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Use of Autochthonous Yeasts and Bacteria in Order to Control *Brettanomyces bruxellensis* in Wine

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Received: 20 October 2017; Accepted: 1 December 2017; Published: 5 December 2017

Abstract: Biocontrol strategies for the limitation of undesired microbial developments in foods and beverages represent a keystone toward the goal of more sustainable food systems. *Brettanomyces bruxellensis* is a wine spoilage microorganism that produces several compounds that are detrimental for the organoleptic quality of the wine, including some classes of volatile phenols. To control the proliferation of this yeast, sulfur dioxide is commonly employed, but the efficiency of this compound depends on the *B. bruxellensis* strain; and it is subject to wine composition and may induce the entrance in a viable, but nonculturable state of yeasts. Moreover, it can also elicit allergic reactions in humans. In recent years, biological alternatives to sulfur dioxide such as the use of yeasts and lactic acid bacteria starter cultures as biocontrol agents are being investigated. The controlled inoculation of starter cultures allows secure, fast and complete alcoholic and malolactic fermentations, limiting the residual nutrients that *B. bruxellensis* utilizes to survive and grow in wine. The current study is focused on the assessment of the effect of autochthonous yeasts and bacterial strains from the Apulia Region on the development of *B. bruxellensis* in wine, in terms of both growth and volatile phenols' production. The investigation evidences the positive role of indigenous mixed cultures in the control of this spoilage yeast, either co-inoculating different strains of *Saccharomyces cerevisiae*, *S. cerevisiae*/non-*Saccharomyces* or co-inoculating *S. cerevisiae*/*Oenococcus oeni*. Our findings expand the existing knowledge of the application of protechnological microbial diversity and of non-*Saccharomyces* as a biocontrol agent in oenology. We report a further demonstration of the interest in selecting indigenous strains as a strategic tool for winemakers interested in the improvement of regional wines.

Keywords: *Brettanomyces bruxellensis*; volatile phenols; biocontrol; *Saccharomyces cerevisiae*; non-*Saccharomyces*; *Oenococcus oeni*; wine

1. Introduction

Wine spoilage microbes comprise one of the most relevant problems for the wine industry, due to undesirable products that can lead to considerable economic losses [1,2]. Yeasts belonging to the genus *Brettanomyces*/*Dekkera*, in particular *Brettanomyces bruxellensis*, are generally known as some of the main enological spoilage microbes [3,4]. The growth of these yeasts usually leads to the production of volatile phenols that negatively affect the aroma of wine [5,6]. Volatile phenols, especially 4-ethylphenol, are associated with disagreeable flavors, described as "phenolic", "horse

sweat", "stable", "leather" or "animals" [6,7]. Ethylphenols derive from hydroxycinnamic acids, as a consequence of sequential conversion of specific hydroxycinnamic acids (ferulic and p-coumaric acids): first, the hydroxycinnamate decarboxylase transforms these hydroxycinnamic acids into vinylphenols; then, they are respectively reduced to 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) by the vinylphenol reductase [8,9]. Their organoleptic threshold in red wine is 605 and 110 mg/L, respectively for 4-EP and 4-EG [5,10]. Several authors have developed specific analytical methods to check metabolites responsible for wine spoilage [11] or to evaluate the population of these contaminant yeasts [12,13].

Usually, the concentration of *B. bruxellensis* is low during the first steps of winemaking, due to slow growth [14]. In addition, during alcoholic fermentation (AF), the metabolic activity of *S. cerevisiae* inhibits its development, and its population remains low. Nevertheless, the period from the end of AF to the beginning of malolactic fermentation (MLF) is considered a critical point at which *B. bruxellensis* can grow. During this period, there are available nutrients, and there is no competition with other microorganisms. Ageing is also an important period in which *B. bruxellensis* may develop [15]. Sulphur dioxide (SO_2) is commonly employed in order to avoid the growth of this yeast, but the efficiency of this chemical compound is variable among *B. bruxellensis* strains and depends also on the wine composition. Besides, SO_2 can induce the entrance in a viable, but non-culturable (VBNC) state of yeasts [2,16,17]. Moreover, it can also elicit allergic reactions in humans. During the VBNC state, the cells of *B. bruxellensis* show reduced size and metabolic activity; furthermore, they cannot reproduce on solid media [18]. As a consequence, yeasts are undetectable by classical cultivable microbiological analysis. *Brettanomyces* can begin to grow at the end of alcoholic fermentation (AF), thanks to residual sugars, low molecular SO_2 concentration and semi-aerobic conditions [1,2,19]. Several studies have focused attention on the control of *B. bruxellensis*, developing several methods to prevent wine depreciation, first of all through the use of SO_2 [20].

