used were purchased from Merck (Merck KGaA, Darmstadt, Germany).

2.3.2. Hydrogen Sulfide (H_2S) Production

H_2S production of all the 124 yeast isolates was evaluated on BIGGY agar (Bismuth Sulphite Glucose Glycine Yeast; Thermo Fisher Scientific, Waltham, MA, USA). The yeast strains were aerobically cultured in YEPD broth at 28 °C. Successively, each yeast strain was spread on the surface of BIGGY agar medium, and plates were incubated at 15 °C for 5 days. On this medium, H_2S-negative strains showed white colonies, while H_2S-producing colonies were characterized by a brown or dark brown color. For results, the following chromatic scale was considered: 0 (white colonies, no hydrogen sulfide production), 1 (cream colonies), 2 (light brown colonies), 3 (brown colonies), 4 (dark brown or black colonies, very intensive hydrogen sulfide production) [31]. The test was performed in triplicate.

2.4. Technological Characterization

Four selected *S. cerevisiae* strains (CS1C; CS2C; CSB21B; 41CM) were subjected to technological characterization trials. In all the tests, the industrial lager brewing *S. pastorianus* Weihenstephan 34/70 (Fachhochschule Weihenstephan, Freising, Germany) was used as reference strain [32].

2.4.1. Yeast Culture Preparation

The yeast strains were cultured aerobically in 100 mL of YEPD broth into Erlenmeyer flasks (working volume of 250 mL) at 18 °C for 24 h maintained under stirring using a digital orbital shaker (Heathrow Scientific, IL, USA) set at 150 rpm. After incubation, the broth cultures (BC) were centrifuged (5000 rpm for 10 min at 4 °C) to obtain the cell pellet (CP).

2.4.2. Flocculation Assay

Flocculation was estimated according to Soares [33], with some modification. The CP was deflocculated in 250 mM EDTA solution, washed with 250 mM NaCl solution and suspended in the same solution to a final concentration of about 10^8 cells/mL. The OD_{600} of this suspension (OD_d) was measured using a basic BioSpectrometer (Eppendorf, Hamburg, Germany).

Flocculation was then induced by adding $CaCl_2$ (4 mM final concentration) to 5 mL of the cell suspension in a 10 mL test tube. The tube was shaken at oscillation rate of 50/min for 5 min and then left standing vertically.

After 10 and 30 min at 15 °C, 1 mL of suspension was removed just below the meniscus and mixed with 1 mL of 250 mM EDTA (pH 8.0). Finally, the OD_{600} of this suspension (OD_f) was measured using a basic BioSpectrometer (Eppendorf, Hamburg, Germany).

Flocculation (%) was calculated using the following formula:

$$100\ (OD_d - OD_f)/OD_d$$

(OD_d: optical density of deflocculated cell suspension. OD_f: optical density after the flocculation phase). The test was performed in triplicate.

All the chemical compounds used were purchased from Merck (Merck KGaA, Darmstadt, Germany).

2.5. Fermentation Trials

The CP of each yeast strain was washed with physiological solution (0.9% *w*/*v* NaCl), and the inoculum was made at a concentration of 10^6 cfu/mL in 200 mL of MBW using Erlenmeyer flasks (working volume of 250 mL) plugged with a special glass device (Müller valve) containing pure sulfuric acid. The fermentations were performed at 15 °C and their progress was monitored daily by measuring the mass loss due to CO_2 release until a constant weight was recorded for 3 consecutive days [34]. Sugar concentrations (maltose and glucose), pH and the alcohol content evolution were monitored during the fermentation. Maltose and glucose were determined using a colorimetric kit (Merck KGaA) according to the manufacturer's instructions. pH was measured using a pHmeter (Crison basic 20, Barcelona, Spain). Alcohol was determined according to the method of AOAC (Association of Official Analytical Chemists) [35].

At the end of the fermentation process (14 days), the fermented products were centrifuged at 3000 rpm for 15 min, decarbonized through agitation and subjected to chemical analysis. The fermentations were carried out in triplicate.

