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Industrial-Level Brewing Using Oenological *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* as Mixed-Inoculum

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Abstract: The development of new food processes and formulations begins at the laboratory stage, progresses through pilot plant trials, and culminates in industrial production. Although the positive effects in terms of sensory characteristics and qualitative differentiation have been widely studied at laboratory level, fermentations conducted at the industrial level by oenological *Saccharomyces cerevisiae* and non-*Saccharomyces* strains have not been thoroughly investigated. Scaling up to the industrial level is a critical process that involves more than simply increasing the dimensions of the process itself. The purpose of our research was to compare laboratory and industrial-level brewing of a novel craft beer produced with the addition of common unmalted wheat and fermented by *Schizosaccharomyces pombe* and *S. cerevisiae* strains. Fermentation was carried out using *a S. cerevisiae* strain either of oenological origin alone or through sequential inoculations with *S. pombe*. Beers produced with the mixed starter showed greater reproducibility between the two production levels than those fermented by *S. cerevisiae* alone. According to the results, the main differences highlighted between laboratory and industrial-level trials with *S. cerevisiae* alone concerned the extent of starch degradation, fermentation efficiency, and alcohol production, which were higher in brewing at the laboratory level. In contrast, beers produced at industrial level using sequential inoculation received significantly higher scores for foam quantity and persistence, as well as overall olfactory intensity, while scoring significantly lower scores for saltiness and sourness. To our knowledge, this research is the first to explore the use of *Sc. pombe* for industrial beer production.

Keywords: industrial brewing; *Saccharomyces cerevisiae*; *Schizosaccharomyces pombe*; volatile compounds; white beer

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1. Introduction

Food product development is a complex and iterative process that starts with a concept and ends with the production of large amounts for the market [1]. This complex process is known by the term "level-up", which refers, but is not limited to, any increase in the process dimension according to a fixed ratio [2]. Anyone who is preparing a food production process at the industrial level knows that the transition between proof of concept and application is not an easy undertaking. This is because ingredients may behave differently from a level to another and equipment differs not only for dimension but also for technological solutions applied [3]. Formulations made of a few ingredients and processes that consist of just a few steps are easier to scale. In addition, when a food process involves the use of living microorganisms, as for fermented beverages, the hurdles to a successful transition to the industrial level are even greater. Since the microorganisms

usually interact with the environment, their modifications (including a change in scale) could cause unexpected and unpredictable metabolic alterations that result in product changes [2]. As a result, appearance, aroma, taste, and texture of industrial product may significantly differ from the prototype, although such differences are not necessarily negative. In an activity at the industrial level, it is also possible to modify the starting formulation and processing step so that the scaled-up products result more closely to the original ones [3]. Regarding the brewing process, quality and availability of laboratory-level brewing plants have improved considerably as a response to the growing diffusion of home and hobby brewing [4]. This implies that this level, product safety, and quality do not suffer from issues arising from the equipment used. However, challenges in scaling up from laboratory to industrial-level equipment derive from their different performances. When the fixed cereal-water ratio is scaled, the greater extraction efficiency of production-level mash causes the extraction of a wort with a higher sugar concentration, a stronger malty flavor, and a darker color than the wort produced at a smaller level. The same issue concerns the boiling kettle, whose greater extraction efficiency increases the percentage of α-acids converted to their isomerized forms compared to what happen in smaller equipment [4].

The scaling up of a brewing process presents unique challenges compared to general fermentation industries, primarily due to the delicate nature of the brewing process. One significant issue is maintaining the consistency and quality of beer flavors when transitioning from laboratory level to industrial production. Several factors like temperature control, yeast management, and oxygenation are difficult to regulate in larger fermenters, leading to possible modification of the final product. Additionally, large-level beer fermentation is more prone to contamination, as beer is a relatively low-alcohol product and susceptible to spoilage by unwanted microorganisms. Controlling microbial contamination in large batches is challenging, requiring stringent sterilization and monitoring protocols. The introduction of advanced bioreactors and real-time data analysis is helping address these problems, improving both consistency and safety [5].

Research advancements are focusing on optimizing yeast strains to improve their robustness during large-level production. For instance, hybrid models combining mechanistic and data-driven approaches are being developed to predict performance and optimize fermentation parameters across different scales. Computational fluid dynamics (CFD) tools are also being employed to predict how changes in reactor design might affect beer quality, addressing concerns related to temperature and oxygen gradients [6].

The analysis of the literature relating to the industrial brewery production highlights that the following issues have been addressed so far, in particular, the optimization of beer fermentation by encapsulated yeasts [7]; the fate of vitamins such as pyridoxine and folates [8]; gluten-reduction ability in gluten-free brewing [9]; the dry hopping effects [10]; the high gravity brewing [11]; the effects of stirring on fermentation efficiency [12]; the environmental impact [13]; and the economies of level [14]. "Cross-over" applications in the field of food microbiology represent an approach of using a microorganism isolated/used as a starter in a specific traditional fermentation process in new product development and/or to improve quality and safety in another supply chain or other food production [15]. Indeed, *Saccharomyces cerevisiae* and non-*Saccharomyces* starter strains of oenological origin demonstrated that they could brew high-quality novel industrial beer [16,17]. Recent investigations into using *Schizosaccharomyces pombe* for beer production have reaffirmed the challenges previously identified, highlighting the inherent difficulties of employing *Sc. pombe* in fermentation processes [18].