One of the strategies is the use of starter cultures to control the AF and the malolactic fermentation (MLF). The use of starter cultures allows a secure, fast and complete AF and MLF, limiting the residual nutrients that *B. bruxellensis* utilizes to survive and develop in wine. In this context, microbial interactions between *Saccharomyces* spp., non-*Saccharomyces* spp. and LAB are important for the success and safety of wine fermentations [21,22]. The main types of interaction between *Saccharomyces* spp. and non-*Saccharomyces* yeasts are: nutritional limitation or competition [23,24] and the release of toxic compounds as killer toxins into the environment [15,25,26]. On the other hand, there are three types of interaction between yeast and LAB. The most common is bacterial inhibition, followed by stimulation and neutralism [27]. Usually, the MLF starter cultures are inoculated when AF is finished. If a reduced concentration of SO_2 is added, the probability of wine contamination by *B. bruxellensis* increases [4,28]. A recommended strategy to avoid this problem and reduce the time between AF and MLF is the co-inoculation of the malolactic starter culture with the yeast [4,29,30]. Among the starter cultures, many investigations have studied the microbial biodiversity in a regional area in order to select autochthonous starters denoted by different oenological characteristics [31,32] since these microorganisms are potentially adapted to a definite grape must and reflect the "terroir" of a particular area [31,33].

In order to limit the use of SO_2, more and more studies have investigated the efficacy of biological methods to control *B. bruxellensis* and ethylphenols in wine such as the use of different yeasts species and lactic acid bacteria (LAB) [34,35]. Examples of these studies are the investigations of Suárez-Lepe et al. [36] and Morata et al. [35]. They recommended the use of *S. cerevisiae* strains that possess hydroxycinnamate decarboxylase (HCDC+) to carry out the AF. Other biological methods to control *B. bruxellensis* that employ non-*Saccharomyces*-specific strains are being investigated. Oro et al. [37] showed that *Metschnikowia pulcherrima* secreted pulcherriminic acid, which inhibits the growth of *B. bruxellensis*. Moreover, killer toxins able to inhibit the growth of *B. bruxellensis* in wine and secreted by non-*Saccharomyces* strains have been investigated. Mehlomakulu and coworkers [15] identified two killer toxins, CpKT1 and CpKT2, from the wine-isolated yeast *Candida pyralidae*. A similar action was described for the killer toxins isolated from *Kluyveromyces wickerhamii* and *Pichia anomala* [38], *Pichia membranifaciens* [26], *Torulaspora delbrueckii* [25] and *Ustilago maydis* [39].

Furthermore, an early inoculation with a selected LAB culture has proven to be a useful tool for controlling the proliferation of *B. bruxellensis* in wine. Investigations by Gerbaux et al. [40] showed that in the inoculated wines, the MLF started before that in non-inoculated wines, and so, the period between the AF and the MLF was reduced when the LAB culture was inoculated. In these wines, *B. bruxellensis* was not developed, and the concentration of volatile phenols was significantly reduced.

The main objective of this study was to analyze the effect of autochthonous yeasts and bacterial strains of enological interest (*Saccharomyces* spp. and non-*Saccharomyces* and *Oenococcus oeni*) on the growth and the ethylphenols production of an autochthonous strain of *B. bruxellensis*. The suitability of the studied yeast and bacteria strains as biocontrol starters to combat *B. bruxellensis* is discussed.

2. Materials and Methods

2.1. Microbial Strains and Growth Conditions

Autochthonous yeast strains isolated from the Apulia Region located in Southern Italy were: *S. cerevisiae* KT029756, KT029757, KT029758 [32] *Candida zemplinina* KT029748 [32], *Hanseniaspora uvarum* KT029770 [32], *Hanseniaspora guilliermondii* KT029766, *Torulaspora delbrueckii* KT029800 [32], *Metschnikowia pulcherrima* KT029783 [32] and *Brettanomyces bruxellensis* Unifg 8 [4]. In addition, one indigenous strain of *Oenococcus oeni* Unifg 21 [41] was also used. Strains of *O. oeni* were cultivated on de Man Rogosa Sharpe (MRS) broth (pH 5.5), supplemented with 100 mg/L cycloheximide (Sigma, Milan, Italy), after incubation at 28 °C for 72 h. Yeasts (*Saccharomyces* and non-*Saccharomyces*) were grown on yeast extract-peptone-dextrose (YPD) broth, supplemented with 100 mg/L chloramphenicol (Sigma, Milan, Italy) and incubated at 28 °C for 48–72 h.

