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Witbier Fermented by Sequential Inoculation of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*: Influence of Starchy Ingredients and *S. cerevisiae* Strain Used for In-Bottle Refermentation

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Abstract: Great attention has recently been dedicated to the use of non-*Saccharomyces* yeast strains for the development of new beer formulations. However, the effect of the *Saccharomyces* strain used in the refermentation of this type of beer has never been investigated. The research described aimed to optimize the quality of beers fermented by an oenological *Schizosaccharomyces pombe* strain alternately combined with two *S. cerevisiae* strains (WB06, commercial; 9502, of an oenological origin). The influence of both in-bottle refermentation (alternately carried out by one of the two *Saccharomyces cerevisiae* strains used in the sequential first fermentation) and starchy ingredients (three mixtures of 65% of malted barley alternately combined with 35% of unmalted common, durum, or emmer wheat) was studied. The beer formulation was optimized through a two-factor mixed three- and two-level design, where the two factors were the starchy ingredients and the refermenting *S. cerevisiae*. Beers from durum wheat beers refermented by WB06 had the highest alcohol contents. Common wheat beers refermented by 9502 showed the highest antioxidant activity values. The highest overall sensory score was assigned to the beers refermented by 9502. The fitted quadratic model had a good predictive ability for five physicochemical and fourteen sensory characteristics, with an R^2 often higher than 0.9.

Keywords: brewing; design of experiment; non-brewing yeasts; non-*Saccharomyces* strain; quality; refermentation; yeast-mixed cultures; wheat beer

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1. Introduction

Consideration of the role of non-*Saccharomyces* yeasts in brewing processes has changed radically over the last few years. The presence of non-*Saccharomyces* strains in brewery environments as well as in other companies involved in the production of fermented drinks was viewed with great concern due to their negative impact on turbidity, production of off-flavours, and increase in volatile acidity [1]. However, if properly selected, applied, and managed, they have the potential to give distinctive characteristics to the produced beers when compared to products already on the market [2]. Non-*Saccharomyces* yeasts of an oenological origin are now often used in beer fermentation, as they are considered particularly valuable in the production of desirable flavours [3]. They can be used as pure starters (to produce high-flavoured–low alcohol beers), in spontaneous fermentation (to produce lambic/gueuze styles), or in mixed-fermentation (simultaneous or sequential) with *Saccharomyces cerevisiae* strains for both first fermentation and bottle conditioning [4].

Schizosaccharomyces pombe has recently been considered for use in mixed-culture fermentation due to its technological performances: (i) its production of a higher ethanol concentration than other non-*Saccharomyces* yeast due to the metabolization of maltose and

maltotriose [4]; (ii) its contribution to aroma production through the generation of fruity flavours [5]; (iii) its metabolization of L-malic acid [6]; (iv) colour stabilization [7]; and (v) its contribution to the consistency and persistence of foam [4].

Pownall et al. [8] evaluated the fermentation of standard- and high-gravity wort through the simultaneous inoculation of an *S. cerevisiae* starter and a *Schizosaccharomyces pombe* strain. According to their results, the mixed cultures were able to produce esters and higher alcohols in higher concentrations than the parent monocultures. The use of *Schizosaccharomyces pombe* and *S. cerevisiae* yeasts in sequential fermentations has been tested on Kei-apple (*Dovyalis caffra* L.) juice [9], wine [10], and fruit wines [11]. Indeed, to the best of our knowledge, the sequential inoculation of the two yeasts has not yet been applied in beer fermentation, except for in the research of Baiano et al. [12], whose manuscript is still under revision.

The craft beer brewing procedure includes a stage of refermentation in the bottle, generally with the sole addition of sugar. Only recently, craft breweries have started to carry out refermentation by adding not only sugar but also yeasts. The reason is that, although after racking and before bottling, the beer retains a number of viable yeast cells capable of resuming their metabolic activities following priming, further inoculation is essential to ensure regular refermentation. During this stage, biosynthesis of further compounds other than ethanol and carbon dioxide occurs, but the relative amounts of these compounds depend on the inoculated yeast strain. This implies that the choice of the refermenting yeast strain is strategic to give each craft beer its distinctive analytical and sensory profile [13,14]. Understanding what happens during the second fermentation is even more important when the beer comes from a sequential (first) fermentation since it is the product of different balances compared to a beer fermented using a single strain. However, the effects of the yeast strain used for the refermentation of beers obtained in such a way have not been investigated at all. To complicate this picture, there is the influence exerted by the starting cereal matrix on the products of fermentative metabolism. It remains to be understood whether this influence is mitigated by the first fermentation and, therefore, becomes less significant during refermentation. The present work aimed to optimize the quality characteristics (in particular, those related to the concentration of antioxidant compounds and the sensory characteristics) of craft beers fermented by the sequential inoculation of an oenological *Schizosaccharomyces pombe* strain alternately combined with two *S. cerevisiae* strains (WB06, commercial; 9502, of an oenological origin). Since the starting idea was to strongly diversify beers by strengthening the effect exerted by a variety of yeasts, the novelty of the work is the investigation of the influence of refermentation (alternately carried out by one of the two *S. cerevisiae* strains already used in the sequential fermentation) on the quality of beers obtained through sequential (first) fermentation. Moreover, since previous research has shown that the influence of starchy ingredients is greater than that of yeasts, in the present work, the effect of refermentation was tested on three mixtures (65% of malted barley alternately combined with 35% of unmalted common, durum, or emmer wheat species). A two-factor mixed three- and two-level experimental design was applied to optimize the beer formulation, where the two factors were the starchy ingredients, represented by three cereal mixtures, and the refermenting *S. cerevisiae* strains, i.e., WB06 and 9502.