However, the use of *Sc. pombe* in brewing beer has been rarely explored, likely due to its unfavorable aftertaste and certain unique physiological characteristics [19]. To address this gap, the present study aimed to compare laboratory- and industrial-level brewing of a novel industrial beer produced from malted barley and unmalted common wheat and fermented by a mixed starter consisting of a *Sc. pombe* and *S. cerevisiae* of oenological origin. To the best of our knowledge, this investigation is the first one concerning the application of *Sc. pombe* for the industrial brewing of an industrial beer

2. Materials and Methods

2.1. Materials and Equipment

Laboratory-level (LSC) and industrial (CSC) brewing trials were compared. The laboratory-level trials were performed at the University of Foggia in a 20-L all-in-one Braumeister plant (Speidel Tank-und Behälterbau GmbH, Ofterdingen, Germany, Available online: https://www.speidels-braumeister.de/en/braumeister/10-20-50-litre-braumeister.html [accessed on 21 August 2024]). The industrial brewing trials were carried out at Officine Birrai, a brewpub located in Lecce, Italy, using a 10 HL semi-automatic plant with pneumatic valves, PLC management, two twin vats for mashing and boiling-whirlpool, a filter vat, tube-in-tube exchanger, and steam heating (Multi-Brew, Simatec, Vaie, Italy). Both the laboratory-level and industrial brewing trials used a cereal mixture made of 65% Pilsner barley malt (Agroalimentare Sud, Melfi, Italy) and 35% unmalted common wheat cv. Risciola (Soc. Cooperativa Agricola Valleverde, Bovino, Italy). Two yeast strains were used: *S. cerevisiae* ITEM9502, hereinafter referred to as 9502, isolated from Susumaniello grape [20], and the oenological *Sc. pombe* strain ITEM 6956, hereinafter referred to as G18, selected for its ability to metabolize maltose and maltotriose. The two oenological strains belong to the ITEM Microbial Culture Collection of CNR-ISPA (http://www.ispacnr.it/collezioni-microbiche [accessed on 21 August 2024]). The strains were grown on YPD medium (1% [*w*/*v*] yeast extract, 2% [*w*/*v*] peptone; 2% [*w*/*v*] glucose; 2% [*w*/*v*] agar [Oxoid, Hampshire, UK]) and maintained at 4 °C for further analysis. Liquid cultures were grown in liquid YPD at 26 °C for 48 h.

2.2. Brewing Procedures

2.2.1. LSC Trials

The beer formulation included in LSC trials (Figure S1) included 30 L water; 8.25 kg cereal mixture; 15 g dried hop cones cv. Cascade (6.7% α-acid content); 7.5 g bitter orange peels; and 3.75 g coriander (all the flavoring agents supplied by Birramia, Querceta, Italy).

The cereals were coarsely ground with a two-roller mill and added to the mashing water (conductivity 390 ± 10 µS/cm,) previously heated at 52 °C. The mashing process followed these steps: protein rest (55 °C; 10 min); β-amylase rest (63 °C; 45 min); α-amylase rest (70 °C; 45 min); and mash-off (78 °C; 15 min). During mashing, the wort pH was adjusted by addition of lactic acid (80% *v*/*v*). The wort was then boiled for 65 min. The hop was added to the wort 5 and 50 min (half and half) after the boil started. Coriander and bitter orange peels were added 60 min after the boil started. At the end of boiling, the wort was cooled at room temperature and submitted to whirlpool. The final pH was 5.4 ± 0.2.

Thirty liters of wort (original gravity 1.048 ± 0.001) were inoculated with *S. cerevisiae* 9502 (~1×10^7 cells/mL) and fermented at 20.0 ± 0.5 °C for 21 ± 1 days until a final gravity of 1.010 ± 0.002 was reached. The resulting beers were hereafter referred to as LSC-9502.

Another 30 liters of the same wort were submitted to sequential inoculation as follows: the wort was first inoculated with *Sc. pombe* G18 (~1×10^7 cells/mL), and after 48 h-fermentation (intermediate gravity 1.030 ± 0.003), it was inoculated with *S. cerevisiae* 9502 (~1×10^7 cells/mL). Fermentations were carried out at 20 ± 1 °C for 21 ± 1 days until a final gravity value of 1.010 ± 0.002 was reached. The resulting beer was hereafter referred to as LSC-G18-9502. At that point, the temperature was lowered to 4 °C for two days to facilitate clarification, and after racking, LSC-9502 and LSC-G18-9502 beers were packaged into 500 mL glass brown bottles.

2.2.2. CSC Trials

The CSC trials (Figure S2) were performed with the ingredients in the same proportions described for the LSC tests but on a larger scale. The following amounts of ingredients were used: 10 HL water; 275 kg cereal mixture; 500 g hop pellets cv. Cascade; 500 g bitter orange peels; and 250 g coriander. The coarsely ground cereals were added to the mashing water (conductivity of 350 ± 10 µS/cm) previously heated at 52 °C. The mashing steps were as follows: protein rest (55 °C; 12 min); β-amylase rest (64 °C; 40 min); α-amylase rest

(72 °C; 20 min); and mash-off (78 °C; 2 min). During mashing, the wort pH was adjusted by the addition of lactic acid (80% *v*/*v*). The wort was then boiled for 75 min. Hops were added to the wort 15 and 60 min (half and half) after the boil started, while coriander and bitter orange peels were added 5 min before the end of boiling. After boiling, the wort was cooled at room temperature and submitted to whirlpool. The final pH value was 5.5 ± 0.2.

The wort was then inoculated with *S. cerevisiae* 9502 alone (CSC-9502) or in sequential inoculation with *Sc. pombe* G18 (CSC-G18-9502) with the same microbial concentrations used for LSC trials. The fermentation step was carried out at 20 ± 0.5 °C for 10 days. Successively, the temperature was lowered to 10 ± 1 °C until the beer was transferred into tanks. Finally, the beers were packed into 24 L-polykegs with bags (PolyKeg®, Grumello del Monte, Italy). Bottles and polykegs were stored at 20 ± 2 °C for about 30 days and then kept at 4 °C until analyses. The adjustments to the formulation and process steps were intended to mitigate the different performances between the equipment used in laboratory production and that used in industrial production.