2.5.1. Chemical Analysis

pH of each sample was measured using a pHmeter (Crison basic 20, Barcelona, Spain). The determination of the alcohol content of fermented products was carried out using a distillation procedure and measurement of specific gravity of the distillate based on the standards of the Association of Official Analytical Chemists [35]. Glycerol, acetaldehyde, succinic acid, acetic acid, citric acid, total SO_2, L-malic acid and D-lactic acid were determined using enzymatic kits (Steroglass, Perugia, Italy) according to the manufacturer's instructions. Diacetyl (2,3-butanedione) was spectrophotometrically determined according to the method of Alvarez et al. [36] using a BioSpectrometer basic (Eppendorf, Hamburg, Germany). All the analyses were carried out in triplicate.

2.5.2. Antioxidant activity (TEAC)

For the antioxidant activity (AA), the CP obtained from 5 mL of BC was washed with physiological solution (0.9% *w*/*v* NaCl) and was suspended in 500 μL of methanol, and after 12 h of storage at −20 °C, the suspension was centrifuged (10,000 rpm for 15 min at 4 °C) to obtain the cell extract (CE).

The AA was evaluated using the ABTS (2,2 azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) method according to Iorizzo et al. [37] and by DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) assay according to Molyneux [38].

For total cell protein extraction, 5 mL of the CP matrices was suspended in 1 mL of NaOH (0.1 M) sonicated (100 W, 25 kHz frequency, 30 min, duty cycle 50%, 25 °C) according to Liu et al. [39] using an ultrasonic homogenizer (Labsonic M, Sartorius; Varedo, Italy) and incubated at 95 °C for 20 min; the supernatant was centrifuged at 13,000 rpm for 5 min and was used to estimate the amount of total protein.

The suspension was used for protein measurement according to Di Martino et al. [40] using a BioSpectrometer (Eppendorf; Milan, Italy), and the cell protein concentration (μg/mL) was calculated by means of a calibration curve where bovine serum albumin (BSA) was used as a standard. The data of antioxidant activity were expressed as ratio μg Trolox equivalents/mg cell protein (BSA equivalents). All the chemical reagents used in the experiments were purchased from Merck (Merck KGaA, Darmstadt, Germany).

2.6. Statistical Analysis

All experiments were carried out in triplicates. The results were expressed as the mean ± SD. Microbial count levels and chemical parameters were analyzed by ANOVA (IBM SPSS Statistics 21).

3. Results

3.1. Molecular Identification

Based on the analysis of 28S rDNA using the primers U1 and U2, all the 124 yeast isolates turned out to belong to the *S. cerevisiae* species. The taxonomic placement at a species level was assigned to rDNA gene sequences with closest BLASTn matches to organisms located in GenBank [29] at 98% aligned similarity or greater. Based on the chromogenic test on BIGGY agar and the cryotolerance test (ability to grow at 4 °C and a greater capacity to ferment at 15 °C), CSB21B, CS1C, CS2C and 41CM *S. cerevisiae* strains were selected for further technological characterization. Genetic analysis of the HO/chromosome IV gene using the specific primers ScHO-F and ScHO-R allowed us to confirm that the four selected strains belonged to *S. cerevisiae* species (data not shown).

3.2. Technological and Physiological Properties

3.2.1. Flocculation Ability

The results of the flocculation capacity (%) of the four selected *S. cerevisiae* strains and *S. pastorianus* W-34/70 are shown graphically in Figure 1 and numerically in Table S1 (Supplementary Materials). After 10 min, the flocculation values ranged from 3.0% (CS1C) to 24.0% (CSB21B) with high variability among the various yeasts; instead, after 30 min, there were minor variabilities, except for the 41CM strain, which showed lower flocculation (30.3%) compared to the other strains with a flocculation capacity above 50%

