Selected *Schizosaccharomyces pombe* Strains Have Characteristics That Are Beneficial for Winemaking

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Abstract

At present, wine is generally produced using *Saccharomyces* yeast followed by *Oenococcus* bacteria to complete malolactic fermentation. This method has some unsolved problems, such as the management of highly acidic musts and the production of potentially toxic products including biogenic amines and ethyl carbamate. Here we explore the potential of the fission yeast *Schizosaccharomyces pombe* to solve these problems. We characterise an extensive worldwide collection of *S. pombe* strains according to classic biochemical parameters of oenological interest. We identify three genetically different *S. pombe* strains that appear suitable for winemaking. These strains compare favourably to standard *Saccharomyces cerevisiae* winemaking strains, in that they perform effective malic acid deacidification and significantly reduce levels of biogenic amines and ethyl carbamate precursors without the need for any secondary bacterial malolactic fermentation. These findings indicate that the use of certain *S. pombe* strains could be advantageous for winemaking in regions where malic acid is problematic, and these strains also show superior performance with respect to food safety.

Introduction

Several research teams are studying the winemaking potential of non-*Saccharomyces* yeast strains [1]. For example, fission yeasts from the genus *Schizosaccharomyces* show more rapid malic acid deacidification, by converting malic acid to ethanol and CO₂ [2–4]. *Schizosaccharomyces pombe* possesses several remarkable metabolic properties that may be useful in modern quality winemaking [2,5–6], including a malic dehydrogenase activity, high autolytic polysaccharides release [7], ability of gluconic acid reduction [8–11], urease activity [12,13], elevated production of pyruvic acid and colour improvement [14,15], as well as low production of biogenic amines and ethyl carbamate [6,16].
However, *S. pombe* type strains are not currently employed because of specific off-flavours commonly associated with their metabolism. Indeed, *Schizosaccharomyces* strains are commonly isolated from wines suffering from strong organoleptic and chemical deviations including the appearance of acetic acid, acetaldehyde, acetoin and ethyl acetate [17–21]. These undesirable properties of the commonly-used strains have been assumed to be general properties of the species, and only one commercial strain of *S. pombe* is currently available on the market [22]. The exploration of the winemaking properties of other *S. pombe* strains has generally been overlooked. This omission partly reflects the absence of any specific processes to select strains that are appropriate for winemaking [5], and difficulties with isolating *S. pombe* from environmental samples [23]. Given this situation, it has been difficult to obtain a representative strain collection of this species. However, guided by a recent analysis of genetic and phenotypic diversity of 161 *S. pombe* strains [24], we can now conduct a more extensive survey of the utility of this species for winemaking.

The present study examines the winemaking potential of 75 *S. pombe* strains that are available from different microorganism type culture collections, or have been collected by us [5,25]. We apply classic selection parameters used in traditional fermentative *Saccharomyces* yeasts, along with more advanced and specific parameters for *S. pombe*. The results indicate that at least three *S. pombe* strains can potentially provide a viable and attractive alternative to the application of genetically modified *Saccharomyces* species [26–29] whose use is restricted in food industry by European legislation [30].

**Materials and Methods**

**Microorganisms**

The following yeasts were used for the experimental fermentations: 75 *S. pombe* strains, including the non-clonal 54 strains recently described [24], the three mating types of the standard laboratory strain: Leupold’s 972 (h−), 975 (h+) and 968 (h90) (JB22, JB32 and JB50), strain V1 collected by us [5], and 17 strains collected from traditional Chinese breweries by Feng-Yan Bai (unpublished). The following *S. cerevisiae* strains were used as controls: 87 and 88 from the Spanish Type Culture collection. Data for all strains are provided in Table 1.

**First phase of *S. pombe* strain selection**

Starter cultures were grown from 100 μL of each yeast suspension, cultivated in 5 mL volumes of YEPD at 25°C for 24 h. This procedure was performed in triplicate before the final inoculation of 1 mL in the fermentative medium. 1 mL (10⁸ CFU/mL) of these starters cultures were then inoculated into tubes containing 9 mL of sterilised concentrated must (Dream Fruits S.A., Quero, Toledo, Spain), which was diluted to 212 g/L glucose + fructose and enriched with 4 g/L malic acid (Panreac, Barcelona, Spain) (final pH 3.1) to simulate acidic musts where the use of *S. pombe* is more recommended in order to increase wine quality [3,5]. After 21 days of fermentation, enzymatic analysis was performed for glucose + fructose, malic acid and acetic acid (S1 Table). This experiment was performed in triplicate for each studied strain.

