1 **Mixed Yeast Communities Contribute to Regionally Distinct Wine Attributes**

2 Diana Lynne Hawkins[1]
3 Jess Ryder[1]
4 Soon A. Lee[1]
5 Katie Parish-Virtue[2]
6 Bruno Fedrizzi[2]
7 Matthew R. Goddard[1,3]
8 Sarah J. Knight[1]
9
10 Affiliations
11 [1]School of Biological Sciences, University of Auckland, Auckland 1142, New Zealand
12 [2]School of Chemical Sciences, University of Auckland, Auckland 1142, New Zealand
13 [3]School of Life and Environmental Sciences, University of Lincoln, Lincoln, LN6 7DL, United
14 Kingdom
15
16 Corresponding author: Diana Hawkins, Diana.L.Hawkins@gmail.com, Tel.: +6421883085
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18 **Abstract**

19 There is evidence that vineyard yeast communities are regionally differentiated, but the extent to which
20 this contributes to wine regional distinctiveness is not yet clear. This study represents the first
21 experimental test of the hypothesis that mixed yeast communities - comprising multiple, region-
22 specific, isolates and species - contribute to regional wine attributes. Yeast isolates were sourced from
23 uninoculated Pinot Noir fermentations from 17 vineyards across Martinborough, Marlborough and
24 Central Otago in New Zealand. New methodologies for preparing representative, mixed species
25 inoculum from these significantly differentiated regional yeast communities in a controlled, replicable
26 manner were developed and used to inoculate Pinot Noir ferments. Twenty-eight yeast-derived aroma
27 compounds were measured in the resulting wines via Headspace Solid-Phase Microextraction coupled
28 with Gas Chromatography-Mass Spectrometry. Yeast community region of origin had a significant
29 impact on wine aroma, explaining ~10% of the observed variation, which is in line with previous
30 reports of the effects of region-specific *S. cerevisiae* isolates on Sauvignon Blanc ferments. This study
31 shows that regionally distinct, mixed yeast communities can modulate wine aroma compounds in a
32 regionally distinct manner and are in line with the hypothesis that there is a microbial component to
33 regional distinctiveness, or *terroir*, for New Zealand Pinot Noir.

34 **Introduction**

35 Wine is well known for its regional distinctiveness, with the same grape varieties grown in different
36 localities exhibiting different attributes. Regional distinctiveness is a point of differentiation for certain
37 consumers, and thus distinctiveness can have economic value for wine producers (Van Leeuwen and
38 Seguin 2006). Historically, regional distinctiveness, or *terroir*, has been attributed to regional
39 differences in climate, soil, annual weather patterns, aspect, and cultural vineyard practices, among
40 other factors, but a role for microbes has not been considered (Van Leeuwen and Seguin 2006;
41 Alexandre 2020). However, an increasing number of studies across a number of countries have
42 demonstrated that viticultural regions harbour regionally distinct microbial communities (Gayevskiy
43 and Goddard 2012; Bokulich *et al.* 2013, 2016; Knight *et al.* 2015; Griggs *et al.* 2021). This, combined
44 with evidence that different species and strains of yeast impart distinct flavours and aromas to wine
45 (Howell *et al.* 2004; Swiegers and Pretorius 2005; Sumby, Grbin and Jiranek 2010; Hall, Durall and
46 Stanley 2011; Hall *et al.* 2017; Tempère *et al.* 2018) suggests that microbes may contribute to a wine's
47 regional distinctiveness, or *terroir*.
48
49 Key wine aroma compounds, such as esters, higher alcohols, carbonyl compounds, sulfur compounds,
50 volatile phenols, and volatile acids, have been directly linked to yeasts' metabolic processes during
51 fermentation (Howell *et al.* 2004; Swiegers and Pretorius 2005; Sumby, Grbin and Jiranek 2010; Zott
52 *et al.* 2011; Franc and Polona Zabukovec and Hans-Josef 2017; Tempère *et al.* 2018; Kinzurik *et al.*
53 2020). The production of these compounds has been found to vary amongst yeast species and strains,
54 resulting in differences in the type and quantity of aroma compounds in wines fermented by different
55 species and strains (Howell *et al.* 2004; Swiegers and Pretorius 2005; Sumby, Grbin and Jiranek 2010;
56 Hall, Durall and Stanley 2011; Hall *et al.* 2017; Tempère *et al.* 2018). Further, in ferments with more
57 than one species or strain of yeast, interactions between yeasts, including metabolite sharing, may
58 further modulate final wine aroma (Bordet *et al.* 2020) and this variance in aroma cannot be replicated
59 by simply blending together the wines produced by individual species or strains (Howell *et al.* 2006;
60 Anfang, Brajkovich and Goddard 2009). If specific combinations of different yeasts produce specific
61 types and amounts of metabolites, and there is evidence for different specific combinations of unique
62 yeasts (communities) in different regions, it is reasonable to predict that this can result in a microbial
63 aspect to *terroir*.
64
65 Many studies report that vineyard yeast are transported to wineries on grapes, are present in grape
66 must, and contribute to wine fermentations (Fleet 2003; Grainger and Tattersall 2016; Martiniuk *et al.*
67 2016; Hall *et al.* 2017; Morrison-Whittle and Goddard 2018). Consequently, uninoculated
68 fermentations are a way of capturing the contributions of local yeast communities during fermentation
69 (Sumby, Grbin and Jiranek 2010; Gayevskiy and Goddard 2012; Bokulich *et al.* 2013; Medina *et al.*
70 2013; Taylor *et al.* 2014; Šuranská, Vránová and Omelková 2016). Such uninoculated fermentations
71 typically comprise multiple yeast species, whose population numbers, species type, and strains are
72 often hard to characterise (Povhe Jemec *et al.* 2001; Selli *et al.* 2005; Goddard 2008; Zott *et al.* 2008;