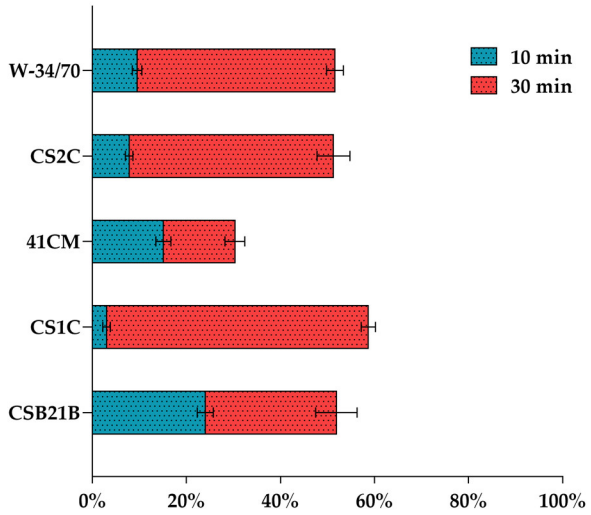

Figure 1. Flocculation capacity (%) of *S. cerevisiae* CSB21B, CS1C, CS2C, 41CM and *S. pastorianus* W-34/70 strains after 10 min and 30 min at 15 °C.

3.2.2. Antioxidant Activity

The results of the antioxidant activity are shown in Table 2. In the ABTS test, Trolox values were higher than those obtained by the DPPH method, highlighting a greater capacity of the ABTS method to detect the specific antioxidant substances present in the cell extract of yeasts.

Data obtained using the ABTS method showed significant differences, with values between 4.56 (41CM) and 6.48 (W-34/70) μg Trolox Eq./mg cell protein.

Instead, data obtained by the DPPH method showed no meaningful differences, with values comprised between 0.85 (CS2C) and 0.98 (CSB21B) μg Trolox Eq./mg BSA Eq.

Table 2. Antioxidant activity of *S. cerevisiae* CSB21B, CS1C, CS2C, 41CM and *S. pastorianus* W-34/70 strains by DPPH and ABTS methods. Data are expressed as means ± standard deviation obtained in three independent experiments ($n = 3$); lower case letters indicate significant differences within row ($p < 0.05$).

Antioxidant Activity (μg Trolox Eq./mg Cell Protein)	CSB21B	CS1C	41CM	CS2C	W-34/70
ABTS	5.91 ± 0.14 ^{b}	4.93 ± 0.11 ^{c}	4.56 ± 0.26 ^{d}	5.13 ± 0.20 ^{c}	6.48 ± 0.26 ^{a}
DPPH	0.98 ± 0.08 ^{a}	0.95 ± 0.06 ^{a}	0.93 ± 0.03 ^{a}	0.85 ± 0.05 ^{a}	0.96 ± 0.09 ^{a}

3.2.3. Fermentation Kinetics and Chemical Composition of Fermented Products

All the *S. cerevisiae* strains featured fermentation kinetics and final amounts of ethyl alcohol similar to the S. *pastorianus* W-34/70 reference strain. In Table S2, the values of the ethanol produced during alcoholic fermentation are reported. In all tests, the fermentation activity of the starter yeasts ended after 14 days.

The fermentation kinetics of glucose and maltose (Figure 2) were also similar among the different starters used, with differences almost always statistically insignificant. All the numerical data are shown in Tables S3 and S4.