**Yeasts used in the second phase of *S. pombe* strain selection**

The yeast strains used in the second selection phase included *S. pombe* IFI936/CECT12774 and IFI935/CECT1376 and *S. cerevisiae* IFI87/CECT12512 and IFI88/CECT12513 from the Spanish Type Culture collection. The *S. pombe* strains JB899/Y470, JB873/NCYC3422 and
| Bähler laboratory strain name | strain ID(s) | location collected | substrate | date collected | reference |
|-------------------------------|-------------|--------------------|-----------|----------------|-----------|
| JB4                           | CBS5557; CCY44-6-1; CBS10393; DBVP6275; JCM8262; NBRC1608; NNCYC683 | Spain | Listan grapes | ND | NA |
| JB22                          | Leupolds 972 (h-); CBS10395; NNCYC1430 | France | rotten grapes | 1947 | NA |
| JB32                          | Leupolds 975 (h+) | France | ND | ND | NA |
| JB50                          | Leupolds 968 (h90) | France | ND | ND | NA |
| JB758                         | NOTT30; NNCYC936; CBS10394 | Sri Lanka | fermenting toddy | 1979 | PMID:25665008 |
| JB759                         | NOTT33; Y0036; CBS10498 | South Africa | beverage | ND | PMID:25665008 |
| JB760                         | NOTT50; DBVP62812 | Italy (Sicily) | grape must treated with SO2 | 1966 | PMID:25665008 |
| JB762                         | NOTT75; CBS358; DBVP6374 | ND | ND | 1987 | PMID:25665008 |
| JB837                         | NOTT1; UWOPS92.229.4 | Mexico | Tequila | 1992 | PMID:25665008 |
| JB838                         | NOTT2; UWOPS94.422.2 | Mexico | Tequila | 1994 | PMID:25665008 |
| JB840                         | NOTT4; UFMGR435; CBS10458 | Brazil (Aracaju) | frozen pulp of Eugenia uniflora ("pitanga", Myrtaceae) | 1999 | PMID:25665008 |
| JB841                         | NOTT5; UFMGA1263; CBS10469 | Brazil (Vicosa) | must of Brazilian cachaça | 1996 | PMID:25665008 |
| JB842                         | NOTT6; UFMGA602; CBS10460 | Brazil (Belo Horizonte) | must of Brazilian cachaça | 1996 | PMID:25665008 |
| JB845                         | NOTT9; UFMGR434; CBS10476 | Brazil (Aracaju) | frozen pulp of Eugenia uniflora ("pitanga", Myrtaceae) | 1999 | PMID:25665008 |
| JB846                         | NOTT10; UFMGA826; CBS10465 | Brazil (Belo Horizonte) | must of Brazilian cachaça | 2000 | PMID:25665008 |
| JB848                         | NOTT12; UFMGR428; CBS10475 | Brazil (Aracaju) | frozen pulp of Eugenia uniflora ("pitanga", Myrtaceae) | 1999 | PMID:25665008 |
| JB852                         | NOTT16; UFMGA529; CBS10462 | Brazil (Belo Horizonte) | must of Brazilian cachaça | 1996 | PMID:25665008 |
| JB853                         | NOTT17; UFMGA1000; CBS10465 | Brazil (Belo Horizonte) | must of Brazilian cachaça | 1996 | PMID:25665008 |
| JB854                         | NOTT18; UFMGR427; CBS10474 | Brazil (Aracaju) | frozen pulp of Eugenia uniflora ("pitanga", Myrtaceae) | 1999 | PMID:25665008 |
| JB858                         | NOTT22; UFMGA738; CBS10464 | Brazil (Belo Horizonte) | must of Brazilian cachaça | 1996 | PMID:25665008 |
| JB862                         | NOTT26; NNCYC380; CBS10392 | ND | raw cane sugar | 1953 | PMID:25665008 |
| JB864                         | NOTT28*; ATCC24751; CBS10391; NNCYC132 | Africa | African Milet Beer | 1921 | PMID:25665008 |
| JB870                         | NOTT34; Y0037; CBS10499 | South Africa | wine | ND | PMID:25665008 |
| JB872                         | NOTT36; CBS2775; IF00347; NBR0347; NNCYC3418 | Japan | fermenting molasses | 1957 | PMID:25665008 |
| JB873                         | NOTT37; CBS6680; DBVP6448; NNCYC3422 | Poland | apple | 1965 | PMID:25665008 |
| JB874                         | NOTT38; CBS6682; DBVP6376; NNCYC3421 | South Africa | millet beer | 1965 | PMID:25665008 |
| JB875                         | NOTT39; CBS7335 | Spain | alpechin (water which oozes from a heap of olives) | 1988 | PMID:25665008 |
| JB878                         | NOTT42; DBVP62804 | Malta | wine | 1963 | PMID:25665008 |
| JB879                         | NOTT43; DBVP62805 | Malta | wine | 1963 | PMID:25665008 |
| JB884                         | NOTT48; DBVP62810 | Malta | wine | 1963 | PMID:25665008 |
| JB899                         | NOTT63; Y4707 | ND | ND | ND | PMID:25665008 |
| JB900                         | NOTT64; Y831 | South Africa | industrial glucose | ND | PMID:25665008 |
| JB902                         | NOTT66; CBS374 | Netherlands (Delft) | molasses | 1928 | PMID:25665008 |
| JB910                         | NOTT74; DBVP66449; CBS1062; IF00344; NRRLFY-1358 | ND | cane sugar molasses | ND | PMID:25665008 |
| JB913                         | NOTT77; CBS1058 | Indonesia | molasses | 1949 | PMID:25665008 |
| JB914                         | NOTT78; CBS357; DBVP6280; CLIB834 | Jamaica | molasses | 1912 | PMID:25665008 |

(Continued)
| Bähler laboratory strain name | strain ID(s) | location collected | substrate | date collected | reference |
|------------------------------|--------------|--------------------|-----------|----------------|-----------|
| JB916                        | NOTT80;CBS352;DBVPG6373 | Indonesia | arrack factory (distilled alcoholic drink made from sap of the coconut flower) | 1923 | PMID:25665008 |
| JB917                        | NOTT81;CBS1057;DBVPG6375 | Sweden | brewer's yeast | 1933 | PMID:25665008 |
| JB918                        | NOTT82;CBS1059 | Mauritius | cane sugar | 1949 | PMID:25665008 |
| JB929                        | NOTT93;CBS1063;DBVPG6450; NRRLY-1362 | ND | cane sugar molasses | 1934 | PMID:25665008 |
| JB930                        | NOTT94;FRR2208 | Australia (Nundah) | glace syrup | ND | PMID:25665008 |
| JB931                        | NOTT95;FRR2535 | ND | raspberry cordial concentrate | 1983 | PMID:25665008 |
| JB934                        | CLIB837 | France | winemaking concentrate | ND | PMID:25665008 |
| JB938                        | CLIB841 | France | winemaking | ND | PMID:25665008 |
| JB939                        | CLIB842 | Spain | winemaking | ND | PMID:25665008 |
| JB942                        | CLIB845 | France | winemaking | ND | PMID:25665008 |
| JB943                        | CLIB846 | France | winemaking | ND | PMID:25665008 |
| JB953                        | PHAFF65-116 | Australia (Barossa Valley) | exudate of a Eucalyptus Tree | ND | PMID:25665008 |
| JB1110                       | YHL266;Zimmer1987_0209 | ND | red current jelly | 1977 | PMID:25665008 |
| JB1117                       | YHL281;Zimmer1987_0208 | Holland | malt | 1975 | PMID:25665008 |
| JB1154                       | AWR1875 | Australia (Barossa Valley) | ND | ND | PMID:25665008 |
| JB1171                       | CECT12622;NOTT123;IFI356 | ND | grape juice | 1982 | PMID:25665008 |
| JB1174                       | CECT12918;NOTT126;IFI2139 | ND | grape juice | 1982 | PMID:25665008 |
| JB1180                       | Kambucha_YFS76;NOTT132 | ND | ND | ND | PMID:25665008 |
| JB1197                       | NBRC0340;NOTT159;AJ14275;IFO0340;JCM1846 | ND | ND | 1983 | PMID:25665008 |
| JB1205                       | NBRC10568;NOTT1167 | ND | ND | ND | PMID:25665008 |
| JB1206                       | NBRC10569;NOTT1168 | ND | ND | ND | PMID:25665008 |
| JB1207                       | NBRC10570;NOTT1169 | ND | ND | ND | PMID:25665008 |
| JB1468                       | GZLJ3.34 | Guizhou Laojiao Distillery, Changshun, Guizhou | Dec. 2012 | NA | |
| JB1508                       | Santiago Benito V1; HE963293 | Spain | Organic Honey | 2012 | PMID:24929740 |
| JB1469                       | GZLJ3.36 | Guizhou Laojiao Distillery, Changshun, Guizhou | Dec. 2012 | NA | |
| JB1470                       | GZLJ3.41 | Guizhou Laojiao Distillery, Changshun, Guizhou | Dec. 2012 | NA | |
| JB1471                       | GZLJ5.6 | Guizhou Laojiao Distillery, Changshun, Guizhou | Dec. 2012 | NA | |
| JB1472                       | GZLJ5.28 | Guizhou Laojiao Distillery, Changshun, Guizhou | Dec. 2012 | NA | |
| JB1473                       | QT11.6 | Guicheng Laojiao Distillery, Qitai, Xinjiang | Sept. 2013 | NA | |
| JB1474                       | XEBLK30.8 | Xiaoerbulake Distillery, Xinyuan, Xinjiang | Sept. 2013 | NA | |
| JB1475                       | XEBLK1.1 | Xiaoerbulake Distillery, Xinyuan, Xinjiang | Sept. 2013 | NA | |
JB917/CBS1057 were selected during the first phase of the study. The strain V1 was selected in a previous study [5].