2.2. Vinification Assays

Fermentations were performed in 5-L tanks containing 4.5 L of "Nero di Troia" red must (sugar 220 g/L, titratable acidity 5.0, pH 3.5) at 20 °C. All samples were inoculated with *B. bruxellensis* Unifg 8 with the aim of simulating a natural contamination (1×10^3 cfu/mL),and then inoculated with different combinations of starter cultures (Table 1) to obtain an initial cell density of 2×10^6 cfu/mL, for *S. cerevisiae*, non-*Saccharomyces* and *O. oeni*. All fermentations were carried out in triplicate.

Table 1. Microorganisms employed in the different grape must fermentations.

Sample Codes	Inoculated Strains
Sc56 (control)	*S. cerevisiae* KT029756
Sc56 + Sc57	*S. cerevisiae* KT029756 + *S. cerevisiae* KT029757
Sc56 + Sc58	*S. cerevisiae* KT029756 + *S. cerevisiae* KT029758
Sc56 + Sc57 + Sc58	*S. cerevisiae* KT029756 + *S. cerevisiae* KT029758 + *S. cerevisiae* KT029757
Sc56 + Cz	*S. cerevisiae* KT029756 + *C. zemplinina* KT029748
Sc56 + Hg	*S. cerevisiae* KT029756 + *H. guilliermondii* KT029766
Sc56 + Hu	*S. cerevisiae* KT029756 + *H. uvarum* KT029770
Sc56 + Td	*S. cerevisiae* KT029756 + *T. delbrueckii* KT029800
Sc56 + Mp	*S. cerevisiae* KT029756 + *M. pulcherrima* KT029783
Sc56 + Oo	*S. cerevisiae* KT029756 + *O. oeni* Unifg 21

2.3. Monitoring of B. bruxellensis Growth

B. bruxellensis Unifg 8 growth was monitored on modified WLN agar medium [42] (Wallerstein Laboratory Nutrient media 60 g/L, sorbic acid 0.25 g/L, trehalose 5 g/L, p-coumaric acid 100 mg/L, agar 15 g/L, cycloheximide 30 mg/L and pH 5.5) after 7 and 21 days from the inoculation time. WLN plates were incubated over a period of 5–11 days at 30 °C.

2.4. Volatile Phenol Production and Quantification

All standards (4-EG, 4-EP, 2-optanol) and chemical reagents (dichloromethane, methanol and ethanol, all HPLC gradient-grade) were purchased from Sigma–Aldrich. Pure water was obtained from a Milli-Q purification system (Millipore, Burlington, MA, USA). The extraction of volatile compounds was carried out with a solid phase extraction (SPE) procedure, with tubes LiChrolut® EN 200 mg, 3 mL, standard PP (polypropylene) (Marck, Darmstadt, Germany). Vac Elut 20 station equipment from Varian (Palo Alto, Santa Clara, CA, USA) was used. Tubes were conditioned according to [43], with some modifications: tubes were rinsed with 4 mL of dichloromethane–hexane (2:3), 4 mL of methanol and, finally, 4 mL of a water-ethanol mixture (12%, *v*/*v*). Fifty milliliters of wine, containing 1 ppm of 2-octanol as the internal standard, were passed through, with a flow rate of 2 mL/min. Afterwards, the sorbent was dried by letting air pass through it. The analytes blocked in the stationary phase were eluted with 1.3 mL of dichloromethane-hexane (2:3). All samples were stocked at −20 °C before gas chromatographic analysis.

A 6890N series gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) with an Agilent 5973 mass selective detector (MSD) and equipped with an HP-INNOWAX capillary column (60 m × 0.25 mm I.D., 0.25-µm film thicknesses, J & W Scientific Inc., Folsom, CA, USA) was used. The injection was realized in the splitless mode at 250 °C. Helium (1.0 mL/min) was used as the carrier gas. The initial temperature of the column oven was 40 °C, and was increased to 230 °C at 2.5 °C/min. Electron impact mode (70 eV) was used to record the spectra. Each volatile compound concentration (ppm) was obtained by normalizing its peak area with the internal standard. Each volatile compound was identified by comparing mass spectra with those of the data system library (NIST 98, $P > 90\%$). All analyses were carried out in triplicate.

2.5. Statistical Analysis

One-way analysis of variance (ANOVA) with a 95% confidence level was carried out to test for statistically-significant differences between samples for each sampling time (Tukey test, $\alpha = 0.05$).