73 Medina *et al.* 2013; Šuranská, Vránová and Omelková 2016; Bagheri, Bauer and Setati 2017). Multiple
74 yeast species of varying ethanol tolerances are present at the beginning of uninoculated fermentations
75 (Povhe Jemec *et al.* 2001; Selli *et al.* 2005; Di Maro, Ercolini and Coppola 2007; Goddard 2008; Zott
76 *et al.* 2008; Bokulich *et al.* 2013; Medina *et al.* 2013; Šuranská, Vránová and Omelková 2016; Bagheri,
77 Bauer and Setati 2017; Stefanini and Cavalieri 2018). As fermentations progress, in addition to
78 metabolites that modulate wine aroma, some yeast species produce toxins and ethanol allowing them to
79 outcompete others (De Deken 1966; Young and Yagiu 1978; Povhe Jemec *et al.* 2001; Goddard 2008;
80 Ciani and Comitini 2015; Šuranská, Vránová and Omelková 2016; Tempère *et al.* 2018). If present,
81 *Saccharomyces* species, particularly *S. cerevisiae*, are responsible for the fermentation of most sugars
82 due to their ability to produce and tolerate increasing ethanol and elevated temperatures (Swiegers and
83 Pretorius 2005; Thomson *et al.* 2005; Di Maro, Ercolini and Coppola 2007; Goddard 2008; Šuranská,
84 Vránová and Omelková 2016; Varela and Borneman 2017; Englezos *et al.* 2018). Consequently, the
85 diversity of species is typically greater at the early stages of fermentation (Selli *et al.* 2005; Goddard
86 2008; Zott *et al.* 2008).
87
88 There are some compelling studies that have shown correlations between regional differences in grape
89 microbiomes and wine metabolomes (e.g. Bokulich *et al.* 2016 Drumonde-Neves *et al.* 2017) but
90 correlation does not demonstrate causation as another region-specific factor may have driven
91 differences in both the microbiomes and wine chemistry. However, objective, controlled direct
92 experiments to test whether there is a microbial aspect to *terroir* are limited (Alexandre 2020).
93 Empirical tests of whether the entire grape associated microbiome contributes to regional wine
94 attributes would be impossible as most grape microbes do not grow on artificial laboratory media. One
95 estimate is that 95% of wine grape associated fungi do not grow on standard media (Taylor *et al.*
96 2014), and therefore it is not currently possible to isolate and grow the total microbial community from
97 the fruits or juice to derive an experimental inoculum to conduct such tests. However, most yeast
98 components of the grape microbiome are able to be cultured in the laboratory. Yeast communities that
99 derive from the local environment are abundant in spontaneous ferments (Taylor *et al.* 2014; Morrison-
100 Whittle and Goddard 2018) but also contribute to inoculated ferments that are sulfured, and thus locally
101 derived yeast communities variously contribute to fermentation (Povhe Jemec *et al.* 2001; Selli *et al.*
102 2005; Goddard 2008; Zott *et al.* 2008; Medina *et al.* 2013; Šuranská, Vránová and Omelková 2016;
103 Bagheri, Bauer and Setati 2017).
104
105 Sauvignon Blanc fermented by regionally distinct populations of *Saccharomyces cerevisiae*, the work
106 horse of wine fermentation, provided the first and only experimental evidence of microbially driven
107 regional distinctions in wine phenotypes that we are aware of (Knight and Goddard 2015; Knight *et al.*
108 2015); however, as discussed, wine fermentation is more complex (Selli *et al.* 2005; Goddard 2008;
109 Zott *et al.* 2008) and we are aware of no tests as to whether regionally distinct yeast communities
110 produce different wine chemistries or not.
111

The Martinborough, Marlborough and Central Otago regions represent 85% of New Zealand's Pinot Noir production (New Zealand Wine Growers 2022a) and are known to vary by climate, soil, geography and crucially vineyard associated yeast communities (Knight and Goddard 2015; Morrison-Whittle and Goddard 2015). Thus, NZ Pinot Noir provides an excellent system to test and quantify whether mixed yeast communities contribute to wine regional distinctiveness. To evaluate the impact that mixed yeast communities have on wine aroma, yeasts were isolated from each of the regions and representative communities were reconstructed and then inoculated into a standardised Pinot Noir grape juice. Here we test the hypothesis that mixed yeast communities - comprising multiple, region-specific, culturable isolates and species – contribute to regional wine attributes. While simplified from the true complexity of the fruit and ferment microbial environment, using representative culturable yeast communities enables these naturally occurring ecosystems to be emulated in a controlled manner (De Roy *et al.* 2014; Ponomarova and Patil 2015). This not only allows objective empirical tests of this hypothesis but also provide potential practical tools for winemakers. As far as we are aware, this is the first time mixed yeast communities have been objectively tested for their contribution to *terroir*.