2.3. Chemical Analyses of Beer

The alcohol content (*v*/*v* %) was determined as previously described [21]. The organic acids, glycerol, and sugars (maltodextrin, sucrose, maltose, maltotriose, glucose, and fructose) were analyzed by an 1100 HPLC-DAD system (Agilent, Santa Clara, CA, USA) through a 300 mm × 7.7 mm × 8 µm Hi-Plex H column (Agilent Technologies, Santa Clara, CA, USA) [22,23]. The extraction of volatile compounds was carried out according to the method described by Palombi and coworkers by using a micro-extraction solid phase (SPME) technique [24]. VOCs were firstly identified based on their mass spectra using NIST14 library and secondly with the retention data of commercially available standards and MS data reported in the literature. Data were expressed as Relative Peak Area (RAP) (area peak compound/area peak internal standard) × 100 [25]. Analyses were performed in duplicate.

2.4. Sensory Analysis

A panel of 13 trained panelists, aged between 18 and 70 years, analyzed the experimental beers. A quantitative descriptive analysis (QDA) was performed as described by Baiano et al. [21] with some modifications. The panelists evaluated and assigned a score to five visual (color, amount, persistence of foam, color, and turbidity of the liquid fraction), eight olfactory (overall olfactory intensity [OOI], olfactory finesse [OF], malty, hoppy, floral, fruity, spicy, and yeast), four gustatory (sweetness, bitterness, saltiness, and sourness), and three tactile (alcoholic, effervescence, and body/fullness) characteristics and to the overall sensory quality (OSQ). Color was evaluated on the following four-point scales: 1 (white), 2 (rose), 3 (cream), and 4 (capuchin) for foam; 1 (pale straw yellow), 2 (straw yellow), 3 (golden yellow), and 4 (amber) for the liquid fraction. A 0–5 level was used. Concerning foam, 0 corresponded to white, while 5 indicated a capuchin color. For the liquid fraction, 0 corresponded to a pale straw yellow and 5 to the amber color.

2.5. Statistical Analysis

Averages and standard deviations were calculated on six replicates. (The beers obtained from the two replicates of the brewing process were submitted to three analytical replicates, at least.) A one-way ANOVA followed by LSD test ($p < 0.05$) was applied to evaluate each analytical parameter, the statistical significance of the differences between laboratory and industrial brewing. The principal component analysis (PCA) and the linear regression analysis were also applied to verify relationships among beer samples and parameters. The statistical analyses were performed through Statistica for Windows V. 7.0. (Statsoft, Tulsa, OK, USA).

3. Results and Discussion

3.1. Chemical Characterization of Beers

The alcohol content, organic acid, and sugar profiles of the beers are shown in Table 1, which also details the differences between lab- and industrial-level production for each formulation. In the pairwise comparison of beers fermented by *S. cerevisiae* 9502, it is evident that the alcohol content was slightly higher in laboratory beers, with values comparable to the typical characteristics of a standard white beer (4.5–5.5%), as stated by the BJCP style guidelines [26]. Significant effects of brewing dimensions were also detected on the organic acid metabolism of the yeast. Succinic and acetic acids affect the beer taste and are responsible for beer saltiness/bitterness and vinegar flavor, respectively [27,28]. However, succinic acid can also positively affect sensory properties due to its contribution to forming fruity esters, such as methyl-, ethyl-, and diethyl succinate [29]. The higher concentration of succinic acid in lab beers was due to both aeration and the maintenance of a fermentation temperature of approximately 20 °C for a longer time than in an industrial process. Succinic acid production is known to increase with temperature and during aerobic fermentation [30–32]. The higher acetic acid content at the lab level could be the result of either acetaldehyde oxidation or oxidative decarboxylation of pyruvic acid. Concerning the beer sugar profiles, maltodextrins are non-fermentable carbohydrates produced during mashing through starch hydrolysis. The interest in their amounts in beer depends on their influence on the organoleptic properties of the beer [33] since they are responsible for palate fullness.

The pairwise comparison of beers produced with common wheat and fermented by *S. cerevisiae* 9502 substantially confirmed the differences between laboratory and industrial levels, as previously observed for common wheat beers, with a few clarifications. First, it should be underlined the gap in alcohol content between the lab and industrial beers was greater, favoring the former. This is consistent with the low maltodextrin content of the LSC-9502 beers and can be explained by the significant differences in starch-degrading enzymes among the common old wheat varieties, as reported by Alfeo et al. [34]. Additionally, fructose and glycerol concentrations were higher in laboratory-produced beers.

Compared to the common wheat-based beers fermented by *S. cerevisiae* 9502, fewer differences were observed between laboratory and industrial scale for common wheat-based beers submitted to sequential fermentation. Indeed, LSC-G18-9502 and CSC-G18-9502 exhibited similar concentrations of citric, lactic, and acetic acids, as well as maltose. Moreover, maltotriose contents were very low in both LSC and CSC beers, due to *Sc. pombe* ability to metabolize this sugar [35].

The dataset, including all chemical parameters, was analyzed using PCA (Figure 1). The two first factors explained over 75% of the total variance. A clear separation can be observed between LSC and CSC beers. Beers produced at the laboratory level were located in the section of the plane characterized by negative values of Factor 1, while those produced at the industrial level were placed in the half of the plane identified by positive values of Factor 1. Within each type of beer, another point of interest concerned the overall extent of the differences between the two production scales, which could be measured based on their relative distance in the factorial plane.