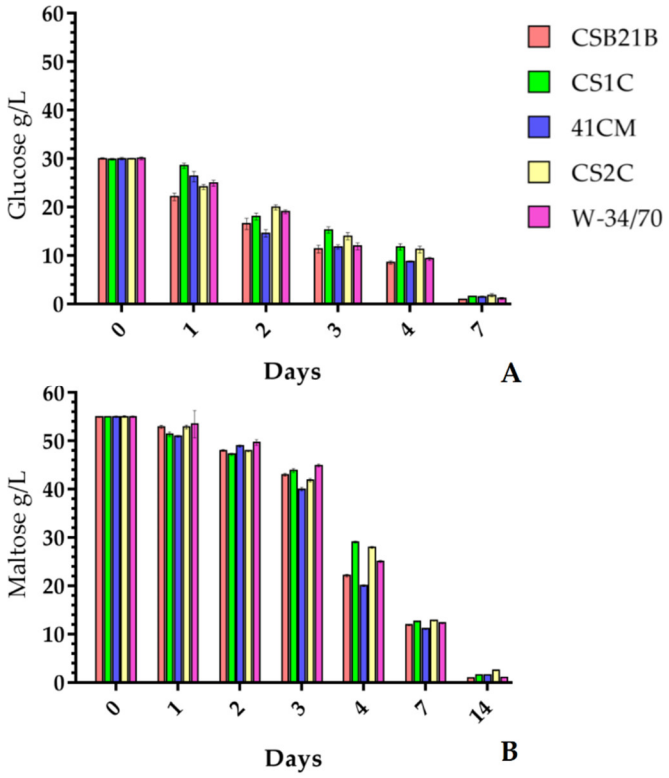

Figure 2. Fermentation kinetics of glucose (**A**) and maltose (**B**) in MBW inoculated with *S. cerevisiae* CSB21B, CS1C, CS2C, 41CM and *S. pastorianus* W-34/70.

The results of chemical characteristics of fermented products are shown in Table 3. pH values during alcoholic fermentation were similar in the different tests. In table S5, the pH values during alcoholic fermentation are reported. Instead, meaningful differences often occurred in regard to the amounts of every single organic acid present in the different fermented products.

In particular, in the case of acetic acid (range 7.7–13.0 mg/L), there were non-significant differences in the products obtained using the 41CM, CS2C and S. *pastorianus* W-34/70 starters.

As for the acetaldehyde production, the values ranged from 6.2 mg/L (41CM) to 16.5 mg/L (CSB21B), with non-meaningful differences between the 41CM and S. *pastorianus* W-34/70 strains. The glycerol concentrations in the fermented products ranged from 1.7 to 2.4 g/L; data showed a low variability among the selected yeast strains in terms of ability to produce such compounds. Moreover, in regard to the production of diacetyl, there were significant differences between the four selected yeasts and the reference strain, with values between 201.3 μg/L (CSB21B) and 466.3 μg/L (CS2C).

Table 3. Chemical characteristics of fermented products obtained by using *S.cerevisiae* CSB21B, CS1C, CS2C, 41CM and *S. pastorianus* W-34/70 as starter cultures. Data are expressed as means ± standard deviation obtained in three independent experiments ($n = 3$); lower case letters indicate significant differences within row ($p < 0.05$).

	CSB21B	CS1C	41CM	CS2C	W-34/70
pH	3.4 ± 0.2 ^{a}	3.4 ± 0.1 ^{a}	3.1 ± 0.2 ^{a}	3.5 ± 0.3 ^{a}	3.6 ± 0.2 ^{a}
SO_2 (mg/L)	20.1 ± 1.6 ^{a}	13.4 ± 0.9 ^{b}	7.0 ± 0.2 ^{c}	2.0 ± 0.1 ^{d}	8.6 ± 0.6 ^{c}
Acetaldehyde (mg/L)	16.5 ± 0.7 ^{a}	9.8 ± 0.4 ^{c}	6.2 ± 0.5 ^{d}	11.7 ± 0.9 ^{b}	6.6 ± 0.3 ^{d}
Acetic acid (mg/L)	11.6 ± 1.2 ^{a}	13.0 ± 1.5 ^{a}	8.2 ± 0.4 ^{b}	8.1 ± 0.6 ^{b}	7.7 ± 0.4 ^{c}
Alcohol (% vol)	4.4 ± 0.2 ^{a}	4.2 ± 0.2 ^{a}	4.3 ± 0.4 ^{a}	4.0 ± 0.2 ^{a}	4.2 ± 0.3 ^{a}
Citric acid (mg/L)	6.2 ± 0.8 ^{c}	12.0 ± 0.7 ^{a}	12.2 ± 1.1 ^{a}	11.1 ± 1.1 ^{a}	9.6 ± 0.4 ^{b}
Diacetyl (μg/L)	201.3 ± 18.0 ^{d}	282.7 ± 20.1 ^{c}	293.7 ± 11.9 ^{c}	466.3 ± 7.2 ^{a}	364.7 ± 23.4 ^{b}
Glycerol (g/L)	2.4 ± 0.2 ^{a}	1.7 ± 0.2 ^{b}	1.7 ± 0.1 ^{b}	2.0 ± 0.2 ^{a}	1.7 ± 0.2 ^{b}
D-lactic acid (mg/L)	44.0 ± 1.7 ^{c}	74.1 ± 3.2 ^{a}	44.2 ± 3.9 ^{c}	40.7 ± 2.0 ^{c}	54.4 ± 4.7 ^{b}
L-malic acid (mg/L)	29.0 ± 1.3 ^{a}	22.8 ± 1.5 ^{b}	10.6 ± 0.4 ^{d}	17.4 ± 0.8 ^{c}	8.7 ± 0.2 ^{d}
Succinic acid (mg/L)	47.8 ± 2.9 ^{e}	91.1 ± 2.8 ^{d}	126.0 ± 3.8 ^{b}	147.0 ± 8.6 ^{a}	112.0 ± 4.0 ^{c}