2. Materials and Methods

2.1. Experimental Design

Experimental brewing was performed according to a two-factor mixed 3-level and 2-level experimental design in which one factor was the unmalted wheat species contained in the brewed cereal mixture, while the other factor was the yeast used for in-bottle refermentation. Concerning the first factor, three wheat species were considered: common wheat cv. *Risciola* (C), durum wheat cv. *Dauno III* (D), and emmer (E), grown in an experimental field in the province of Foggia (Italy). Three cereal mixtures were prepared, each made of 65% malted barley (M) supplied by Agroalimentare Sud (Melfi, Italy) and 35%

of one of the above-mentioned unmalted wheat. The mixtures will be hereinafter indicated by MC, MD, and ME, respectively. Regarding the second factor, two yeast strains were alternately used for in-bottle refermentation: a commercial *S. cerevisiae* (WB06) supplied by Lesaffre Italia (Sissa Trecasali, Italy), usually employed in wheat beer production and the *S. cerevisiae* ITEM9502 (hereinafter referred to as 9502) isolated from the *Susumaniello* grape, whose characteristics are described by Tristezza et al. [15] and which is stored in the ITEM Microbial Culture Collection of CNR-ISPA (http://www.ispacnr.it/collezioni-microbiche (accessed on 24 May 2024)).

2.2. Brewing Protocol

A formulation suitable to produce ~100 L of beer was prepared with the following ingredients: 135 L of water (115 L for mashing and 20 L for sparging); 16.25 kg of Pilsner barley malt; 8.75 kg of unmalted wheat; 200 g of dried hop cones cv. Cascade (6.7% α-acid content); 100 g of bitter orange peels; and 50 g of coriander seeds. (All the flavouring agents were supplied by Birramia, Querceta, Italy). The experimental brewing trials were carried out in a 20 L Braumeister plant (Speidel Tank-und Behälterbau GmbH, Ofterdingen, Germany).

Malted and unmalted cereals were coarsely ground with a 2-roller mill (Albrigi Luigi, Stallavena, Italy) and added to the mashing water (with a conductivity of 420 ± 10 µS/cm) previously heated at 47 °C. The mashing steps were the following: protein rest (54 °C; 10 min); β-amylase rest (63 °C; 50 min); α-amylase rest (70 °C; 50 min); and mash-off (81 °C; 15 min). The spent grain was sparged with water at 81 °C, and the latter was added to the wort (a final pH of 5.2 ± 0.2). Hops were added 5 and 50 min (half and half) after the wort started boiling, while coriander and bitter orange peels were added 5 min before the end of boiling (the total boiling time was 65 min).

The wort resulting from the mashing of the three malted barley–unmalted wheat mixtures (MC, MD, and ME) were cooled and whirlpooled. The original gravity values were 1.048 ± 0.001, 1.057 ± 0.003, and 1.048 ± 0.001, respectively. The three wort types were submitted to sequential fermentation, as described below. MC, MD, and ME wort samples were inoculated with *Schizosaccharomyces pombe* G18 (~1×10^7 cells/mL), a strain isolated from grape must and selected for its ability to metabolize maltose and maltotriose, belonging to the ITEM Microbial Culture Collection of CNR-ISPA (http://www.ispacnr.it/collezioni-microbiche). The inoculated wort types were fermented at 23 ± 2 °C until an intermediate gravity of 1.030 ± 0.003, 1.043 ± 0.006, and 1.022 ± 0.004, respectively, was reached. (This happened within 48 h.) At this point, each wort beer was divided into two halves, one of which was fermented with *S. cerevisiae* WB06 (~1×10^7 cells/mL) and the other fermented with *S. cerevisiae* 9502 (~1×10^7 cells/mL). Fermentations were carried out at 23 ± 2 °C for 22 ± 2 days until a final gravity value of 1.010 ± 0.002 was reached. Then, the two halves separately fermented were brought together, mixed until a uniform product was obtained, and kept at 4 ± 1 °C for 2 days. Finally, each beer (MC, MD, and ME) was again divided into two halves, one of which was inoculated with *S. cerevisiae* WB06 (~1×10^6 cells/mL), while the other one was inoculated with *S. cerevisiae* 9502 (~1×10^6 cells/mL), primed with sucrose (8 g/L), and packaged into 750 mL glass brown bottles. The beer bottles were first conditioned at 20 ± 2 °C for 30 days and then stored at 5 ± 1 °C for another month until analyses were carried out. Combining the three types of beers obtained from the first fermentation with the two *S. cerevisiae* strains used for refermentation, six types of final beers were produced: MC-9502, MD-9502, ME-9502, MC-WB06, MD-WB06, and ME-WB06. For each type of beer, two technological replicates were performed.