The shortest distance between LSC and CSC beers obtained by sequential fermentation of *Sc. pombe/S. cerevisiae* represented an index of the greater reproducibility of their production process compared to those of the beers fermented by *S. cerevisiae* alone (Figure 1A), suggesting that the interactions between the two yeast strains were more influential than the differences caused by the production scale. The comparison of Figure 1A,B highlighted the peculiar characteristics of each beer. In fact, LSC-9502 had the highest alcohol percentage and the highest amounts of lactic acid, LSC-G18-9502 showed the highest glycerol content, and CSC-9502 and CSC-G18-9502 had no characterizing compounds. The analysis of the volatile compound dataset (Figure 2A) further confirmed the scalability of the brewing process involving the sequential fermentation of *Sc. pombe/S. cerevisiae.*

Table 1. Alcohol content, organic acid, and sugar composition of the beers. Comparison between laboratory and industrial level. LSC: laboratory-scale; CSC: industrial-scale; 9502: *S. cerevisiae* strain *Sc. pombe* strain; nd: not detected. Different letters indicate significant differences at $p < 0.05$ by LSD multiple-range test; s: significant; ns: not significant.

Beers	Alcohol Content (%)	Organic Acids (g/L)					Sugars (g/L)					
		Citric	Malic	Succinic	Lactic	Acetic	Maltodextrin	Maltotriose	Maltose	Glucose	Fructose	Glycerol
LSC-9502	6.23 ± 0.04^{b}	0.75 ± 0.03^{a}	0.81 ± 0.09^{a}	4.91 ± 0.15^{b}	1.14 ± 0.04^{b}	2.43 ± 0.17^{b}	10.60 ± 1.69^{a}	2.91 ± 0.14^{a}	2.32 ± 0.08^{a}	3.53 ± 0.21^{b}	0.83 ± 0.09^{b}	3.39 ± 0.21^{b}
CSC-9502	4.36 ± 0.25^{a}	1.15 ± 0.15^{b}	2.03 ± 0.15^{b}	1.13 ± 0.07^{a}	0.49 ± 0.04^{a}	1.28 ± 0.12^{a}	29.64 ± 2.30^{b}	20.42 ± 1.68^{b}	2.97 ± 0.23^{b}	nd ^{a}	0.41 ± 0.05^{a}	1.76 ± 0.15^{a}
Significance	s	s	s	s	s	s	s	s	s	s	s	s
LSC-G18-9502	5.25 ± 0.05^{b}	1.34 ± 0.21^{a}	1.01 ± 0.08^{a}	1.61 ± 0.09^{b}	0.65 ± 007^{a}	1.49 ± 0.17^{a}	13.5 ± 0.14^{a}	0.54 ± 0.10^{b}	0.90 ± 0.09^{a}	0.32 ± 0.06^{a}	1.00 ± 0.09^{b}	4.34 ± 0.33^{b}
CSC-G18-9502	4.52 ± 0.29^{a}	1.24 ± 0.11^{a}	2.57 ± 0.20^{b}	0.93 ± 0.10^{a}	0.49 ± 0.05^{a}	1.18 ± 0.11^{a}	23.59 ± 1.5^{b}	nd ^{a}	0.94 ± 0.07^{a}	0.54 ± 0.02^{b}	0.38 ± 0.03^{a}	2.85 ± 0.09^{a}
Significance	s	ns	s	s	ns	ns	s	s	ns	s	s	s

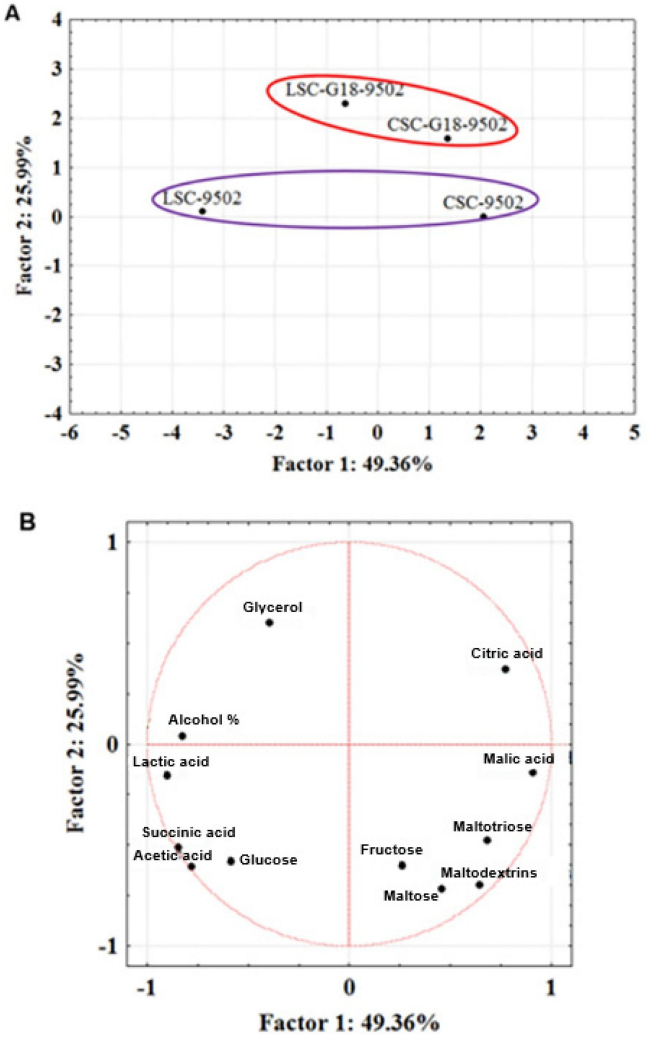

Figure 1. Biplot of scores (**A**) and loadings (**B**) obtained with the application of PCA to alcohol, organic acid, and sugar contents along the first and second component.