3. Results

In order to characterize the enological interaction between several autochthonous resources (*Saccharomyces* spp., non-*Saccharomyces* spp. and *Oenococcus* spp.) and "Brett" spoilage during winemaking, the ability of *B. bruxellensis* to grow has been investigated. At the beginning of AF (*t*0), volatile phenols resulted in being lower than the detection limit in all samples analyzed (Tables 2–4). 4-EG and 4-EP after seven days from the beginning of AF are lower than the sensory perception threshold in all samples analyzed, respectively 0.110 and 0.605 ppm in red wine [10]. It is mandatory to underline that we used the sensory thresholds as a measure to describe the microbial spoilage potential and not to provide information on sensory perception. In fact, it is well assumed, in the scientific literature, that the final sensory properties are a function of the complex compositions of the different wines, due to the different masking and cooperative effects between molecules [44].

Table 2. Volatile phenol content (ppm) of grape musts spiked with *B. bruxellensis* Unifg 8 and inoculated with different combinations of three *S. cerevisiae* strains during alcoholic fermentation (AF): *t*0, day of inoculation; *t*7, 7 days from the inoculation; *t*21, 21 days from the inoculation (nd, volatile phenol not detected). Results of the ANOVA analysis are shown as letters on the data corresponding to the concentration of volatile phenols: different letters on the same column indicate significant differences of 95%.

Samples	4-Ethylguaiacol (ppm)			4-Ethylphenol (ppm)		
	***t*0**	***t*7**	***t*21**	***t*0**	***t*7**	***t*21**
Sc56 (control)	nd	0.0529 ± 0.0001 [a]	0.3655 ± 0.0879 [a]	nd	0.1073 ± 0.0014 [a]	1.2244 ± 0.0052 [a]
Sc56 + Sc57	nd	0.0507 ± 0.0002 [a]	0.3314 ± 0.0047 [a]	nd	0.1174 ± 0.0087 [a]	0.5870 ± 0.0465 [b]
Sc56 + Sc58	nd	0.0984 ± 0.0114 [a]	0.3468 ± 0.0859 [a]	nd	0.1388 ± 0.0234 [a]	0.7760 ± 0.1694 [c]
Sc56 + Sc57 + Sc58	nd	0.0147 ± 0.0063 [a]	0.1190 ± 0.0259 [b]	nd	0.0745 ± 0.0051 [b]	0.1616 ± 0.0008 [d]

Table 3. Volatile phenol content (ppm) of grape musts spiked with *B. bruxellensis* Unifg 8 and inoculated with different combinations of *S. cerevisiae* KT029756 with non-*Saccharomyces* strains (*Candida zemplinina* KT029748, *Hanseniaspora uvarum* KT029770, *Hanseniaspora guilliermondii* KT029766, *Torulaspora delbrueckii* KT029800 and *Metschnikowia pulcherrima* KT029783) during AF: *t*0, day of inoculation; *t*7, 7 days from the inoculation; *t*21, 21 days from the inoculation (nd, volatile phenol not detected). Results of the ANOVA analysis are shown as letters on the data corresponding to the concentration of volatile phenols: different letters on the same column indicate significant differences of 95%.

Samples	4-Ethylguaiacol (ppm)			4-Ethylphenol (ppm)		
	***t*0**	***t*7**	***t*21**	***t*0**	***t*7**	***t*21**
Sc56 (control)	nd	0.0529 ± 0.0001 [a]	0.3655 ± 0.0879 [a,b]	nd	0.1073 ± 0.0014 [a]	1.2244 ± 0.0052 [a]
Sc56 + Cz	nd	0.0008 ± 0.0001 [a,b]	0.2168 ± 0.0178 [b]	nd	0.0674 ± 0.0001 [a]	0.5720 ± 0.0025 [b]
Sc56 + Hg	nd	0.0109 ± 0.0044 [a,b]	0.1665 ± 0.0121 [b]	nd	0.0745 ± 0.0007 [a]	0.3011 ± 0.0227 [c]
Sc56 + Hu	nd	0.0060 ± 0.0043 [a,b]	0.1877 ± 0.0458 [b]	nd	0.0648 ± 0.0459 [a]	0.6295 ± 0.2456 [b]
Sc56 + Td	nd	0.0929 ± 0.0125 [b]	0.3098 ± 0.0290 [a]	nd	0.1449 ± 0.0216 [a]	0.7158 ± 0.1459 [b]
Sc56 + Mp	nd	0.0698 ± 0.0091 [a]	0.3721 ± 0.0342 [a]	nd	0.1125 ± 0.0058 [a]	0.7110 ± 0.0293 [b]

Table 4. Volatile phenol content (ppm) of grape musts spiked with *B. bruxellensis* Unifg 8 and inoculated with *S. cerevisiae* KT029756 or co-inoculated with *S. cerevisiae* KT029756/*O. oeni* Unifg 21 during AF: *t*0, day of inoculation; *t*7, 7 days from the inoculation; *t*21, 21 days from the inoculation (nd, volatile phenol not detected). Results of the ANOVA analysis are shown as letters on the data corresponding to the concentration of volatile phenols: different letters on the same column indicate significant differences of 95%.