4. Discussion

In our study, preliminary screening for the selection of yeasts was based on the assessment of hydrogen sulfide production and on the fermentative ability at 15 °C, which falls within the fermentation temperatures of lager beers [41,42].

H_2S is a metabolite containing a sulfide group; it has a low sensory threshold (50–80 μg/L) and can be produced by yeasts in different amounts during fermentation, thus contributing to off-flavors [43]. In our study, the capacity to produce H_2S was assessed by growing yeast cells on Biggy agar [44]. *S. cerevisiae* CSB21B, CS1C, CS2C and 41CM strains did not produce detectable quantities of H_2S by this semi-quantitative technique; therefore, they were considered non-producers or low producers of H_2S.

In the field of lager beer production, it is important that yeasts exert their metabolic activities at low temperatures [13,45].

Our results highlighted the ability of the four *S. cerevisiae* strains to ferment at 15 °C, similarly to the reference strain of *S. pastorianus* W-34/70.

Flocculation ability describes the aggregation capacity of yeast cells [46], and the number of yeast cells suspended during fermentation affects the wort fermentation velocity, beer flavor, maturation, cell crop formation and filtration [47]. The four selected *S. cerevisiae* strains showed meaningful differences in terms of flocculation capacity. Several studies have shown that this property is highly strain-dependent and is affected by genetic, physiological and environmental factors [48–51]. The flocculation phase is a positive event when it occurs at the end of the fermentation process. However, when yeasts tend to aggregate and precipitate too early, there may be a slowdown or a blockage of alcoholic fermentation. In these cases, the obtained beers contain excessive residual sugars and unsatisfactory flavor characteristics.

Oxidation reactions, which already occur during the early stages of brewing and in post-fermentation, can have a negative impact on the color and flavor stability and cause

the formation of off-flavor compounds, thus reducing the shelf life of beers [52,53]. During the fermentation, yeast cells will upregulate antioxidant-related genes to mitigate the deleterious effects of reactive oxygen species (ROS) [54]. Oxidative stress typically leads to the formation of radical oxygen, which damages biological macromolecules [55]. To prevent oxidative damages, the yeast cell activates an oxidative stress response, consisting of both enzymatic (peroxidases, catalases and superoxide dismutases) and non-enzymatic antioxidant systems [56].

Yeasts synthesize several bioactive antioxidant compounds such as torulahoidin, citric acid, coenzyme Q, glutathione, thioredoxin, various forms of tocopherols, riboflavin (vitamin B2), yeast-derived cell wall β-glucans, cytochrome C, etc. that may contrast oxidative phenomena during beer maturation [57–63].

Industrial beers after alcoholic fermentation are filtered and pasteurized to remove the yeast and stabilize the beer prior to packaging. However, craft beers are not filtered and pasteurized. In these products, the presence of antioxidant substances, secreted by the cell or released after its death, helps the beer preservation by delaying oxidative degeneration [57,64,65]. Our study showed promising results in terms of the ability of the selected yeasts to act as natural antioxidants.