3.2. Volatile Organic Compounds

Beer is a beverage rich in alcohols and esters, which are formed during alcoholic fermentation [36]. Higher alcohols, considered the most abundant aromatic compounds in beer, are produced from amino acids through the Ehrlich route or from carbohydrate metabolism [37]. Esters are formed by yeast via an enzyme-catalyzed reaction between acyl-CoA and higher alcohols. These compounds play a crucial role in the production of various beverages, such as wine, whiskey, and beer, due to their low perception threshold. Table 2 presented the normalized area of volatile compounds produced by the selected *S. cerevisiae* strain, both single and co-inoculum fermentation with *Sc. pombe* G18, under different experimental conditions. The volatile profiles of single fermentations showed significant differences, depending on the type of cereal used, compared to those with mixed-starter fermentations. Laboratory-scale fermentation using single inoculation was characterized by higher values across all volatile compound classes. The esters class was the most represented, with values varying from 3.78 (LSC-95029) to 0.82 (LSC-G18-9502) mg/L. Among the volatile compounds, esters were the most represented class in all beers, followed by alcohols. Among the various compounds, esters and alcohols play a crucial role in the organoleptic characteristics of alcoholic beverages [38]. Specifically, esters, such as isoamyl acetate, ethyl hexanoate, and ethyl octanoate (associated with banana, sweet, and sour apple notes, respectively), positively contributed to the aroma with fruity notes due to their low perception threshold [39–41].

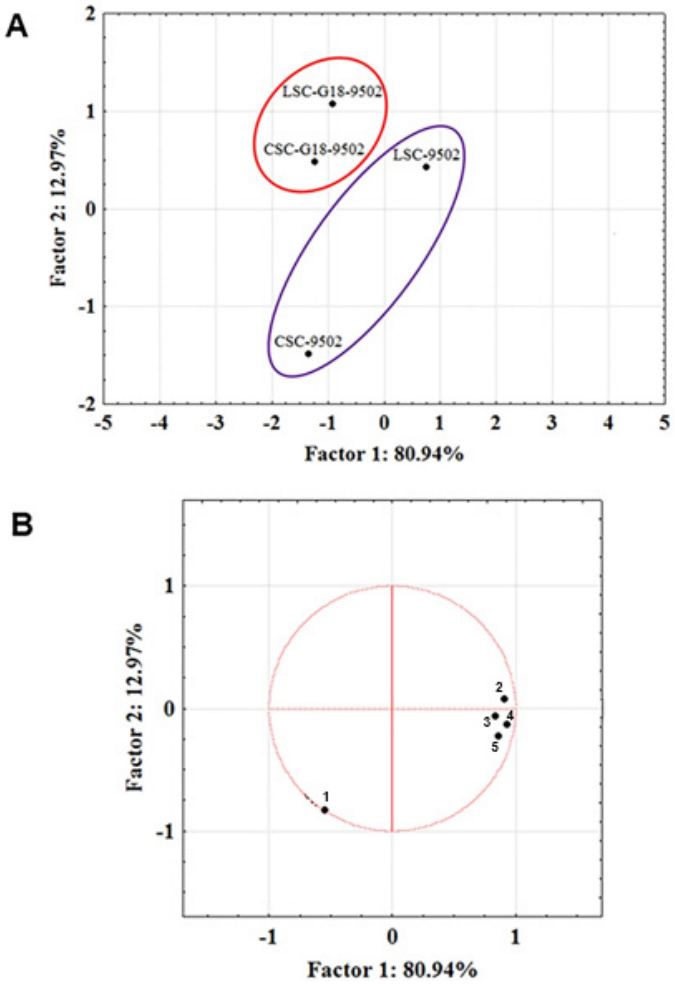

Figure 2. Biplot of scores (**A**) and loadings (**B**) obtained with the application of PCA to the various classes of volatile compounds along the first and second component. 1, total hydrocarbons; 2, total alcohols; 3. Total terpenes/norisoprenoids; 4, total aldehydes/ketons; 5, total esters.

The total higher alcohol content ranged from 0.67 (LSC-9502) to 0.12 mg/L (CSC-G18-9502). When present in amounts below 300 mg/L, higher alcohols impart floral, pleasant, and refreshing notes and contribute a desirable warming character that adds complexity to the beer. However, higher concentrations result in a burning sensation and an alcohol or solvent aroma [42]. Based on the pairwise comparisons, the LSC samples had significantly higher contents of alcohols, while the sole hydrocarbon detected, i.e., styrene, was found only in CSC beers and in very low concentrations, regardless of the product formulation. Styrene can be found in many commercial wheat beers because of both thermal decarboxylation of cinnamic acid during the boiling step and enzymatic decarboxylation during fermentation. The absence of styrene from LSC beers could be explained by their higher aeration that facilitated the evaporation of this aromatic compound [43]. The eerpenes, norispoprenoids, and aldehydes/ketons were highly concentrated in LSC beers except for beers produced by sequential fermentation, where concentrations were similar in laboratory and industrial beers. The organic acids were highly concentrated in LSC beers, except for industrial beers produced by sequential fermentation. Regarding total volatile concentration, the beer samples were ranked in the following decreasing order: LSC-9502, CSC-9502, LSC-G18-9502, and CSC-G18-9502. To visualize the differences in volatile compounds depending on the different experimental conditions tested (raw materials, fermentation conditions, and type of production level), a PCA was carried out using the mean of the repetitions.

Table 2. Volatile compounds (mg/L) of beers: comparison between laboratory and industrial level. LSC: laboratory-scale; CSC: industrial-scale; 9502: *S. cerevisiae* strain; G18: *Sc. pombe* strains; nd: not detected. In line for each type of beer. Different letters indicate significant differences at $p < 0.05$ by LSD multiple range test; s: significant; ns: not significant.