2.3. Physicochemical Analyses of Beers

The pH values, soluble solids (Brix), carbon dioxide content (mg CO_2/L), alcohol content (%), and total and volatile acidity (as g lactic acid/L and g acetic acid/L, respectively) were determined, as described in Baiano et al. [16]. Colour (expressed as srm) was

determined at 430 nm, according to the European Brewery Convention [17], on previously degassed and filtered (0.45 µm) beers.

The total antioxidant content (TAC mg gallic acid equivalents/L) was measured through the Folin–Ciocalteu assay [18] and quantified on a calibration curve of gallic acid (20–1000 mg/L range). The beer antioxidant activity (AA) was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical–scavenging activity [19] and expressed as a percentage of DPPH remaining (% DPPH). The key to understanding the data relating to DPPH is the following: low values of the % of stable radical DPPH indicate high values of antioxidant activity. The phenolic profile was studied by a 1100 HPLC-DAD system (Agilent, Santa Clara, CA, USA) through a 100 × 4.6 mm × 3 µm RP-C18 Gemini column (Phenomenex, Aschaffenburg, Germany) [20]. Peak identification was carried out by comparing their retention times and spectra with those of 18 pure standards, while the quantitative analysis was made on calibration lines built by an injection of known amounts of the same standards. The sum of the concentrations of all phenolic compounds (HPLC-TPC) was also calculated.

2.4. Sensory Analysis of Beers

The experimental beers were analysed by a panel of 10 trained panellists between 22 and 65 years old. A Quantitative Descriptive Analysis (QDA) was performed as described by Baiano et al. [16]. The panellists evaluated and assigned a score to each of the five visual (colour, amount, and persistence of foam; colour and turbidity of the liquid fraction), nine olfactory (overall olfactory intensity—OOI; olfactory finesse—OF, i.e., the overall flavour elegance; and the following flavours: malty, hoppy, floral, fruity, spicy, yeast, and aromatic herbs), four gustatory (sweetness, bitterness, saltiness, and sourness), and 3 tactile (alcoholic, effervescence, and body/fullness) characteristics and the overall sensory quality (OSQ) of each beer after swallowing. Colour was evaluated on the following 4-point scales: 1 (white), 2 (rose), 3 (cream), and 4 (capuchin) for foam and 1 (pale straw yellow), 2 (straw yellow), 3 (golden yellow), and 4 (amber) for the liquid fraction. The other descriptors and the overall sensory quality were evaluated on a 5-point scale.

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 two-way ANOVA followed by an LSD test ($p < 0.01$) was applied to evaluate the statistical significance of the differences induced by the brewing of the three unmalted wheat species (MC, MD, and ME) and the use of the two *S. cerevisiae* yeast strains (9502 and WB06) in refermentation among the resulting beers for each analytical parameter. A Principal Component Analysis (PCA) was also applied to verify if the relationships among beer samples and parameters allowed homogeneous grouping (by wheat species, refermenting yeast, or both). The Pearson correlation coefficients (R, $p < 0.05$) were calculated between couples of parameters and are reported in the Supplementary Materials as Table S1. The main significant correlations are discussed in the text. A multiple regression analysis in which each dependent variable (i.e., the physicochemical and sensory characteristics that showed significant differences among the six samples) were fitted to the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_{ii}^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i X_j \quad (1)$$

where Y is the dependent variable; β_0 is a constant; β_i are the linear coefficients; β_{ii} are the quadratic coefficients; and β_{ij} are the interactive coefficients. ANOVA was used to evaluate the quality of the fitted model and individuate the significant single and interactive factors ($p < 0.05$), while the overall predictive capability of the model was evaluated by the coefficient of determination (R^2). The statistical analyses were performed through Statistica for Windows V. 7.0. (Statsoft, Tulsa, OK, USA).

3. Results and Discussion

3.1. Effects of Starchy Material and Refermenting S. cerevisiae Strain on Physico-Chemical Parameters

As can be inferred from the data in Table 1, the composition of the starting cereal mixture exerted statistically significant effects on all considered variables. The beers produced with malted barley and unmalted common wheat showed a paler colour, a lower dry matter, and a lower total antioxidant content but also higher carbon dioxide and total acidity contents than the other beers. MD beers had both the highest alcohol content and the highest soluble solid content due to the highest original gravity of MD wort. The presence of emmer in the starchy material made the colour of the beer slightly darker than the other two unmalted cereals due to the use of hulled grains [21] and was able to maximize the concentration of total antioxidants in the finished beer. However, the presence of almost intact glumes (the roller mill was not able to ground them) accounted for the lowest total phenolic content (HPLC-TPC) of the ME beers. The Pearson correlation coefficients between TAC and AA (−0.955) and between HPLC-TPC and AA (around 0) indicate that most of the beer antioxidant activity depends on compounds other than polyphenols, such as reducing compounds released by yeast following cell autolysis [22]. The high R-value between colour and TAC (0.873) or AA (−0.993) indicates that darker beers had higher antioxidant properties, in agreement with the findings of Granato et al. [23]. In contrast with Dadic and Van Gheluwe [24], a possible protective role of phenolic compounds with respect to colour loss was not observed (a Pearson coefficient of around 0). Furthermore, attention must be focused on the alcohol/carbon dioxide ratios of the three beers, which were equal to 1.40, 2.41, and 1.58 for MC, MD, and ME, respectively. As can be inferred from both these data and the wort's original gravity values, the highest ratio was obtained from the fermentation of the wort with the highest initial sugar content. The wort's original gravity probably also affected the biosynthesis of alcoholic fermentation by-products. In fact, in agreement with the findings of Zentou et al. [25], the concentration of compounds responsible for volatile acidity was higher in MD beers, which were derived from higher original gravity wort.