Samples	4-Ethylguaiacol (ppm)			4-Ethylphenol (ppm)		
	***t*0**	***t*7**	***t*21**	***t*0**	***t*7**	***t*21**
Sc56 (control)	nd	0.0529 ± 0.0001 [a]	0.3655 ± 0.0879 [a]	nd	0.1073 ± 0.0014 [a]	1.2244 ± 0.0052 [a]
Sc56 + Oo	nd	0.0151 ± 0.0007 [a]	0.0703 ± 0.0311 [b]	nd	0.0737 ± 0.0042 [a]	0.1540 ± 0.0471 [b]

After 21 days from the beginning of AF, 4-EG increased in sample Sc56 (control), Sc56 + Sc57 and Sc56 + Sc58 (about 0.3 ppm, see Table 2 for details). In sample Sc56 + Sc57 + Sc58, 4-EG content is significantly lower (i.e., combination of 3 *S. cerevisiae* strains) than that reported for the other *Saccharomyces* spp. investigated (about 0. 12 ppm). All samples show higher 4-EG amount than the sensory perception threshold; nevertheless, the interaction of three different *S. cerevisiae* led to a significant reduction of 4-EG, from 0.3 ppm (Sc56, Sc56 + Sc57, Sc56 + Sc58) to 0.12 ppm (Sc56 + Sc57 + Sc58).

Similar results were obtained in the 4-EP production. This compound was not detected at the beginning of AF (*t*0). After seven days from the beginning of AF, all *Saccharomyces* spp. combinations led to an increase of 4-EP (0.1 ppm); sample SC56 + SC57 + Sc58 showed the lowest amount of 4-EP (0.07 ppm). Finally, after 21 days, sample Sc56 showed a high amount of 4-EP, 1.2 ppm, two-fold the sensory perception threshold. Samples Sc56 + Sc57 and Sc56 + Sc58 presented an increase of 4-EP, about 0.6–0.7 ppm; those value are comparable with the sensory perception threshold (0.6 ppm). Sample Sc56 + Sc57 + Sc58 presented the lowest 4-EP concentration, 0.16 ppm. The simultaneous presence of the three strains of *S. cerevisiae* (Sc56 + Sc57 + Sc58) led to a drastic reduction of volatile phenols, in particular for 4-EP, resulting in a lower concentration than the sensory perception threshold.

The production of volatile phenols can be correlated with the *B. bruxellensis* population. Cell densities of the different fermentations are reported in Tables 5–7. The *B. bruxellensis* Unifg 8 population was stable during the first weeks of AF (*t*7). After 21 days from the beginning of AF, it increased in samples Sc56, Sc56 + Sc57 and Sc56 + Sc58, respectively 4.8, 5.7 and 6.3 lg cfu/mL, while its cell density was significantly lower in sample Sc56 + Sc57 + Sc58, with a cell density of 3.3 lg cfu/mL.

Table 5. *B. bruxellensis* Unifg 8 cell count (cfu/mL) on Wallerstein Laboratory Nutrient (WLN) agar medium from grape musts inoculated with different combinations of *S. cerevisiae* strains during AF: *t*0, day of inoculation; *t*7, 7 days from the inoculation; *t*21, 21 days from the inoculation. Results of the ANOVA analysis are shown as letters on the data corresponding to the *B. bruxellensis* cell count: different letters on the same column indicate significant differences of 95%.