Compounds	*Sign*	LSC-9502		CSC-9502		*Sign.*	LSC-G18-9502		CSC-G18-9502		*Sign*
		Mean	SD	Mean	SD		Mean	SD	Mean	SD	
ESTERS											
Ethyl Acetate	**s**	0.22 ^{b}	0.06	0.02 ^{a}	0	**s**	0.05 ^{a}	0.01	0.01 ^{a}	0.01	**ns**
Isoamyl Acetate	**s**	0.09 ^{a}	0.04	0.06 ^{a}	0.01	**ns**	0.02 ^{a}	0	0.02 ^{a}	0	**ns**
Ethyl Hexanoate	**s**	1.36 ^{b}	0.18	0.10 ^{a}	0.02	**s**	0.06 ^{a}	0.01	0.21 ^{b}	0.02	**s**
Ethyl Heptanoate	**ns**	0.01 ^{a}	0	0.01 ^{a}	0	**ns**	nd ^{a}		nd ^{a}		**ns**
Methyl Octanoate	**s**	0.01 ^{a}	0	0.01 ^{a}	0	**ns**	nd ^{a}		nd ^{a}		**ns**
Ethyl Octanoate	**ns**	0.58 ^{a}	0.31	0.98 ^{a}	0.34	**ns**	0.23 ^{a}	0.03	0.51 ^{a}	0.20	**ns**
Methyl Decanoate	**ns**	0.05 ^{a}	0	0.03 ^{a}	0.01	**ns**	nd ^{a}		nd ^{a}		**ns**
Ethyl Decanoate	**ns**	0.02 ^{a}	0	0.77 ^{b}	0.31	**s**	0.28 ^{b}	0.05	0.07 ^{a}	0.03	**s**
Diethyl Succinate	**s**	0.97 ^{b}	0.18	nd ^{a}		**s**	0.07 ^{b}	0.01	nd ^{a}		**s**
Ethyl-9-Decenoate	**ns**	0.07 ^{a}	0.06	0.01 ^{a}	0	**ns**	0.04 ^{a}	0.03	nd ^{a}		**ns**
Phenyl Acetate	**s**	0.09 ^{a}	0.06	0.07 ^{a}	0.02	**ns**	0.01 ^{a}	0.00	0.03 ^{a}	0.02	**ns**
Ethyl Dodecanoate	**s**	0.32 ^{b}	0.01	0.07 ^{a}	0	**s**	nd ^{a}		0.01 ^{a}	0	**ns**
Hexyl Acetate	**s**	nd ^{a}		nd ^{a}		**ns**	nd ^{a}		nd ^{a}		**ns**
Ethyl Lactate	**ns**	nd ^{a}		nd ^{a}		**ns**	0.06 ^{b}	0	nd ^{a}		**s**
TOTAL	***s***	***3.78 ^{a}***	***0.43***	***2.12 ^{a}***	***0.68***	***ns***	***0.82 ^{a}***	***0.06***	***0.87 ^{a}***	***0.28***	***ns***
ALCOHOLS											
2-Methyl-1-Propanol	**ns**	0.04 ^{a}	0.02	0.01 ^{a}	0	**ns**	0.02 ^{b}	0	nd ^{a}		**s**
1-Propanol	**ns**	nd ^{a}		nd ^{a}		**ns**	0.01 ^{b}	0	nd ^{a}		**s**
2 + 3 Methyl-1-Butanol	**s**	0.57 ^{b}	0.07	nd ^{a}		**s**	0.47 ^{b}	0.06	nd ^{a}		**s**
1-Hexanol	**ns**	nd ^{a}		nd ^{a}		**ns**	0.02 ^{b}	0	nd ^{a}		**s**
2-Ethyl-1-Hexanol	**ns**	nd ^{a}		nd ^{a}		**ns**	nd ^{a}		nd ^{a}		**ns**
Phenyl Ethanol	**s**	0.06 ^{a}	0.01	0.08 ^{a}	0.02	**ns**	0.05 ^{a}	0	0.06 ^{b}	0	**s**
1-Butanol	**s**	nd ^{a}		nd ^{a}		**ns**	nd ^{a}		nd ^{a}		**ns**
3-Methyl-1-Butanol	**ns**	nd ^{a}		0.19 ^{b}	0.02	**s**	nd ^{a}		0.05 ^{b}	0.02	**s**
TOTAL	***s***	***0.67 ^{b}***	***0.04***	***0.28 ^{a}***	***0.05***	***s***	***0.58 ^{b}***	***0.08***	***0.12 ^{a}***	***0.01***	***s***
TERPENES/NORISOPRENOIDS											
β-Mircene	**s**	nd ^{a}		nd ^{a}		**ns**	nd ^{a}		nd ^{a}		**ns**
D-Limonene	**ns**	0.01 ^{b}	0	nd ^{a}		**s**	0.02 ^{b}	0	nd ^{a}		**s**
2-Bornanone	**s**	0.05 ^{a}	0.02	nd ^{a}		**ns**	nd ^{a}		nd ^{a}		**ns**
Terpinen-4-Ol	**s**	0.04 ^{b}	0	0.01 ^{a}	0	**s**	nd ^{a}		nd ^{a}		**ns**
α-Terpineol	**s**	0.47 ^{b}	0.02	nd ^{a}		**s**	nd ^{a}		nd ^{a}		**ns**
Citronellol	**s**	0.16 ^{b}	0.03	nd ^{a}		**s**	nd ^{a}		nd ^{a}		**ns**
β-Damascenone	**s**	0.09 ^{b}	0	nd ^{a}		**s**	nd ^{a}		nd ^{a}		**ns**
p-Cymene	**ns**	nd ^{a}		nd ^{a}		**ns**	0.03 ^{b}	0	nd ^{a}		**s**
Linalool	**ns**	nd ^{a}		nd ^{a}		**ns**	nd ^{a}		0.13 ^{a}	0.06	**ns**
TOTAL	***s***	***0.81 ^{b}***	***0.03***	***0.02 ^{a}***	***0***	***s***	***0.05 ^{a}***	***0.01***	***0.15 ^{a}***	***0.06***	***ns***
ALDEHYDES/KETONS											
2-Octanone	**s**	0.03 ^{a}	0.01	0.01 ^{a}	0	**ns**	0.01 ^{a}	0	0.01 ^{a}	0	**ns**
Nonanal	**s**	0.03 ^{b}	0.01	nd ^{a}		**s**	nd ^{a}		0.01 ^{a}	0	**ns**
Furfural	**ns**	nd ^{a}		nd ^{a}		**ns**	0.01 ^{b}	0	nd ^{a}		**s**
Benzaldehyde	**ns**	0.01 ^{a}	0	0.01 ^{a}	0	**ns**	nd ^{a}		0.01 ^{a}	0	**ns**
TOTAL	***s***	***0.07 ^{b}***	***0***	***0.02 ^{a}***	***0***	***s***	***0.02 ^{a}***	***0.01***	***0.03 ^{a}***	***0***	***ns***