Table 1. Influence of wheat species and *S. cerevisiae* strain used for refermentation in bottles on physical and chemical parameters.

Factors	Colour (srm)	Alcohol Content (%)	CO_2 (g/L)	Soluble Solids °Bx)	Dry Matter (%)	pH	Total Acidity (g Lactic acid/L)	Volatile Acidity (g Acetic acid/L)	Total Antioxidant Content (mg Gallic acid/L)	HPLC-TPC (mg/L)	Antioxidant Activity (% DPPH)
					Effect of Starchy Ingredient						
MC	3.216 ^{a}	5.81 ^{a}	4.16 ^{c}	3.50 ^{b}	3.33 ^{a}	4.13 ^{c}	2.09 ^{c}	0.57 ^{b}	353 ^{a}	98.71 ^{b}	60.6 ^{c}
MD	4.017 ^{b}	7.07 ^{b}	2.93 ^{a}	3.72 ^{c}	3.93 ^{c}	3.88 ^{a}	2.07 ^{b}	0.62 ^{c}	474 ^{b}	138.28 ^{c}	52.3 ^{b}
ME	5.977 ^{c}	5.80 ^{a}	3.66 ^{b}	3.03 ^{a}	3.54 ^{b}	4.03 ^{b}	1.98 ^{a}	0.54 ^{a}	578 ^{c}	94.08 ^{a}	36.8 ^{a}
Significance	*	*	*	*	*	*	*	*	*	*	*
					Effect of In-bottle Yeast Strain						
9502	4.398 ^{a}	6.19 ^{a}	2.97 ^{a}	3.85 ^{b}	3.72 ^{b}	4.01 ^{a}	2.02 ^{a}	0.58 ^{b}	467 ^{a}	108.49 ^{a}	50.6 ^{b}
WB06	4.409 ^{a}	6.27 ^{b}	4.19 ^{b}	3.58 ^{a}	3.48 ^{a}	4.01 ^{a}	2.08 ^{b}	0.57 ^{a}	470 ^{a}	112.22 ^{a}	49.2 ^{a}
Significance	ns	*	*	*	*	ns	*	*	ns	ns	*

In columns, different letters indicate significant differences at $p < 0.01$ by LSD multiple range test. The asterisks indicate significant differences at $p < 0.01$ by LSD multiple range test. ns: not significant at $p < 0.01$ by LSD multiple range test; MC: malted barley (65%)–unmalted common wheat (35%); MD: malted barley (65%)–unmalted durum wheat (35%); ME: malted barley (65%)–unmalted emmer (35%); 9502: *S. cerevisiae* isolated from grape must; WB06: commercial *S. cerevisiae*. HPLC-TPC: sum of the concentrations of phenolic compounds.

The refermenting yeast exerted statistically significant effects on most variables, except for colour, pH, TAC, and HPLC-TPC (Table 1). Both *S. cerevisiae* yeasts were able to conduct efficient fermentation, although the commercial starter produced slightly more alcohol and CO_2 and left slightly less unfermented sugars than the oenological strain. Analogously, very slight differences were observed between the two yeasts with regard to antioxidant activity and total acidity (higher in beers refermented by the WB06 strain) and volatile acidity (higher in beers refermented by the 9502 strain).

In agreement with Wannenmacher et al. [26], the main groups of phenolic compounds in our experimental beers were phenolic acids, tannins, flavones, and flavonols. Eighteen phenolic compounds were present in all beers but in different concentrations depending

on the starting cereal mixtures. As an example, the use of cereal mixtures determined a great variability in the ratio between the two barley main phenolic acids, p-coumaric acid and ferulic acid (from 0.65 to 1.21), which is instead genetically determined [27]. This means that the starchy ingredient significantly contributed to the differentiation of the phenolic profiles of beers (Table 2). MD beers clearly stood out from the others due to the higher concentrations of 10 of the 18 identified compounds, namely, gallic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, chlorogenic acid (together with MC), ferulic acid, epigallocatechin, epicatechingallate, rutin, and resveratrol (together with MC). The other two beers were further distinguished for their content of 4-hydroxybenzoic acid and catechin (respectively, lower and higher in ME beers) and their concentrations of p-coumaric and rosmarinic acid (higher in ME beers and lower in MC beers).

Table 2. Influence of wheat species and *S. cerevisiae* strain used for refermentation in bottles on the beer phenolic profiles.