Samples	lg cfu/mL		
	***t*0**	***t*7**	***t*21**
Sc56 (control)	4.00 ± 0.06 [a]	4.23 ± 0.05 [ac]	6.32 ± 0.06 [a]
Sc56 + Sc57	4.62 ± 0.08 [b]	4.13 ± 0.06 [a]	4.85 ± 0.06 [b]
Sc56 + Sc58	3.73 ± 0.04 [c]	4.48 ± 0.02 [b]	5.69 ± 0.10 [c]
Sc56 + Sc57 + Sc58	4.62 ± 0.13 [b]	4.3 ± 0.05 [c]	3.30 ± 0.03 [d]

Table 6. *B. bruxellensis* Unifg 8 cell count (cfu/mL) on WLN agar medium from grape musts inoculated with different combinations of *S. cerevisiae* KT029756 with non-*Saccharomyces* strains (*Candida zemplinina* KT029748, *Hanseniaspora uvarum* KT029770, *Hanseniaspora guilliermondii* KT029766, *Torulaspora delbrueckii* KT029800 and *Metschnikowia pulcherrima* KT029783) during AF: *t*0, day of inoculation; *t*7, 7 days from the inoculation; *t*21, 21 days from the inoculation. Results of the ANOVA analysis are shown as letters on the data corresponding to the *B. bruxellensis* cell count: different letters on the same column indicate significant differences of 95%.

Samples	lg cfu/mL		
	***t*0**	***t*7**	***t*21**
Sc56 (control)	4.00 ± 0.06 [a]	4.23 ± 0.05 [ac]	6.32 ± 0.06 [a]
Sc56 + Cz	4.62 ± 0.05 [a]	3.10 ± 0.04 [b]	5.62 ± 0.06 [a]
Sc56 + Hg	3.78 ± 0.06 [a]	3.90 ± 0.01 [a,b,c]	4.60 ± 0.02 [a]
Sc56 + Hu	4.62 ± 0.08 [a]	3.25 ± 0.05 [ab]	5.51 ± 0.07 [a]
Sc56 + Td	4.62 ± 0.03 [a]	4.30 ± 0.03 [ac]	5.64 ± 0.10 [a]
Sc56 + Mp	4.62 ± 0.05 [a]	4.41 ± 0.05 [c]	4.59 ± 0.03 [a]

Table 7. *B. bruxellensis* Unifg 8 cell count (cfu/mL) on WLN agar medium from grape musts inoculated with different combinations of *S. cerevisiae* KT029756 or co-inoculated with *S. cerevisiae* KT029756/ *O. oeni* Unifg 21 during AF: *t*0, day of inoculation; *t*7, 7 days from the inoculation; *t*21, 21 days from the inoculation. Results of the ANOVA analysis are shown as letters on the data corresponding to the *B. bruxellensis* cell count: different letters on the same column indicate significant differences of 95%.

Samples	lg cfu/mL		
	***t*0**	***t*7**	***t*21**
Sc56 (control)	4.00 ± 0.06 [a]	4.23 ± 0.05 [a]	6.32 ± 0.06 [a]
Sc56 + Oo	4.90 ± 0.04 [a]	3.27 ± 0.13 [a]	4.80 ± 0.06 [a]

The effect on volatile phenol production due to the interaction between *Saccharomyces* spp. and several non-*Saccharomyces* spp. of enological interest, such as *Hanseniaspora* spp., *Candida* spp., *Torulaspora* spp. and *Metschnikowia* spp., was investigated. 4-EG in samples Sc56 + Cz, Sc56 + Hg, Sc56 + Hu, Sc56 + Td and Sc56 + Mp was not detected at the beginning of AF (*t*0), while after seven days, its concentration increased, in a range between 0.0929 and 0.0008 ppm (Table 3). Those values were not significantly different from those reported for sample Sc56 and were below the published sensory threshold. At *t*21, we reported an increase of 4-EG content, in particular for samples Sc56 + Td and Sc56 + Mp, 0.3 ppm, comparable to those obtained in sample Sc56. The lowest 4-EG was detected in sample Sc56 + Hg and Sc56 + Hu (0.16 and 0.18 ppm, respectively), with significant differences compared to the inoculation of a single *S. cerevisiae* culture and compared to the co-inoculation of *S. cerevisiae* with *C. zemplinina*, *T. delbrueckii* or *M. pulcherrima*. The concentration of 4-EG at *t*21 resulted in being higher than the sensory perception threshold in all cases, nevertheless

we observed an important reduction of volatile phenols in wine obtained with the co-inoculation of *Saccharomyces* spp. and non-*Saccharom*yces; also, this key feature seemed to be species dependent.

A similar trend was reported for the 4-EP production (Table 3). 4-EP could not be found at the beginning of AF; it increased after seven days from 0.06–0.15 ppm keeping below the sensory threshold value. At *t*21, the production of 4-EP ranged from 0.7–0.3 ppm. Only in samples Sc56 + Td and Sc56 + Mp was the concentration higher than the sensory threshold value (about 0.7 ppm). It is important to underline that the interaction between *Saccharomyces* spp. and non-*Saccharomyces* led to a considerable reduction of volatile phenols compared to the results reported in wine inoculated with a single *Saccharomyces* spp. culture starter. Significant differences were found comparing the final amount of 4-EP in sample Sc56 to sample Sc56 + Hg.