Second phase of *S. pombe* strain selection

Microfermentations were performed using 200 ml aliquots of concentrated must (Dream Fruits S.A., Quero, Toledo, Spain), which were diluted to glucose + fructose (G + F) concentrations of 211 g/L, enriched to 4.3 g/L malic acid (Panreac, Barcelona, Spain) (final pH 3.08), with 60 g/L of Actimax Natura (Agrovín S.A., Ciudad Real, Spain) added to provide nutrition. Each microfermentator was inoculated with 1 mL of liquid YEPD medium containing 10⁷ CFU/mL (determined using a Thomas chamber) of one of the above-mentioned yeasts. All fermentations were performed in 250-ml flasks, which were sealed with a Muller valve and filled with 98% H₂SO₄ (Panreac, Barcelona, Spain) to allow the release of CO₂ and to prevent microbial contamination [31]. The temperature was maintained at 25°C. The fermentations proceeded without aeration, oxygen injection or agitation. All fermentations were performed in triplicate.

The glucose + fructose, malic acid, acetic acid and pyruvic acid contents of the fermentations were monitored over a period of 14 days. The pH, urea, citric acid, glycerol, alcohol content, volatile compounds, amino acids and biogenic amine concentrations were determined at the end of the fermentation.

Measurements of biochemical compounds and pH

Glucose + fructose, malic acid, L-lactic acid, acetic acid, pyruvic acid, urea, glycerol and pyruvic acid analyses (Table 2) were conducted using a Y15 Autoanalyser (Biosystems, Barcelona,
Table 3. Final analysis of volatile compounds performed by the studied strains during fermentations. Selected strains are JB899/Y470, JB917/CBS1057, JB873/NCCY3422 and V1, non-selected *S. pombe* strains from the Spanish culture collection (IFI936/CECT12774 and IFI935/CECT1376) and selected *S. cerevisiae* strains (IFI87/CECT12512 and IFI88/CECT12513).

| Compounds                  | 899±0.00a | 917±0.00a | 873±0.00a | V1±0.00a | 935±0.00a | 936±0.00a | 87±0.00a | 88±0.00a |
|----------------------------|-----------|-----------|-----------|----------|-----------|-----------|----------|----------|
| l-Lactic Acid (g/L)        | 0.00      | 0.00      | 0.00      | 0.00     | 0.00      | 0.00      | 0.00     | 0.00     |
| l-Malic Acid (g/L)         | 0.02      | 0.01      | 0.02      | 0.01     | 1.02      | 0.01      | 3.16     | 3.72     |
| Acetic Acid (g/L)          | 0.24      | 0.29      | 0.33      | 0.30     | 0.93      | 0.98      | 0.21     | 0.24     |
| Residual Sugar (g/L)       | 1.77      | 1.89      | 2.02      | 2.12     | 2.11      | 1.99      | 2.22     | 2.31     |
| Glucose (g/L)              | 8.32      | 8.88      | 8.02      | 8.91     | 8.14      | 8.48      | 8.36     | 8.14     |
| pH                        | 3.44      | 3.45      | 3.44      | 3.42     | 3.36      | 3.45      | 3.14     | 3.11     |
| Urea (mg/L)                | 0.42      | 0.48      | 0.39      | 0.44     | 0.42      | 0.51      | 0.37     | 0.25     |
| Citric Acid (g/L)          | 1.27      | 0.29      | 0.29      | 0.28     | 0.28      | 0.27      | 0.28     | 0.28     |
| Alcohol (% v/v)            | 12.15     | 12.04     | 12.26     | 12.02    | 12.24     | 12.13     | 12.43    | 12.44    |

Results are the mean ± SD of three replicates. Means in the same row with the same letter are not significantly different (p < 0.05).

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Alcohol content was determined by using the boiling method GAB Microebu [33]. The pH was measured with a Crison pH Meter Basic 20 (Crison, Barcelona, Spain).

Quantification of volatile compounds

Volatile compounds (Table 3) were quantified by headspace gas chromatography–mass spectrometry (HS-GC-MS). Analyses were carried out using a Perkin-Elmer Clarus 500 gas chromatograph with a flame ionization detector, coupled to a mass spectrometer single quadrupole Clarus 560 S, all coupled to an automatic headspace sampler Turbomatrix 110 Trap (Perkin-Elmer, Massachusetts, USA). The headspace sampler conditions were: temperature of Spanish kits to perform the analyses were obtained from Biosystems [32].