Factors	Phenolics (mg/L)																	
	Gall. Acid	4-HBA	Cat.	Van. Acid	Caff. Acid	Syr. Acid	EC	Chlor. Acid	EGC	Fer. Acid	*p*-C. Acid	Sin. Acid	EG	Rutin	Resv.	Rosm. Acid	Querc.	Kaemp.
Effect of Starchy Ingredient																		
MC	6.44 ^{a}	1.86 ^{b}	4.03 ^{a}	0.90 ^{a}	2.04 ^{a}	4.44 ^{a}	12.28 ^{a}	22.06 ^{b}	9.53 ^{a}	1.93 ^{a}	1.26 ^{a}	6.30 ^{a}	9.94 ^{a}	2.91 ^{a}	1.61 ^{b}	7.62 ^{a}	1.66 ^{b}	1.89 ^{a}
MD	12.19 ^{b}	2.41 ^{c}	4.67 ^{a}	1.51 ^{b}	4.80 ^{b}	4.04 ^{a}	13.71 ^{a}	25.07 ^{b}	15.17 ^{b}	2.14 ^{b}	1.43 ^{ab}	6.07 ^{a}	22.20 ^{b}	8.20 ^{b}	1.51 ^{b}	9.64 ^{ab}	1.36 ^{a}	2.14 ^{a}
ME	5.59 ^{a}	1.09 ^{a}	6.57 ^{b}	0.92 ^{a}	2.20 ^{a}	3.56 ^{a}	11.21 ^{a}	8.84 ^{a}	12.20 ^{a}	1.82 ^{a}	2.20 ^{b}	4.95 ^{a}	9.49 ^{a}	5.13 ^{a}	1.17 ^{a}	10.67 ^{b}	1.27 ^{a}	5.20 ^{b}
Signific.	*	*	*	*	*	ns	ns	*	*	*	*	ns	*	*	*	*	*	*
Effect of In-bottle Yeast Strain																		
9502	8.08 ^{a}	1.75 ^{a}	5.04 ^{a}	1.13 ^{a}	3.11 ^{a}	4.03 ^{a}	12.03 ^{a}	18.54 ^{a}	12.41 ^{a}	1.93 ^{a}	1.72 ^{a}	6.05 ^{a}	12.63 ^{a}	5.11 ^{a}	1.46 ^{a}	9.00 ^{a}	1.42 ^{a}	3.05 ^{a}
WB06	8.07 ^{a}	1.82 ^{a}	5.14 ^{a}	1.09 ^{a}	2.92 ^{a}	4.00 ^{a}	12.77 ^{a}	18.77 ^{a}	12.18 ^{a}	1.99 ^{a}	1.54 ^{a}	5.50 ^{a}	15.13 ^{a}	5.73 ^{a}	1.40 ^{a}	9.62 ^{a}	1.43 ^{a}	3.10 ^{a}
Signific.	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

In columns, different letters indicate significant differences at $p < 0.01$ by LSD multiple range test. The asterisks indicate significant differences at $p < 0.01$ by LSD multiple range test. ns: not significant at $p < 0.01$ by LSD multiple range test; MC: malted barley (65%)–unmalted common wheat (35%); MD: malted barley (65%)–unmalted durum wheat (35%); ME: malted barley (65%)–unmalted emmer (35%); 9502: *S. cerevisiae* isolated from grape must; WB06: commercial *S. cerevisiae*; 4-HBA: 4-hydroxybenzoic acid; Gall.: Gallic; Cat.: Catechin; Van.: Vanillic; Caff.: Caffeic; Syr.: Syringic; EC: Epicatechin; Chlor.: Chlorogenic; EGC: Epigallocatechin; Fer.: Ferulic; *p*-C.: *p*-Coumaric; Sin.: Sinapic; EG: Epicatechingallate; Resv.: Resveratrol; Rosm.: Rosmarinic; Querc.: Quercetin; Kaemp.: Kaempferol.

The two *S. cerevisiae* strains showed the same behaviour towards phenolic absorption and/or biosynthesis.

3.2. Effects of Starchy Material and Refermenting S. cerevisiae Strain on Sensory Characteristics

The results of the sensory evaluation are registered in Table 3. Consistently with the spectrophotometric measurement of beer colour, the trained panel scored ME beers as the darkest, both in terms of foam (R = 0.617) and liquid fraction (R = 0.711). The higher quantity of foam in MC and MD beers could be related to the findings of Depraetere et al. [28] concerning the presence in wheat, especially common wheat, of foam-promoting factors. The beers produced from durum wheat also showed a high persistence of foam, with the two variables highly correlated (R = 0.815). MC beers also obtained the highest scores for OOI and finesse. Hoppy and yeast flavours were probably the main contributors to the overall olfactory intensity of those beers. High scores for olfactory finesse were also attributed to ME beers, thanks to their floral and yeast flavour. MC and ME were also evaluated as the least bitter and the bitterest beers, respectively, although the differences were not due to the content of bitter substances but to the residual sugars present in greater quantities in soft wheat beers, which better masked the flavour contribution of hops. Despite the differences found between the beers produced from different cereal mixtures, they were all assigned the same OSQ score.

Table 3. Influence of wheat species and *S. cerevisiae* strain used for refermentation in bottles on the beer sensory profiles.