In Table 6, the cell population of *B. bruxellensis* Unifg 8 in fermentations Sc56 (control), Sc56 + Cz, Sc56 + Hg, Sc56 + Hu, Sc56 + Td and Sc56 + Mp is reported. The plate counting revealed that the cell density of *B. bruxellensis* decreased during the AF (*t*7), in particular for samples Sc56 + Cz, Sc56 + Hg and Sc56 + Hu (about 3 lg cfu/mL). At the end of AF, the *B. bruxellensis* Unifg 8 population increased, and the values obtained for samples Sc56 + Cz, Sc56 + Hg, Sc56 + Hu, Sc56 + Td and Sc56 + Mp (4–5 lg cfu/mL) were lower than that obtained for sample Sc56 (control) (lg cfu/mL).

Finally, we tested the interaction between *S. cerevisiae* KT029756 and *O. oeni* Unifg 21. In Tables 4 and 7 are reported respectively the volatile phenol production and *B. bruxellensis* Unifg 8 cell count of samples Sc56 (control) and Sc56 + Oo. Sample Sc56 + Oo showed a diminution in the of volatile phenols production after 21 days from the beginning of AF, 0.07 and 0.15 ppm, respectively for 4-EG and 4-EP, compared with fermentation Sc56 (control) (see Table 4). These values were lower than the sensory perception threshold. Furthermore, the *B. bruxellensis* Unifg 8 population seemed to be affected by *O. oeni* Unifg 21, with a cell density of 4.8 lg cfu/mL at the end of AF (Table 7).

4. Discussion

Biocontrol strategies for the limitation of spoilage microorganisms, pathogens and microbial producers of compounds toxic for human health represent a keystone toward the goal of sustainable food systems [45,46]. *B. bruxellensis* is the main spoilage yeast in winemaking, detrimental for wine quality and mainly responsible for volatile phenols release, but also of biogenic amine bioproducts and responsible for considerable economic losses [20,47,48]. In addition to reducing the risks of undesired microbial developments, biocontrol offers opportunities to reduce chemical preservatives such as SO_2, associated with allergic manifestations in humans. In light of increasing interest in the biotechnological potential related to autochthonous microbes associated with spontaneous fermentation, we tested combinations of Apulian autochthonous eukaryotic and prokaryotic strains of enological interest on the spoilage potential of an Apulian autochthonous strain of *B. bruxellensis*. We reported a reduction of volatile phenols in wine obtained with the inoculation of 3 *S. cerevisiae* strains compared with a single inoculation or in two yeast combinations. These results could be explained by the competition between *B. bruxellensis* and *S. cerevisiae* regarding nutrient availability and/or substrate limitation [21] or by the adsorption phenomena of the cell wall from *S. cerevisiae* yeasts [45]. In contrast, considering the results in the single strain trials, our finding seems to exclude the presence of a killer activity of the *S. cerevisiae* strains. The evidence of a cumulative effect using three *S. cerevisiae* strains simultaneously suggests the existence of competitive phenomena. It might also be possible to speculate that reduction is correlated to an increase of yeast autolysis in wine. Autolyzed yeast can affect both the *Brettanomyces* cell population and volatile phenols' production, as reported in other studies [45]. In particular, the yeast cell wall affected the quantity of ethylphenols during winemaking, probably because of the adsorption of the phenolic compounds by the cell wall fragments [45,46].

During mixed fermentation with non-*Saccharomyces* yeasts, the dominance of a yeast strain can be influenced by the competition for nutrients; e.g., for different modalities of nitrogen use. Several positive and negative interactions have been reported in the literature regarding nutrient availability and/or substrate limitation [21,37]. According to these findings, the co-inoculation of *S. cerevisiae* with a non-*Saccharomyces* strain, especially the co-inoculation with *H. guilliermondii* KT029766, allowed a

diminution of *B. bruxellensis* growth compared with the single inoculation of *S. cerevisiae* KT029756 and, consequently, a minor volatile phenol production. Considering non-*Saccharomyces* species, previous studies demonstrated the existence of a specific killer activity that could be used against several *B. bruxellensis* strains in *M. pulcherrima* [37] and in *T. delbrueckii* [25]. Our study assessed, to the best of our knowledge, for the first time, the impact of strains belonging to the species *C. zemplinina*, *H. guilliermondii* and *H. uvarum*, expanding the existing knowledge of the application of non-*Saccharomyces* as a biocontrol agent in enology.