Table 2. Final analysis of fermentations performed by the studied strains. Selected strains are JB899/Y470, JB917/CBS1057, JB873/NCCY3422 and V1, non-selected *S. pombe* strains from the Spanish culture collection (IFI936/CECT12774 and IFI935/CECT1376) and selected *S. cerevisiae* strains (IFI87/CECT12512 and IFI88/CECT12513).

| Compounds                  | 899±0.00a | 917±0.00a | 873±0.00a | V1±0.00a | 935±0.00a | 936±0.00a | 87±0.00a | 88±0.00a |
|----------------------------|-----------|-----------|-----------|----------|-----------|-----------|----------|----------|
| ±2-Phenyl-ethanol          | 21.76     | 22.88     | 21.44     | 23.35    | 24.17     | 24.66     | 28.97    | 29.12    |
| ±1-Propanol [mg/L]         | 11.42     | 10.12     | 13.26     | 10.73    | 18.99     | 19.76     | 28.45    | 31.33    |
| ±Isobutanol [mg/L]         | 9.31      | 8.57      | 9.86      | 8.94     | 12.11     | 13.52     | 17.51    | 18.13    |
| ±1-Butanol [mg/L]          | 5.13      | 5.45      | 5.36      | 5.28     | 7.23      | 7.44      | 7.81     | 7.96     |
| ±3-Methyl-butanol [mg/L]   | 11.36     | 12.16     | 12.19     | 12.28    | 18.44     | 18.88     | 26.12    | 28.06    |
| ±2-Methyl-butanol [mg/L]   | 3.82      | 2.48      | 2.56      | 2.19     | 3.18      | 4.45      | 4.04     | 4.23     |
| ±Isobutyl acetate [mg/L]   | n.d.      | n.d.      | n.d.      | n.d.     | n.d.      | n.d.      | n.d.     | n.d.     |
| ±Ethyl butyrate [mg/L]     | n.d.      | n.d.      | n.d.      | n.d.     | n.d.      | n.d.      | n.d.     | n.d.     |
| ±2-Phenyl-ethanol [mg/L]   | 21.76     | 22.88     | 21.44     | 23.35    | 24.17     | 24.66     | 28.97    | 29.12    |

Results are the mean ± SD of three replicates. Means in the same row with the same letter are not significantly different (p < 0.05), n.d. = not detected.

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thermostating: 80°C, time of thermostating: 45 min, type of trap: Tenax TA, cycles of purge and trap: 4, temperature of trap capture: 45°C, desorption temperature of the trap: 290°C, time of dry trap purge: 10 min, desorption time of trap: 2 min, trap cleaning time: 5 min, needle temperature: 110°C, temperature of HS-GC transfer line: 150°C, vial pressure: 30 psi and constant pressure column: 28 psi. A FFAP capillary column (60 m × 0.25 mm ID x 0.25 μm film thickness) was used. Helium (Air Liquide, España) was used as carrier gas. Gradient analysis was run using the following temperature program: 40°C (3 min); 40–80°C (2°C/min); 80–180°C (3°C/min); and 210°C (5 min). Identification of individual compounds was based on a comparison of the obtained mass spectra of the individual chromatographic peaks with those valid for the standards and available from the National Institute of Standards and Technology (Gaithersburg, MD) software library. We also compared the retention times valid for individual peaks from the wine samples with those of the known volatile components use as standard patterns. To this effect, we used Gas chromatography quality compounds as the sets of the volatile standards (Fluka, Sigma–Aldrich Corp., Buchs SG, Switzerland).

Quantification of biogenic amines

The studied biogenic amines (Table 4) were analysed using a Jasco (Tokyo, Japan) UHPLC chromatograph series X-LCTM, equipped with a Fluorescence detector 3120-FP. Gradients of solvent A (methanol/acetonitrile, 50:50, v/v) and B (sodium acetate /tetrahydrofuran, 99:1, v/v) were used in a C18 (HALO, USA) column (100 mm × 2.1 mm; particle size 2.7 μm) as follows: 60% B (0.25 ml/min) from 0 to 5 min, 60–50% B linear (0.25 ml/min) from 5 to 8 min, 50% B from 8 to 9 min, 50–20% B linear (0.2 ml/min) from 9 to 12 min, 20% B (0.2 ml/min) from 12 to 13 min, 20–60% B linear (0.2 ml/min) from 13 to 14.5 min, and re-equilibration of the column from 14.5 to 17 min. Detection was performed by scanning in the 340–420 nm range. Quantification was performed by comparison against external standards of the studied amines. The different amines were identified by their retention times.

Analytical determinations of amino acids

Selected amino acids (Table 5) were analysed using a Jasco (Tokyo, Japan) UHPLC chromatograph series X-LCTM, equipped with a Fluorescence detector 3120-FP. Gradients of solvent A (methanol/acetonitrile, 50:50, v/v) and B (sodium acetate /tetrahydrofuran, 99:1, v/v) were used in a C18 (HALO, USA) column (100 mm × 2.1 mm; particle size 2.7 μm) as follows: 90% B (0.25 ml/min) from 0 to 6 min, 90–78% B linear (0.2 ml/min) from 6 to 7.5 min, 78% B from 7.5 to 8 min, 78–74% B linear (0.2 ml/min) from 8 to 8.5 min, 74% B (0.2 ml/min) from

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**Table 4. Final analysis of biogenic amines.** Fermentations by selected *S. pombe* strains (JB899/Y470, JB917/CBS1057, JB873/NCYC3422 and V1), non-selected *S. pombe* strains from the Spanish culture collection (936/CECT12774 and IFI 935/CECT1376) and selected *S. cerevisiae* strains (IFI 87/ CECT12512 and IFI 88/CECT12513).

| Compounds            | 899    | 917    | 873    | V1     | 935    | 936    | 87     | 88     |
|----------------------|--------|--------|--------|--------|--------|--------|--------|--------|
| Histamine (mg/L)     | 0.32 ± 0.03a | 0.36 ± 0.04a | 0.32 ± 0.04a | 0.34 ± 0.03a | 0.38 ± 0.04a | 0.36 ± 0.02a | 0.33 ± 0.02a | 0.32 ± 0.03a |
| Tiramine (mg/L)      | 0.16 ± 0.01a | 0.18 ± 0.04a | 0.16 ± 0.02a | 0.17 ± 0.06a | 0.18 ± 0.04a | 0.16 ± 0.02a | 0.16 ± 0.02a | 0.15 ± 0.02a |
| Phenylethylamine (g/L) | n.d.   | n.d.   | n.d.   | n.d.   | n.d.   | n.d.   | n.d.   | n.d.   |
| Putrescine (g/L)     | 0.43 ± 0.03a | 0.46 ± 0.06a | 0.42 ± 0.05a | 0.44 ± 0.04a | 0.43 ± 0.05a | 0.45 ± 0.07a | 0.42 ± 0.03a | 0.41 ± 0.04a |
| Cadaverine (g/L)     | 0.56 ± 0.03ab | 0.55 ± 0.05ab | 0.49 ± 0.05a | 0.52 ± 0.04a | 0.52 ± 0.03a | 0.53 ± 0.03a | 0.63 ± 0.03b | 0.62 ± 0.04b |

Results represent the mean ± SD for three replicates. Means in the same row with the same letter are not significantly different (p < 0.05), n.d. = not detected.