Fact.	Colour		Foam		Turb.	Flavour Characteristics									Gustatory Characteristics				Tactile Characteristics			OSQ
	Foam	Liquid	Am.	Pers.		OOI	OF	Mal.	Hop.	Flo.	Fr.	Spi.	Yea.	A. H.	Sweet.	Bitter.	Saltin.	Sourn.	Alcoh.	Effer.	Body	
Effect of Starchy Ingredient																						
MC	1.1 ^{a}	2.6 ^{a}	4.7 ^{b}	3.8 ^{a}	3.7 ^{a}	4.4 ^{b}	4.4 ^{b}	3.2 ^{a}	3.3 ^{b}	2.5 ^{a}	3.0 ^{a}	2.1 ^{a}	3.0 ^{b}	2.1 ^{a}	2.4 ^{a}	2.8 ^{a}	2.3 ^{a}	2.7 ^{a}	3.0 ^{a}	3.8 ^{a}	3.9 ^{b}	4.4 ^{a}
MD	1.1 ^{a}	2.5 ^{a}	4.5 ^{b}	4.2 ^{b}	3.4 ^{a}	3.9 ^{a}	4.0 ^{a}	3.2 ^{a}	2.8 ^{a}	2.7 $^{a\,b}$	3.0 ^{a}	2.2 ^{a}	2.6 ^{a}	2.2 ^{a}	2.4 ^{a}	2.9 $^{a\,b}$	2.4 ^{a}	2.8 ^{a}	3.0 ^{a}	3.8 ^{a}	3.5 ^{a}	4.4 ^{a}
ME	1.6 ^{b}	3.6 ^{b}	4.0 ^{a}	3.6 ^{a}	3.3 ^{a}	4.0 $^{a\,b}$	4.5 ^{b}	3.5 ^{a}	2.8 ^{a}	2.9 ^{b}	3.3 ^{a}	2.1 ^{a}	3.3 ^{b}	2.1 ^{a}	2.5 ^{a}	3.2 ^{b}	2.5 ^{a}	3.1 ^{b}	3.0 ^{a}	3.9 ^{a}	3.8 ^{b}	4.2 ^{a}
Signific.	*	*	*	*	ns	*	*	ns	*	*	ns	ns	*	ns	ns	*	ns	*	ns	ns	*	ns
Effect of In-bottle Yeast Strain																						
9502	1.3 ^{a}	3.0 ^{a}	4.7 ^{b}	4.2 ^{b}	3.7 ^{b}	4.7 ^{b}	4.7 ^{b}	3.7 ^{b}	3.2b	3.0 ^{b}	3.7 ^{b}	2.0 ^{a}	3.5 ^{b}	2.0 ^{a}	2.2 ^{a}	3.2 ^{b}	2.2 ^{a}	2.8 ^{a}	3.0 ^{a}	4.0 ^{a}	3.8 ^{a}	4.7 ^{b}
WB06	1.3 ^{a}	2.8 ^{a}	4.2 ^{a}	3.6 ^{a}	3.3 ^{a}	3.5 ^{a}	3.9 ^{a}	3.0 ^{a}	2.7 ^{a}	2.4 ^{a}	2.6 ^{a}	2.3 ^{a}	2.4 ^{a}	2.2 ^{a}	2.7 ^{b}	2.8 ^{a}	2.6 ^{b}	2.9 ^{a}	3.0 ^{a}	3.7 ^{a}	3.6 ^{a}	4.0 ^{a}
Signific.	ns	ns	ns	*	*	*	*	*	*	*	*	ns	*	ns	*	*	*	ns	ns	ns	ns	ns

In columns, different letters indicate significant differences at $p < 0.01$ by LSD multiple range test. The asterisks indicate significant differences at $p < 0.01$ by LSD multiple range test. ns: not significant at $p < 0.01$ by LSD multiple range test; Fact.: factors; MC: malted barley (65%)–unmalted common wheat (35%); MD: malted barley (65%)–unmalted durum wheat (35%); ME: malted barley (65%)–unmalted emmer (35%); 9502: *S. cerevisiae* isolated from grape must; WB06: commercial *S. cerevisiae*; Am.: Amount; Pers.: Persistence; Turb.: Turbidity; OOI: Overall Olfactory Intensity; OF: Olfactory Finesse; Mal.: Malty; Hop.: Hoppy; Flo.: Floral; Fr.: Fruity; Spi.: Spicy; Yea.: Yeast; A.H.: Aromatic Herbs; Sweet.: Sweetness; Bitter.: Bitterness; Saltin.: Saltiness; Sourn.: Sourness; Alcoh.: Alcoholic; Effer.: Effervescence; OSQ: Overall Sensory Quality.