Sulfites in beer are not only important antioxidants to prevent beer from oxidative staling, but they can also act as important mask agents for stale flavor by reacting with carbonyl staling compounds to form bisulfite–carbonyl adducts, which are important for beer flavor stability [65,66]. The production of sulfites in beer is affected by yeast strains, fermentation conditions, wort components and sulfites production, which appeared to be yeast-strain-dependent [67]. In fact, our results showed high variability in the SO_2 production.

Diacetyl as a by-product of fermentation is more characteristic of ales than lagers. Diacetyl production and its oxidation state are correlated to fermentation temperatures. Diacetyl is produced in the initial fermentation steps and can be reabsorbed by the yeast and reduced to flavorless compounds. Yeast strains show a distinct difference in their diacetyl reduction ability. In lager beers, which are characterized by fresh and clean flavor profiles, diacetyl is considered almost invariable as an off-flavor at concentrations above the flavor threshold of about 0.1–0.15 mg/L. Diacetyl is synthesized by oxidative decarboxylation of excess α-acetolactate derived by the valine biosynthetic pathway. [68]. Our results showed a high variability regarding the diacetyl production; in particular, *S. cerevisiae* CS1C, CS2C and 41CM strains produced significantly lower amounts of this compound compared to the *S. pastorianus* W-34/70 reference strain.

Yeasts produce acetaldehyde as an alcoholic fermentation intermediate compound with a threshold perception of about 20 mg/L [69], and its presence in beer above the threshold value is associated with green apple, emulsion paint, wine (white wine) and sherry flavor notes at low levels; but at higher concentrations, it can contribute to an off-flavor reminiscent of rotten apples.

Acetaldehyde is also produced by the oxidation of ethanol (alcohol) or even released by yeasts during beer maturation due to the autolysis process [70]. Our study showed that all the selected yeasts produced quantities of this compound below 20 mg/L.

Glycerol has favorable impacts on the quality of beer, as it is linked to density and viscosity, which are classified as mouth-feel attributes. The favorable threshold is 10 g/L in beers [71]. In our fermentation trials, the four selected *S. cerevisiae* and *S. pastorianus* W-34/70 strains produced similar amounts of glycerol.

The sensory effects of beers are determined by the aroma, taste, temperature and texture. As for organic acids, the balance between the sourness and sweetness of a beverage is of high importance. Predominant organic acids in beer are acetic, pyruvic, lactic, malic and citric acid, mainly derived from vegetable raw materials and the tricarboxylic acid cycle metabolism of yeast. The results of our study showed that the measured pH values were similar, but there was high variability in the quantities of each organic acid synthesized by the selected starter yeast.

Acetic acid causes a quality reduction when its content is higher than the taste threshold. The concentration of acetic acid ranges between 57 and 145 mg/L, and its threshold range is from 71 to 200 mg/L in different kinds of beer [69]. The production of acetic acid during alcoholic fermentation depends on the yeast strain, wort composition and fermentation conditions [72,73]. In our tests, by using the MBW as fermentation substrate, all the yeast starters produced amounts of acetic acid below 12 mg/L.

Organic acids are among the most important compounds in beer: they not only have an effect on the sour taste of beer, but some of them can also have other flavor attributes, such as a bitter or salty taste [74]. Moreover, they affect the beer pH [75], quality and flavor stability [76,77].

Citric acid is an organic acid found in beer normally within a concentration range of 50–250 mg/L; it is produced by the yeast oxidative metabolism. Although it contributes to the overall acidity of the beer, citric acid has little impact on the overall flavor. It is sometimes added to increase the acidity of some low-alcohol and non-alcoholic beers where incomplete fermentation fails to increase acidity to an appropriate level [78,79]. Succinate has long been known to be excreted by yeasts during fermentation, and the flavor threshold is 200 mg/L [69]. Two *S. cerevisiae* strains (41CM, CS2C) and the reference strain W-34/70 have produced this acid at concentrations above its threshold value.