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Table 5. Final analysis of amino acids. Fermentations by selected S. pombe strains (JB899/Y470, JB917/CBS1057, JB873/NCYC3422 and V1), non-selected S. pombe strains from the Spanish culture collection (936/CECT12774 and IFI 935/CECT1376) and selected S. cerevisiae strains (IFI 87/CECT12512 and IFI 88/CECT12513) from the Spanish culture collection.

| Compounds (mg/L) | 899 | 917 | 873 | V1 | 935 | 936 | 87 | 88 |
|------------------|-----|-----|-----|----|-----|-----|----|----|
| Aspartic acid    | 10.82±1.51   | 11.96±1.68   | 11.42±1.33   | 12.16±1.78   | 21.52±2.46   | 18.83±2.13   | 7.36±0.68   | 8.12±0.74   |
| Asparagine      | 19.68±1.56   | 20.25±1.72   | 19.93±1.84   | 21.37±2.12   | 30.18±2.89   | 29.06±2.52   | 16.34±1.09   | 17.45±1.14   |
| Serine          | 6.25±0.89    | 6.88±1.02    | 6.47±0.78    | 7.43±0.93    | 11.69±1.36   | 10.78±1.27   | 3.18±0.35    | 3.64±0.48    |
| Histidine       | 81.32±3.59   | 83.29±3.52   | 82.94±2.89   | 84.32±3.46   | 92.35±4.48   | 94.88±4.69   | 65.47±3.18   | 69.34±3.46   |
| Glycine         | 28.42±1.33   | 27.83±1.16   | 28.48±1.26   | 29.68±1.44   | 39.68±1.77   | 41.12±2.24   | 27.43±1.11   | 28.56±1.23   |
| Arginine        | 66.52±4.62   | 64.52±3.96   | 63.14±3.54   | 69.52±5.43   | 78.21±6.22   | 76.52±5.84   | 42.43±3.53   | 40.88±3.17   |
| Threonine       | 53.17±3.46   | 51.19±2.98   | 46.23±2.72   | 49.94±2.56   | 37.46±2.97   | 36.82±2.64   | 24.72±1.82   | 22.58±0.21   |
| Alanine         | 27.31±2.07   | 28.43±2.16   | 32.05±3.08   | 29.86±3.17   | 42.57±3.21   | 43.26±3.45   | 31.68±2.49   | 29.72±2.28   |
| Tyrosine        | 9.82±0.68    | 10.13±0.62   | 11.28±0.86   | 10.22±0.75   | 12.88±0.93   | 13.31±1.14   | 4.48±0.26    | 4.69±0.34    |
| Valine          | 4.73±0.31    | 5.29±0.44    | 4.82±0.35    | 5.18±0.42    | 2.96±0.27    | 3.18±0.31    | 1.25±0.19    | 1.14±0.16    |
| Tryptophan      | 1.41±0.26    | 1.46±0.22    | 1.59±0.28    | 1.54±0.23    | 1.76±0.23    | 1.81±0.29    | 0.52±0.13    | 0.46±0.07    |
| Phenylalanine   | 5.54±0.42    | 5.26±0.38    | 5.69±0.51    | 5.89±0.54    | 7.62±0.86    | 7.98±1.02    | 3.65±0.29    | 3.78±0.33    |
| Isoleucine      | 12.04±0.74   | 12.56±0.78   | 12.64±0.76   | 13.24±0.88   | 8.17±0.63    | 8.35±0.68    | 2.45±0.32    | 2.62±0.36    |
| Leucine         | 15.18±0.93   | 14.69±0.87   | 15.92±1.05   | 15.86±0.92   | 10.22±0.81   | 11.46±0.98   | 3.87±0.68    | 3.98±0.67    |
| Ornithine       | 18.36±1.65   | 17.87±1.42   | 18.45±1.72   | 19.88±1.78   | 19.93±1.86   | 20.98±1.89   | 29.48±2.27   | 30.74±2.36   |
| Lysine          | 12.27±0.96   | 12.59±0.88   | 12.72±0.91   | 14.13±1.02   | 23.56±1.94   | 24.56±2.33   | 6.16±0.85    | 6.77±0.94    |
| Methionine      | 2.68±0.43    | 2.89±0.36    | 2.73±0.41    | 3.27±0.52    | 6.26±0.89    | 6.47±0.97    | 1.23±0.19    | 1.29±0.22    |

Results represent the mean ± SD for three replicates. Means in the same row with the same letter are not significantly different (p < 0.05).

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8.5 to 11 min, 74–50% B linear (0.2 mL/min) from 11 to 15 min, 50% B (0.2 mL/min) from 15 to 17 min, 50–20% B linear (0.2 mL/min) from 17 to 21 min, 20–90% B linear (0.2 mL/min) from 21 to 25 min, and re-equilibration of the column from 25 to 26 min. Detection was performed by scanning in the 340–455 nm range. Quantification was performed by comparison against external standards of the studied amino acids. The different amino acids were identified by their retention times.