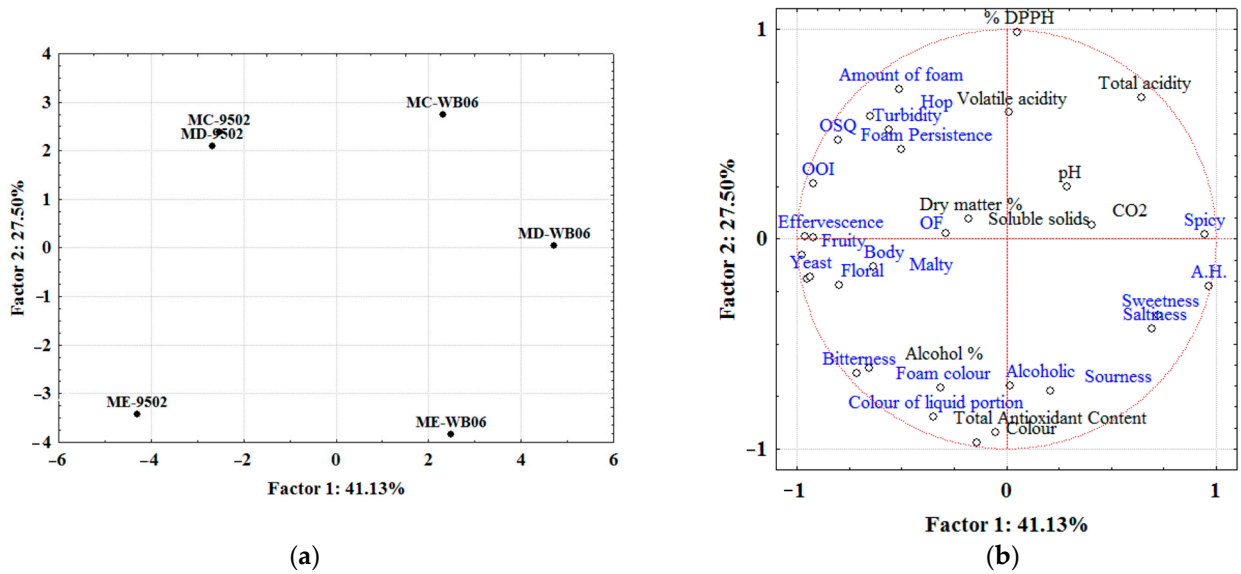
Regarding the effects of the refermenting yeast, the results are quite surprising as the beers refermented by the oenological strain had abundant and persistent foam as well as scores for turbidity, olfactory intensity (malty, hoppy, floral, fruity, and yeast flavours), olfactory finesse, and bitterness higher than those assigned to the beers fermented by the commercial starter, all without causing negative effects on colour, spicy, and aromatic flavours (typical of white beers), alcoholic perception, and effervescence to obtain the highest score for overall sensory quality, too. These results are definitely better than those described by Baiano et al. [29], who compared the performance of the 9502 strain with those of other oenological *S. cerevisiae* yeasts. The reasons for these differences are precisely attributable to the different fermentation and refermentation protocols. In Baiano et al. [29], the oenological yeast was used individually, and refermentation was carried out by inoculating the same yeast strain used for the first fermentation. In the present work, the first fermentation was performed through a sequential inoculation of Sc. pombe, followed by separate fermentation by the two *S. cerevisiae* yeasts, whose products were blended and submitted to refermentation by one of the two strains used in fermentation alternately. This fermentation and refermentation protocol, only apparently more complicated to apply due to the inclusion of additional operations in the usual brewing process, is able to give appreciable characteristics to the finished beer thanks to the contribution of three different yeasts added at different times.

The overall olfactory intensity was strictly and positively correlated to olfactory finesse (R = 0.746) to indicate that the panellists appreciated more flavoured beers. The contributions of hop, floral, fruity, and yeast flavours to OOI were highly significant ($0.520 \leq R \leq 0.785$). Moreover, the olfactory finesse was positively influenced by malty, hop, fruity, and yeast flavours ($0.633 \leq R \leq 0.817$) and negatively affected by spicy and aromatic flavours (R = −0.545 and −0.711, respectively). Finally, the overall sensory quality benefited from the contribution of the following attributes: OOI (R = 0.724, mainly with malty, hop, and yeast flavours); OF (R = 0.717); effervescence (R = 0.681); amount and persistence of foam (R = 0.595 and 0.530); and turbidity (R = 0.513, welcome in beers as it is synonymous with craftsmanship). Saltiness was not perceived as a pleasant factor (R = −0.687 with the overall sensory quality).

3.3. Principal Component Analysis

As can be inferred from Figure 1, the analysis was performed on whole data sets, with the exception of individual phenolics. The first two factors accounted for 68.63% of the total variance. The beer samples are very distant from each other in the factorial plane (Figure 1a), with the exception of those produced with mixtures containing durum

or soft wheat and refermented with *S. cerevisiae* 9502, which are located in the part of the plane characterized by negative values of Factor 1 and positive values of Factor 2. These samples had in common the presence of a remarkable quantity of foam (Figure 1b). The characteristics that made the other beers unique are the following: (i) high total acidity for MC-WB06, positioned in the quadrant corresponding to positive values of both factors; (ii) a remarkable spicy flavour for MD-WB06, placed on the line passing through the 0 of Factor 2 but with positive values of Factor 1; high sourness for ME-WB06, placed in the quadrant corresponding to positive values of Factor 1 and negative values of Factors 2; (iii) more intense colour of foam and liquid fraction for ME-9502, positioned in the quadrant corresponding to negative values of both factors (Figure 1b).



(**a**) (**b**)

Figure 1. Biplot of (**a**) scores and (**b**) loadings obtained with the application of PCA to physicochemical (in black) and sensory data (in blue) sets along the first and second components.

These data highlight the enormous potential for diversification that derives not only from the various combinations of raw materials but, above all, from this new way of managing fermentation and refermentation. Such results are even more interesting as previous research demonstrated the predominant effect of the cereal ingredient compared to that of the yeast when fermentations and refermentations are managed in a more conventional way [28].