Table 2. *Cont.*

Compounds	*Sign*	LSC-9502		CSC-9502		*Sign.*	LSC-G18-9502		CSC-G18-9502		*Sign*
		Mean	SD	Mean	SD		Mean	SD	Mean	SD	
HYDROCARBONS											
Styrene	**s**	nd ^{a}		0.04 ^{b}	0	**s**	nd ^{a}		0.01 ^{b}	0	**s**
TOTAL	***s***	***nd*** ^{a}		***0.04*** ^{b}	***0***	***s***	***nd*** ^{a}		***0.01*** ^{b}	***0***	***s***
ACIDS											
Acetic Acid	**s**	0.04 ^{b}	0.01	nd ^{a}		**s**	nd ^{a}		nd ^{a}		**ns**
Hexanoic Acid	**s**	nd ^{a}		nd ^{a}		**ns**	nd ^{a}		0.02 ^{b}	0	**s**
Octanoic Acid	**s**	0.04 ^{a}	0	0.10 ^{a}	0.02	**ns**	nd ^{a}		0.06 ^{a}	0.02	**ns**
Nonanoic Acid	**s**	0.73 ^{b}	0.25	nd ^{a}		**s**	nd ^{a}		nd ^{a}		**ns**
n-Decanoic Acid	**ns**	0.33 ^{b}	0.02	nd ^{a}		**s**	nd ^{a}		nd ^{a}		**ns**
TOTAL ACIDS	***s***	***1.13*** ^{b}	***0.24***	***0.10*** ^{a}	***0.02***	***s***	***nd*** ^{a}		***0.07*** ^{b}	***0.02***	***s***
TOTAL VOLATILE COMPOUNDS		***6.46***		***2.58***			***1.47***		***1.25***		

According to this analysis (Figure 2), Factors 1 and 2 explained nearly 94% of the total variance. The comparative analysis of Figure 2A,B indicated that the volatile profile of CSC-9502 beers was characterized by the highest hydrocarbon content. The other beer samples exhibited profiles not dominated by specific classes of compounds.

The volatile concentration in the beer samples was detected in the following decreasing order: LSC-9502, CSC-9502, LSC-G18-9502, and CSC-G18-9502.

3.3. Sensory Evaluation of Beers Fermented by Sequential Inoculation

The brewing process involving sequential fermentation demonstrated the highest scalability in terms of stability of the chemical properties between laboratory and industrial scales. Thus, LSC-G18-9502 and CSC-G18-9502 beers were compared from a sensory point of view (Table 3). The LSC and CSC beers showed similar colors of foam and liquid portion, which were respectively evaluated as white and golden yellow. The CSC beers obtained significantly higher scores for quantity and persistence of the foam, probably because of the fermentation stage at 10 ± 1 °C, which increased the solubilization of carbon dioxide in the liquid fraction. From an olfactory perspective, the industrial beers highlighted a significantly higher overall OI, despite having slightly lower concentrations of volatile compounds (1.25 mg/L) in respect to LSC beers (1.47 mg/L).

This result could be explained by the higher concentration in CSC beers of compounds having low threshold values, such as ethyl octanoate and linalool [44,45]. The absence of significant differences in the intensities of the various types of flavor is likely because the two samples differ primarily in the concentrations of alcohols and acids, with both compounds having very high perception thresholds. The highest scores for saltiness and sourness were assigned to LSC-G18-9502 samples, consistent with their higher succinic acid contents. The different alcohol content between the two beers was not perceived on a sensorial level. The similar fullness-observed LSC and CSC beers were probably due to the concentrations of compounds that contribute to these characteristics, namely maltodextrins and glycerol, which are respectively higher in CSC-G18-9502 and LSC-G18-9502. In summary, significant differences between the two scales were found for only 5 of the 21 sensory characteristics considered in the QDA analysis, thus confirming the reproducibility of the production involving the sequential fermentation of *Sc. pombe* and *S. cerevisiae*.

Table 3. Sensory analysis of beers: comparison between laboratory and industrial level. LSC: laboratory-scale; CSC: industrial-scale; 9502: *S. cerevisiae* strain; G18: *Sc. pombe* strain. Different letters indicate significant differences between LS and CS at $p < 0.05$ by LSD multiple-range test; s: significant; ns: not significant; OFI: overall olfactory intensity; OF: olfactory finesse; OSQ: overall sensory quality.