Fermentation in stress conditions

Study of fermentation in stress caused by ethanol, sulphur dioxide and the osmotic stress caused by salts (NaCl and KCl), were performed based on methods described previously for S. cerevisiae [34]. 15 mL sealed test tubes containing 10 mL of sterilized grape must were inoculated with the studied strains. The grape must final sugar concentration was 206 g/L diluted from a concentrated grape must (Dream Fruits S.A., Quero, Toledo, Spain) that was corrected to the respective stressful conditions before yeast inoculation: Ethanol (6%; 10%; 12% and 14%), KCl (0.75 M), NaCl (1.5 M) (both osmotic stress) and KHSO₃ (150 and 300 mg/L) (sulphur dioxide). Ethanol, KCl, NaCl products were from Panreac (Panreac, Barcelona, Spain) and KHSO₃ was from Agrovín S.A. (Alcázar de San Juan, Spain). The testing tubes were inoculated to a cellular density of 5x10⁶ cells/mL with the studied strains: S. pombe strains (JB899/Y470, JB917/CBS1057, JB873/NCYC3422, JB4/NCYC683, JB837/NOTT1 and V1), non-selected S. pombe strains from the Spanish culture collection (936/CECT12774 and IFI 935/CECT1376) and selected S. cerevisiae strains (IFI 87/CECT12512 and IFI 88/CECT12513) from the Spanish culture collection. Final optical density was determined at 640 nm after 24 h of incubation at 25°C by an Y15 spectrophotometer (BioSystems, Barcelona, Spain). The maximal ethanol production was estimated through the fermenting power method [35,36]. In this
case the original concentrated grape must (Dream Fruits S.A., Quero, Toledo, Spain) was diluted to a sugar concentration of 300 g/L, the sealed flasks with Müller trapflasks (Alamo, Madrid, Spain) were incubated at 25°C. All the tests were performed in triplicate.

Sensory analysis

For a sensory analysis, fermentations triplicate with *S. pombe* strains (JB899/Y470, JB873/NCYC3422, JB917/CBS1057, JB4/NCYC683, JB837/NOTT1, 936/CECT12774, IFI935/CECT1376 and V1) and *S. cerevisiae* strains (IFI 87/CECT12512 and IFI88/CECT12513) were performed with microvinifications with similar methodology to previously described [13, 15]. Sealed 1 L flasks with a Müller trapflasks (Alamo, Madrid, Spain) containing 800 ml of sterilised (115°C, 15 min) concentrated must (Dream Fruits S.A., Quero, Toledo, Spain), which was diluted to 202 g/L glucose + fructose and enriched with 4 g/L malic acid (Panreac, Barcelona, Spain) (final pH 3.11). The flasks were inoculated with the studied strains to initial population of 10⁶ CFU/mL. The completion of the fermentation process was verified by weight loss and confirmation that final enzymatic analysis of glucose + fructose was below 3 g/L after fermenting at 25°C. After fermentation, wines were racked and stored for 7 days at 4°C in 750 mL wine bottles. The final product was bottled in 350 mL wine bottles, sealed bottles and stored horizontally in a climate chamber at 4°C for two weeks until sensory evaluation.

Final wines were assessed (in a blind test) by a panel of 10 experienced wine tasters, all members of the staff of the Food Technology Department of the Technical University of Madrid, Spain. Assessments took place in standard sensory analysis chambers with separate booths. Following the generation of a consistent terminology by consensus, one visual descriptor, four aromas and four taste attributes were chosen to describe the wines. Formal assessment consisted of two sessions held on different days where wine tasters tasted all fermented triplicates. The panellists used a 10-cm unstructured scale, from 0 (no character) to 10 (very strong character), to rate the intensity of the 10 attributes.

Statistical analyses

All statistical analyses were performed using PC Statgraphics v. 5 software (Graphics Software Systems, Rockville, MD, USA). The significance was set to p < 0.05 for the ANOVA matrix F value. The multiple range test was used to compare the means.

Results and Discussion

First phase of *S. pombe* strain selection

A specific advantage of *Schizosaccharomyces* yeast in winemaking is that it degrades malic acid. To examine this with our strains, we initiated fermentation of grape must supplemented with malic acid (4 g/L). After fermenting the 75 *S. pombe* strains in test tubes for 21 days, we measured malic acid. We also measured two basic parameters, residual glucose + fructose and acetic acid production, which are typically used in the selection of fermentative yeasts of the genus *Saccharomyces*, the main yeast genus used in winemaking. The final concentrations of glucose + fructose after fermentation were close to 0 g/L (Fig 1a). This finding is in agreement with data reported by other authors, and is indicative of strong fermentative power [37,3]. This property allows the production of dry wines that are most popular in the world market.

The degradation of malic acid, which currently represents the primary industrial application of *Schizosaccharomyces* yeasts, was nearly 100% in all the studied strains (Fig 1b). These data are comparable to those from other authors, who reported malic acid degradation rates ranging between 75 and 100% [38].
Out of the 75 initial *S. pombe* strains tested, however, only five strains (JB4/NCYC683, JB837/UWOPS92.229.4, JB873/NCYC3422, JB899/Y470 and JB917/CBS1057) showed moderate final concentrations of acetic acid (\(<0.4\) g/L) (Fig 1c). According to these results, just 6.5% of the *S. pombe* strains could be used for producing quality wine. In a previous study, a similarly low rate of 5% was reported [5]. Three of these strains (JB837, JB873 and JB899) showed acetic acid concentration lower than 0.3 g/L. These data demonstrate that it is possible to identify *S. pombe* strains that can produce quality wines (Fig 1c).
Comparison between pre-selected *S. pombe* strains and other culture collection strains

After identifying the three *S. pombe* strains that show low acetic acid production, we studied additional features in detail. Microvinifications (200 mL) were performed to verify the acetic acid data and to confirm the proper fermentative ability of the pre-selected strains. In this portion of the study, the pre-selected *S. pombe* strains (JB837, JB873 and JB899) were compared with two *S. cerevisiae* strains (87/CECT12512 and 88/CECT12513) and with three other *S. pombe* strains (V1, 935/CECT1376 and 936/CECT12774) that had been tested previously [5].

**Acetic acid.** The maximal final concentrations of acetic acid were approximately 0.3 g/L (Fig 2.a.1; Table 2) for all selected strains, while the *S. pombe* strains from the Spanish Type Culture Collection produced acetic acid concentrations of up to 1 g/L (Fig 2.b.1; Table 2).

![Fig 2. Fermentation kinetics of acetic acid (1), malic acid (2) and glucose + fructose (3) for (a) selected *S. pombe* strains (JB899/Y470, JB917/CBS1057, JB873/NCYC3422 and V1); (b) non-selected *S. pombe* strains from the collection (935 and 936); (c) selected *S. cerevisiae* strains (87 and 88).](https://plosone.org/doi/10.1371/journal.pone.0151102.g002)
Therefore, when used as a single strain during fermentation, the latter strains are not suitable for the production of quality wines [14,5].