3.4. Multiple Regression Analysis

This paper aimed to write equations that describe the relationships among each measured variable and the factors whose influence has been evaluated (the types of starchy ingredient and refermenting yeast); quantify these effects through the calculation of the corresponding regression coefficients; and evaluate the ability of the models to fit the experimental data. Table 4 lists the analytical parameters for which models with a significant predictive ability ($p < 0.05$) have been found. For each of them, the significant ($p < 0.05$) linear, quadratic, and interactive terms are reported.

Table 4. Estimated regression coefficients and coefficients of determination of the quadratic models that describe the effects of wheat species and *S. cerevisiae* strain used for refermentation on the considered beer variables.

Variables	Intercept	Linear, Quadratic, and Interactive Effects of Wheat Species (W) and *S. cerevisiae* Strains (Y)					R^2 of the Quadratic Models
		W	Y	W^2	Y^2	W * Y	
Colour (srm)	3.184			0.468			0.987
	(0.000)			(0.000)			(0.000)
Alcohol content (%)	2.743				−0.054		0.902
	(0.000)				(0.023)		(0.003)
CO_2 (g/L)	9.428	−5.061	3.828	0.980		−1.306	0.924
	(0.005)	(0.029)	(0.010)	(0.049)		(0.030)	(0.050)
TAC (mg gallic acid/L)	310,056	149.750	−210.917		27.861	37.333	0.995
	(0.001)	(0.000)	(0.001)		(0.001)	(0.017)	(0.000)
% DPPH	63.885			−2.991			0.961
	(0.000)			(0.000)			(0.000)
Foam amount	6.531			−0.231	0.172	−0.671	0.905
	(0.000)			(0.040)	(0.053)	(0.009)	(0.031)
Colour liquid portion	2.268				0.139		0.733
	(0.000)				(0.007)		(0.007)
Turbidity	4.111		−0.256				0.620
	(0.000)		(0.031)				(0.031)
Overall flavour intensity	4.153		−1.139				0.860
	(0.000)		(0.001)				(0.002)
Olfactory finesse	4.207					−0.367	0.711
	(0.000)					(0.014)	(0.042)
Floral flavour	3.627				0.132	−0.613	0.915
	(0.000)				(0.052)	(0.003)	(0.007)
Fruity flavour	5.250	−0.649	−1.539		0.194	−0.269	0.980
	(0.000)	(0.008)	(0.002)		(0.010)	(0.010)	(0.000)
Malty flavour	2.814					−0.297	0.706
	(0.000)					(0.032)	(0.045)
Yeast flavour	2.965	−2.208		0.583	−0.193		0.926
	(0.008)	(0.028)		(0.021)	(0.001)		(0.004)
Sweetness	3.422				0.104		0.759
	(0.000)				(0.008)		(0.021)
Bitterness	3.142	−0.502			−0.082		0.914
	(0.000)	(0.011)			(0.001)		(0.002)
Sourness	2.514					0.075	0.644
	(0.000)					(0.024)	(0.024)
Alcoholic	3.001				−0.012	0.018	0.737
	(0.000)				(0.010)	(0.026)	(0.029)
Effervescence	3.534				−0.058		0.876
	(0.000)				(0.001)		(0.001)

* *p*-values are reported in brackets.

According to the statistical data (Table 4), the quadratic models well described most of the five physico-chemical and fourteen sensory characteristics, with an R^2 higher than 0.9 for ten variables. Sourness and turbidity were not as well predicted as the other characteristics, with R^2 values of 0.644 and 0.620, respectively. Based on these results, the beer parameters can be ideally divided into the following four groups based on the factor(s) capable of predicting them: (1) the single wheat species factor, affected by two parameters, namely, colour (spectrophotometrically determined) and AA; (2) the single yeast strain factor affected by five beer characteristics, i.e., alcohol content, the colour of the liquid fraction, turbidity, OOI, and effervescence; (3) the only interactive effects of wheat species and yeast strains, which affected the malty flavour and sourness; and (4) the contribution of both factors for other variables.

4. Conclusions

The starchy matrix is confirmed to exert a very strong influence on the characteristics of beer. As well as identifying a precise beer style, the use of a mixture of malted and non-malted grains instead of malt alone can represent a strategy to counterbalance the reduced phenolic diversity due to barley domestication. The proposed fermentation and

refermentation protocols were able to give appreciable characteristics to the finished beer thanks to the contribution of three different yeasts added at different times. Moreover, this unconventional management of yeast inoculation could be considered a powerful tool for beer diversification, allowing yeasts of an oenological origin, therefore, not completely adapted to the brewing ingredients, to impart physical, chemical, and organoleptic characteristics, even superior to those of the selected commercial starters. The exception was represented by the phenolic profiles, which were not affected by yeasts. The other physical, chemical, and sensory characteristics allowed the clustering of beers by both the starchy ingredient and fermenting yeast. The multiple regression analysis produced models with high predictive capacity for most beer characteristics, thus promising to be a useful tool for planning a quality beer in advance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/beverages10030051/s1, Table S1: Pearson correlation coefficients ($p < 0.05$).

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Informed Consent Statement: The craft beer objects of this research are products made with procedures/ingredients (yeasts included) characterized in terms of hygiene and safety (they are not novel foods); thus, they do not present risks to the health of users. The sensory study was performed using human volunteers who were previously asked to sign an informed consent form. An appropriate protocol for protecting the rights and privacy of all participants was utilized.

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