Beers	Colour		Foam		Turbidity	Flavor								Gustatory Characteristics				Tactile Characteristics			OSQ
	Foam	Liquid	Amount	Persistence		OOI	OF	Malty	Hoppy	Floral	Fruity	Spicy	Yeast	Sweetness	Bitterness	Saltiness	Sourness	Alcoholic	Effervescence	Body/Fullness	
LSC-G18-9502	$1.0 \pm 0_{a}$	$2.7 \pm 0.5_{a}$	$2.3 \pm 0.5_{a}$	$2.2 \pm 0.4_{a}$	$3.7 \pm 0.5_{a}$	$2.5 \pm 0.5_{a}$	$3.8 \pm 0.8_{a}$	$2.8 \pm 0.4_{a}$	$2.8 \pm 0.4_{a}$	$2.2 \pm 0.4_{a}$	$2.5 \pm 0.5_{a}$	$2.7 \pm 0.5_{a}$	$2.5 \pm 0.5_{a}$	$2.2 \pm 0.4_{a}$	$3.2 \pm 0.8_{a}$	$2.7 \pm 1.0_{b}$	$3.2 \pm 0.4_{b}$	$2.8 \pm 0.8_{a}$	$3.0 \pm 0.8_{a}$	$3.0 \pm 0.9_{a}$	$3.0 \pm 0.8_{a}$
CSC-G18-9502	$1.0 \pm 0_{a}$	$2.8 \pm 1.4_{a}$	$3.4 \pm 0.8_{b}$	$3.5 \pm 1.0_{b}$	$3.4 \pm 1.1_{a}$	$3.4 \pm 1.0_{b}$	$3.8 \pm 0.9_{a}$	$3.2 \pm 1.0_{a}$	$2.6 \pm 1.4_{a}$	$2.7 \pm 1.4_{a}$	$3.1 \pm 1.5_{a}$	$2.2 \pm 1.5_{a}$	$2.3 \pm 1.5_{a}$	$2.8 \pm 0.6_{a}$	$2.2 \pm 0.8_{a}$	$0.8 \pm 0.4_{a}$	$1.4 \pm 0.4_{a}$	$2.5 \pm 1.2_{a}$	$2.8 \pm 0.6_{a}$	$2.5 \pm 0.8_{a}$	$4.2 \pm 0.7_{a}$
Significance	ns	ns	s	s	ns	s	ns	ns	ns	ns	ns	ns	ns	ns	ns	s	s	ns	ns	ns	ns

The two production levels were compared by linear regression further to confirm our findings. Since linear regression analysis has been used in comparison studies [46], it was applied to all the analytical results to detect any bias concerning the industrial-level reproducibility of the laboratory-level designed process. The analysis produced the following R values ($p < 0.05$): 0.932 for the regression analysis applied to LSC-G18-9502 and CSC-G18-9502 and 0.742 for that applied to LSC-9502 and CSC-9502 overall dataset. (Figure 3).

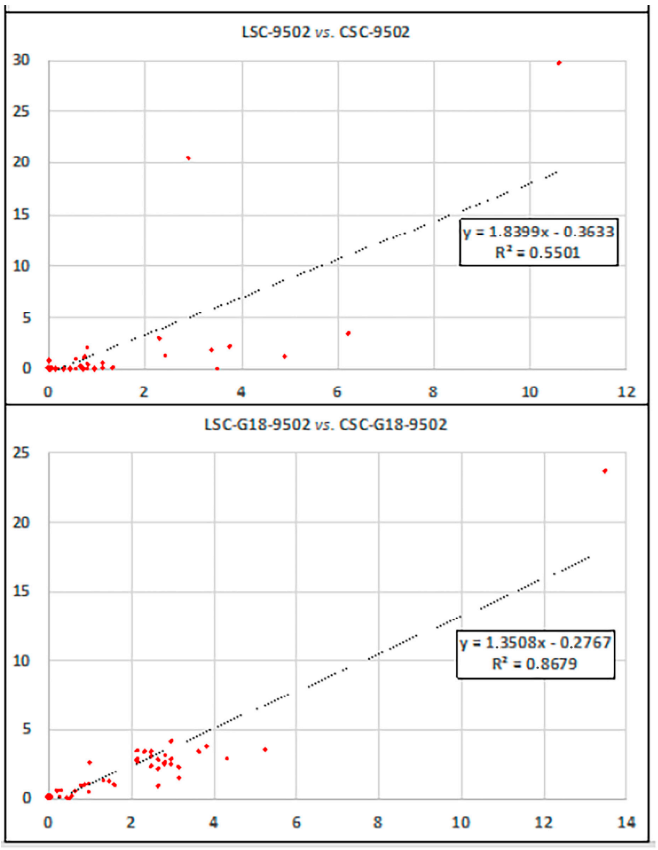

Figure 3. Graphic representation of linear regression analysis between laboratory and industrial production based on the whole datasets. The analytical data corresponding to the beers produced at laboratory and industrial levels are reported on the x- and y-axis, respectively.

These results demonstrate higher reproducibility in the production process involving the sequential inoculation of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* compared to the brewing process using only the *S. cerevisiae* strain. In the latter process, regression analysis indicated that the main bias is related to the fate of carbohydrates, including varying extent of starch degradation, fermentation efficiency, and alcohol production. However, other investigations highlighted differences between production levels depending on the different equipment solutions, which can affect mass and heat transfer phenomena or impact the microbial systems involved [2,10,12].

4. Conclusions

The transition of the brewing process from the laboratory to the industrial level cannot be considered simply a change of the procedure dimension according to a fixed ratio: The shift from one scale to another may require adjustments in formulation and/or production processes. To assess the feasibility of brewing scalability, we studied the production of novel beer for ingredients (unmalted common wheat) or type of fermentation (performed by an oenological *S. cerevisiae* alone or in sequential inoculation with a *Sc. pombe* strain). Differences were observed between those produced at the laboratory and industrial level,

with the extent of the gap depending on the type of fermentation. The reproducibility of beer characteristics was higher in beer produced by sequential inoculation and was also better for sensory characteristics compared to chemical parameters. Furthermore, a linear regression model was used to evaluate brewing reproducibility from laboratory to industrial levels, showing better performance for brewing with sequential yeast inoculation. In conclusion, for the first time, we have used a mixed inoculum of *Sc. pombe*/*S. cerevisiae*, both of oenological origin, to produce beer at an industrial scale, offering natural means for product diversification and the potential to create safer beers, continuing the historical role of yeasts in enhancing food safety.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app14198609/s1, Figure S1: Schematic diagram of LSC production process; Figure S2: Schematic diagram of CSC production process.

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