**Malic acid.** The malic acid degradation was almost complete for most of the studied *S. pombe* strains (Fig 2.a.2 and 2.b.2; Table 2), although the degradation kinetics differed. The two *S. cerevisiae* strains degraded 11–24% of the initial malic acid content in the must (Fig 2.c.2; Table 2). Several authors have proposed similarly high malic acid degradation for yeast belonging to other genera than *Schizosaccharomyces* [5, 39–43]. The malic acid reduction influenced the final pH of the wine (Table 2), as *S. pombe* fermentations showed up to 0.34 higher pH values than *S. cerevisiae* fermentations.

**Sugar consumption kinetics.** The consumption kinetics of glucose + fructose was more rapid in the *S. cerevisiae* strains than in most studied *S. pombe* strains (Fig 2.a.3, 2.b.3 and 2.c.3; Table 2). Similar results have been described before [6]. Nevertheless, differences in degradation kinetics of glucose + fructose between the studied *S. pombe* strains were evident (Fig 2.a.3 and 2.b.3).

**Pyruvic acid.** All studied *S. pombe* strains produced more pyruvic acid than the *S. cerevisiae* yeasts (Fig 3). Strain JB873 yielded a maximum pyruvic acid concentration of 487 mg/L after 48 h of fermentation, which is a slightly higher concentration than those obtained in previous studies [5,6]. The other *S. pombe* strains provided a maximum pyruvic acid content that varied from 254 to 276 mg/L, except for the case of *S. pombe* JB899 that reached a pyruvic acid content of 374 mg/L (Fig 3). Previous pyruvic acid studies with *S. cerevisiae* and non-*Saccharomyces* species other than *Schizosaccharomyces* showed maximum values of about 150 mg/L during the first stages of fermentation [43]. These values are substantially lower than those obtained for the studied *S. pombe* strains. There is a strong correlation between the amount of

![Fig 3. Fermentation kinetics of pyruvic acid for selected *S. pombe* strains (JB899/Y470, JB917/CBS1057, JB873/NCYC3422 and V1), non-selected *S. pombe* strains from the collection (935 and 936) and selected *S. cerevisiae* strains (87 and 88).](image)

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pyruvic acid released by yeasts and the formation of vitisin A [3,5,15]; vitisin A is a stable pigment that influences wine colour quality. Thus, the selected strains with high pyruvate production rates such as JB873 and JB899 may be of interest in red wine production.

Glycerol. Schizosaccharomyces species have previously been reported to produce more glycerol than Saccharomyces species [15]. In this study, final levels of glycerol varied from 8.02 g/L to 8.91 g/L (Table 2). Even though two S. pombe strains showed the highest levels (V1 and JB917), several differences were observed between the studied strains, and not in every case were the values higher than those obtained for the two S. cerevisiae strains. Increased glycerol content has been described as one of the main contributors of non-Saccharomyces strains on wine quality [1,44].

Ethanol. Among all strains tested, the final ethanol levels varied from 12.04 to 12.44 (% vol/vol) (Table 2). The fermentations involving S. pombe produced very similar ethanol levels to the S. cerevisiae strains we examined (Table 2). Among the S. pombe strains, only slight differences in final ethanol levels of less than 0.24 (% vol/vol) were evident. In contrast other authors have shown that some non-Saccharomyces types of yeast produce lower ethanol yields than Saccharomyces [45–47]. The sugar metabolism can also be used to produce compounds other than ethanol, such as glycerol or pyruvic acid, or to increase the yeast biomass [48,49]. Previous studies with S. pombe showed similar results to those obtained here [13]. Other authors observed lower final ethanol levels using other non-Saccharomyces species under specific high aeration conditions [50,51].

Urea. The urea content of the finished wines was lower in the fermentations involving S. pombe, with values less than 0.5 mg/L (Table 2). This result can be attributed to the special ability of the Schizosaccharomyces genus to produce urease [12,52]. This enzymatic activity also could reduce the initial level of precursors for ethyl carbamate (one of the most toxic compounds reported in wine) [3,15]. This factor is becoming increasingly important as ethyl carbamate is a known carcinogen that is present in a variety of fermented foods [53].

Citric acid and lactic acid. No differences were evident with respect to citric and lactic acid (Table 2), as no wine performed malolactic fermentation [6] or Lachancea thermotolerans species were used [54]. Note that after a traditional malolactic fermentation process by lactic bacteria, all citric acid could be converted into acetic acid; this collateral effect increases the final acetic acid content [6] and consequently slightly reduces wine quality.

Volatile aromas. Higher alcohols were produced in higher total concentrations by S. cerevisiae fermentations (Table 3). Some differences were observed between the studied S. pombe strains (Table 3). Other authors described non-Saccharomyces yeasts as lower producers of higher alcohols than S. cerevisiae [42,43,54–57], and much strain variability has been reported [58,59]. This finding could be of interest in facilitating the making of wines with typicity for specific grape varieties or to increase wine complexity [60]. Similarly, the tested S. pombe strains produced less esters than the S. cerevisiae strains. No differences between the S. pombe and S. cerevisiae strains were observed with respect to compounds considered negative, such as ethyl acetate and diacetyl. However, those compounds could increase after a malolactic fermentation process performed by lactic bacteria [15]. Significant differences in compounds such as acetaldehyde and ethyl acetate were evident between the different S. pombe strains.

Biogenic amines. During the past years, harmful effects of biogenic amines [61–64] have been demonstrated, which now constitutes a serious matter in food safety that must be taken into account. A histamine value of 2 mg/L is considered the maximum level [65] in some countries. All studied strains reported values below that threshold (Table 4). The study shows that S. pombe does not produce higher levels of biogenic amines than S. cerevisiae. Subtle statistical differences were observed in the case of cadaverine (Table 4), but this biogenic amine is not produced by yeasts, and the slight differences could reflect that some strains remove biogenic
amines during the fermentation process; similar processes have been described before [66]. However, most biogenic amines are produced during wine ageing and malolactic fermentation [67], as they are compounds produced primarily by lactic acid bacteria [68,69]. Thus, the wines that still contain malic acid (Fermentations by *S. cerevisiae*) could increase their biogenic amine contents later on [6,16].