S. cerevisiae has the ability to degrade or form malic acid during fermentation [80,81]. Malic acid is converted by the malic enzyme into pyruvic acid [82,83]. In beers, the flavor threshold of malic acid is 200 mg/L [69].

S. cerevisiae strains produce a low amount of D-lactic acid during alcoholic fermentation [84]. Levels up to 100 mg/L have been associated with a low enzymatic activity of pyruvate decarboxylase [85]. Lactic acid in beer has a flavor threshold of 400 mg/L [69]. All the yeasts used in our study produced amounts of malic and lactic acids well below their threshold levels.

5. Conclusions

Wider availability of new yeasts and a better understanding of their physiology can have a significant and direct impact on the brewing industry, providing a valuable opportunity for brewers to characterize and diversify their products, especially in craft beer production.

S. cerevisiae is a ubiquitous species in nature, but its origin and biogeography distribution has not been clarified yet [86–89]. Vineyard and grape berry surfaces provide a physical environment in which complex microbial communities can establish themselves. The enormous variability of these ecosystems can be a bio-reserve of yeasts with functional characteristics applicable in the field of fermented beverages. Production of lager-type beers requires a set of essential phenotypes, including the ability to flocculate and ferment at low temperatures. The fermentation dynamics and chemical parameters examined showed an overall good potential of *S. cerevisiae* CSB21B, CS1C, CS2C and 41CM strains as a starter for lager beer production.

The relationship between adaptation and natural selection of yeasts in the brewing environment is far from being fully clarified.

Previous studies have shown that the "hybrid species" *S. pastorianus* has inherited, above all, the characteristics of alcohol tolerance and fermentative vigor from the parent *S. cerevisiae*.

Our results showed that some specific strains of *S. cerevisiae* also possess good cryotolerance and develop fermentative dynamics similar to the reference strain *S. pastorianus* W-34/70 in a model system (MBW) at 15 °C.

The good potential of these yeasts does not axiomatically mean that they are suitable to be used in brewing.

Additional studies are needed to further validate these findings. These selected yeasts will be employed on a pilot scale in the production of lager beers at temperatures below 15 degrees to evaluate some fermentation dynamics in natural beer wort with the

addition of hops and verify their influence on the chemical and sensory characteristics of the beers produced, especially with reference to volatile compounds (higher alcohols, terpenes, esters).

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/pr9091628/s1, Table S1: Flocculation capacity (%) of *S. cerevisiae* CSB21B, CS1C, CS2C and 41CM and *S. pastorianus* W-34/70 strains after 10 min and 30 min at 15 °C. Table S2: Alcohol production during fermentation in MBW by *S. cerevisiae* CSB21B, CS1C, CS2C, 41CM and S. pastorianus W-34/70. Table S3: Maltose values during fermentation in MBW inoculated with *S. cerevisiae* CSB21B, CS1C, CS2C, 41CM and *S. pastorianus* W-34/70. Table S4: Glucose values during fermentation in MBW inoculated with *S. cerevisiae* CSB21B, CS1C, CS2C, 41CM and *S. pastorianus* W-34/70. Table S5: pH values during alcoholic fermentation in MBW inoculated with *S. cerevisiae* CSB21B, CS1C, CS2C, 41CM and *S. pastorianus* W-34/70.

Author Contributions: Conceptualization, M.I. and B.T.; methodology, B.T., F.L. and F.C.; software, F.L.; validation, A.G. and M.F.; formal analysis, B.T. and F.L.; investigation, M.I. and R.A.; resources, R.C.; data curation, G.A.; writing—original draft preparation, M.I. and B.T.; writing—review and editing, M.I., M.F., B.T. and F.L.; supervision, R.C.; funding acquisition, R.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was carried out in the context of the scientific project ADAPT: influence of agro-climatic condition conditions on the microbiome and genetic expression of grapevines for the production of red wines: a multidisciplinary approach. PRIN: Research Projects of National Interest; funded by the Italian Ministry for Universities and Research (MIUR), grant number 2017M83XFJ-(CUP H34I19000590001).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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