In a usual winemaking process, the period that takes place from the end of AF to the start of MLF is particularly conducive to the development of *B. bruxellensis* because the wine is not protected by SO_2, and there is no competition from other wine microorganisms; the *S. cerevisiae* population is decreasing; and the indigenous LAB are not yet established. Yeast and bacteria co-inoculation permits a reduction in overall vinification time, and this is generally advantageous to the winery from a cost perspective; and also, microbiologically, the wines are safe and stable sooner [29,49]. According to these findings, the co-inoculation of *S. cerevisiae* KT029756 with *O. oeni* Unifg 21 hindered *B. bruxellensis* Unifg 8 growth, thus permitting a reasonable reduction in the volatile phenol production compared with the single inoculation of *S. cerevisiae*. On the one hand, it is possible to speculate that the effect of *O. oeni* development is due to a lesser nutrient availability. On the other hand, the decrease in volatile phenols might be also partially due to the capacity of selected *O. oeni* strains to degrade tartaric acid ester-bound hydroxycinnamic acids impacting the production of volatile phenols [50].

The effects of a given microbial regimen on the size of the *B. bruxellensis* population is not always sufficient to explain the changes in volatile phenol concentration. This evidence led us to speculate that, in several cases, there exist direct and/or indirect impacts of the protechnological strains used on the biosynthesis of volatile phenols in the *B. bruxellensis* strain used in the present study. It is important to highlight that this study was limited to only one *B. bruxellensis* biotype. In light of the existing strain-dependent characteristics in microbial interaction, in future studies, we aim to verify the behavior using other strains representative of the Apulian *B. bruxellensis* diversity [4].

Our study provides original biological information to improve biocontrol options in winemaking for reducing the risks associated with *B. bruxellensis* spoilage potential. In addition, our findings highlight the importance of autochthonous microbial diversity to design tailored microbial starter cultures as a strategy to cope with specific regional issues [51–53] and a trend to avoid the occurrence of spontaneous fermentations that can lead to increased risks for human health [54].

5. Conclusions

Biocontrol applications for the limitation of undesired microbial growth in foods and beverages represent important tools in the framework of more sustainable food systems. In the current investigation, we report original advances to design biocontrol strategies against *B. bruxellensis* in enology. Furthermore, we describe for the first time the effect of the inoculation of autochthonous yeasts and bacterial strains from the Apulia Region on *B. bruxellensis*, in terms of both growth and volatile phenols' production. Our results suggest that the use of a mixed starter of different strains of *S. cerevisiae* or a mix of specific strains of non-*Saccharomyces* with *S. cerevisiae* are efficient strategies in the control of this spoilage yeast and the volatile phenols' production in wine. The co-inoculation of indigenous *S. cerevisiae* with *O. oeni* resulted also in a valuable method to reduce the *B. bruxellensis* development. This work highlights the importance of the autochthonous microbial resources and potential value of mixed cultures to be used as biocontrol starters against spoilage microorganisms.

Acknowledgments: This research was supported by the Apulia Region in the framework of the Projects "Sviluppo di approcci microbiologici innovativi per il miglioramento della qualità di vini tipici Regionali—NEWINE (Bando "Ricerca e sperimentazione in Agricoltura" and Project Code PRS_042), "Biotecnologie degli alimenti per l'innovazione e la competitività delle principali filiere regionali: estensione della conservabilità e aspetti funzionali—BIOTECA" (Bando "Aiuti a Sostegno Cluster Tecnologici Regionali"; Project Code QCBRAJ6). Vittorio Capozzi was supported by Fondo di Sviluppo e Coesione 2007–2013—Accordo di Programma Quadro Ricerca Regione Puglia "Programma regionale a sostegno della specializzazione

intelligente e della sostenibilità sociale ed ambientale—FutureInResearch". Pasquale Russo was supported by a grant of the Apulian Region in the framework of the "Perform Tech (Puglia Emerging Food Technology)" project (Practice Code LPIJ9P2). Carmen Berbegal was supported by "Programa Atracció de Talent Valencia Campus 2015 de la Universitat de València".

Author Contributions: Giuseppe Spano, Pasquale Russo, Vittorio Capozzi, and Sandra Pati conceived and designed the experiments. Carmen Berbegal, Carmela Garofalo, Pasquale Russo, Vittorio Capozzi, and Sandra Pati performed the experiments. Carmen Berbegal, and Carmela Garofalo wrote the paper. All authors critically read the paper.

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

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