**Amino acids.** *S. pombe* fermentations showed higher final levels in most amino acids (Table 5). Similar results were obtained before, as it has been described as demanding less nitrogen [14] and releasing more nitrogen [7] than *S. cerevisiae*. However some differences were observed among the studied strains (Table 5). *S. cerevisiae* fermentations produced a higher final level in ornithine. The observed differences in threonine, valine, isoleucine and leucine explain the differences measured for higher alcohols (Table 5), because they are precursors of 1-propanol, isobutanol, 2-methylbutanol and 3-methylbutanol, respectively (Table 5) [43]. The statistical differences reported for histidine, tyrosine and lysine show that *S. pombe* fermentations increase the content of some biogenic amine precursors [65,67]. Nevertheless, the transformation of some of those precursors to biogenic amines takes place during the malolactic fermentation, therefore the wines fermented by *S. pombe* did not run a serious risk of increasing the levels of histamine or tyramine since they do not need any longer a malolactic fermentation [16].

**Fermentations in stress conditions**

Several authors have reported how important is to study yeast performance under stress conditions [70,71]. After performing the stress tests conditions for the selected strains (Table 6) for some parameters described before in literature [34]. We observed that different phenotypic cases were detected for the studied strains in the studied parameters. Other authors have reported before the importance of phenotypic performance in yeasts [72,73]. According to these results strain *S. pombe* 936/CECT12774 would develop better than the others *S. pombe* under circumstances similar to KCl test and strain JB837/NOTT1 would not be able to perform properly. In the NaCl test strains JB873/NCYC3422, JB4/NCYC683, 935/CECT1376 and 936/CECT12774 performed slightly better than the others *S. pombe*. For the ethanol resistance tests, strains JB873/NCYC3422, 935/CECT1376 and 936/CECT12774 could perform better than the others. In the case of KHSO₃ test, strains JB873/NCYC3422, JB4/NCYC683, 935/CECT1376

| Compounds (mg/L) | 899 | 917 | 873 | 4 | 837 | V1 | 935 | 936 | 87 | 88 |
|------------------|-----|-----|-----|---|-----|----|-----|-----|----|----|
| KCl (0.75 M)     | 1   | 1   | 1   | 2 | 2   | 0  | 1   | 2   | 3  | 3  |
| NaCl (1.5 M)     | 0   | 0   | 0   | 1 | 1   | 0  | 1   | 1   | 1  | 1  |
| Ethanol 6% (% v/v)| 1   | 1   | 1   | 2 | 2   | 1  | 1   | 1   | 2  | 3  |
| Ethanol 10% (% v/v)| 0 | 0   | 0   | 0 | 0   | 0  | 1   | 1   | 1  | 1  |
| Ethanol 12% (% v/v)| 0 | 0   | 0   | 0 | 0   | 0  | 0   | 0   | 1  | 1  |
| Ethanol 14% (% v/v)| 0 | 0   | 0   | 0 | 0   | 0  | 0   | 0   | 0  | 0  |
| KHSO₃ (150 mg/L) | 2   | 2   | 3   | 3 | 3   | 1  | 2   | 3   | 3  | 3  |
| KHSO₃ (300 mg/L) | 1   | 1   | 2   | 2 | 2   | 0  | 1   | 2   | 2  | 3  |
| Fermenting power | 11.21| 11.39| 12.36| 12.42| 10.68| 11.33| 12.44| 12.61| 13.81| 13.68|
| (Ethanol (% v/v)) | ±0.18b| ±0.24b| ±0.21c| ±0.23c| ±0.28a| ±0.22b| ±0.31c| ±0.26c| ±0.29d| ±0.33d|

Results represent the mean ± SD for three replicates. Means in the same row with the same letter are not significantly different (p < 0.05).

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and 936/CECT12774 showed to be more resistant. The highest fermenting powers were obtained by strains JB873/NCYC3422, JB4/NCYC683, 935/CECT1376 and 936/CECT12774 among the \textit{S. pombe} strains. Even though the values were better for the \textit{S. cerevisiae} controls, these differences could be related to the slower developing kinetic describe for \textit{S. pombe} than \textit{S. cerevisiae} that was observed in the previous trials and reported by other authors [6]. In fact \textit{S. pombe} has been described before as highly resistant to some stress conditions [23].

**Sensory evaluation**

A sensory evaluation was performed to verify that the selected strains had the potential to produce wines that were pleasant to drink. Fig 4 shows a spider web diagram of the average scores of some olfactory and taste attributes. Large differences in the perception of acidity were recorded; this result agrees with acidity parameters explained above in the previous fermentations. Slight differences were reported regarding to sweetness even though all fermentations depleted successfully all the sugars, this can be explained by differences in acid-sweet balance. Serious faults were reported for non-selected \textit{S. pombe} strains IFI935/CECT1376 and 936/CECT12774 regarding to high acetic acid and reduction characters. Fermentations by selected \textit{S. pombe} strains JB899/Y470 and JB873/NCYC3422 received the best scores from all tasters while non-selected IFI935/CECT1376 and 936/CECT12774 received the lowest scores, \textit{S. cerevisiae} strains IFI87/CECT12512 and IFI88/CECT12513 received moderate scores in overall impression scores related to excessive high acidity for the tasters. These results show that \textit{S. pombe} can perform better than \textit{S. cerevisiae} under very acidic conditions. The above data show that all fermentations with \textit{S. pombe} achieved the main goals related to malic acid deacidification from a very acidic must.

**Conclusions**

Among 75 \textit{S. pombe} strains tested, we have discovered three strains with highly promising winemaking properties. These strains could be used to produce wines with low levels of malic acid.
acid, acetic acid, ethyl carbamate and biogenic amines, and with an appropriate volatile aroma profile. Because these \textit{S. pombe} strains produced similar ethanol as the budding yeast strains we examined, this species may well have untapped potential for the producing of ethanol as a fuel (‘bioethanol’).

**Supporting Information**

SI Table. First Selection Phase Results. (XLSX)

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**Author Contributions**

Conceived and designed the experiments: SB DJ FC FP JB FB. Performed the experiments: AB DJ FC FP JB FB SB. Analyzed the data: AB DJ FC FP JB SB. Contributed reagents/materials/analysis tools: SB JB DJ FB. Wrote the paper: AB DJ FC FP JB FB SB.

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