Methodology

Regional yeast community isolation

Fruit was collected from six Pinot Noir vineyards in each of the three geographic regions tested approximately two days before commercial harvest (Figure 1). Within each vineyard site, grapes were collected and pooled from nine focal vines that captured the topological variability observed. Fruit was collected into sterile plastic bags using snips sterilised with Trigene (10% v/v), chilled to 4°C and transported to the University of Auckland for processing.

In the laboratory, a total of 20kg of fruit from each vineyard site was weighed, hand destemmed using sterile gloves and combined into sterile 20 L fermentation vessels for each vineyard. The grapes were crushed by hand within the vessel and the starting Brix and yeast assimilable nitrogen (YAN) were measured. The YAN was adjusted to a minimum of 200mg/L using Diammonium Phosphate; if the YAN was above 200mg/L no Diammonium Phosphate addition was made. The ferments were warmed to 20 ± 3 °C to initiate uninoculated fermentation and the ferment weights (El Haloui, Picque and Corrieu 1988).

Brix and temperature were monitored daily to track fermentation progress. A 10mL sample to capture the yeast communities present early in these ferments were taken after a reduction of 2° Brix. The composition of the yeast community changes dramatically during fermentation and reduces in diversity (Goddard *et al.* 2010). By sampling as the ferment begins to accelerate, we aimed to capture the widest diversity of isolates that actively metabolise during fermentation, while excluding those that were incidentally present on the grapes but do not contribute to fermentation. Unfortunately, the desired sampling time point for vineyard CFRP in Central Otago was missed, which reduced the number of

150 regional yeast communities from this region to five, rather than six. Samples were stored in 15% (v/v)
151 glycerol at -80 °C to preserve the yeast communities prior to isolation.
152
153 Frozen must samples were thawed, and serial dilutions were plated onto YPD (1% Yeast Extract, 2%
154 Peptone, 2% Glucose, 2% Agar) and incubated at 28 °C for 48 hours. Single colonies were selected in
155 an unbiased manner using a grid-like process until 96 individual isolates were obtained from each
156 sample, or all viable single colonies were selected, whichever occurred first. This resulted in a mixed
157 yeast community of up to 96 individual yeast isolates from each vineyard site. Individual isolates were
158 stored in 96-well culture plates in 15% glycerol at -80°C until further analysis.
159
160 ***Taxonomic identification of representative yeast communities***
161 Frozen yeast isolates were revived in liquid YPD and DNA was extracted using 1.25 mg/mL
162 Zymolyase in 1.2 M sorbitol and 0.1 M KH_2PO_4 at pH 7.2 at 37 °C for 30 minutes followed by 10
163 minutes at 95°C and 1 minute at 15°C to lyse the cells (Knight and Goddard 2015). The ITS1-5.8S
164 rRNA-ITS2 region was amplified via PCR using the ITS1 and ITS4 primers (White *et al.* 1990)
165 following (Goddard 2008). Following amplification, *HaeIII* and *Hinf1* restriction enzymes were used
166 separately to digest the ITS amplicons (Esteve-Zarzoso *et al.* 1999). The digested ITS fragments were
167 visualised by gel electrophoresis and isolates were grouped into cohorts based on visual assessment of
168 band patterns (Esteve-Zarzoso *et al.* 1999).
169
170 Three individuals from each cohort were selected for Sanger sequencing such that isolates from each
171 geographic region were equally represented. If individuals within a cohort were not found across all
172 three regions, three individuals were taken from three different vineyard sites to avoid selecting clones.
173 PCR amplification of the D1/D2 26S rRNA region was performed following Gayevskiy and Goddard
174 (2012), amplicons were cleaned via NucleoSpin Gel and a PCR Clean-Up kit (Macherey-Nagel) and
175 sequenced via Dye Terminator Sanger Sequencing at Auckland Genomics at the University of
176 Auckland. Once sequenced, the DNA fragments were subjected to BLAST analyses against the NCBI
177 nucleotide database to identify the species of the microorganism in question.
178
179 ***Lab-scale fermentation to test contribution of regional yeast communities to terroir***
180 *Grape must preparation and sterilisation*
181 Pinot Noir juice and solids for the fermentation trials were prepared from frozen commercially
182 produced Pinot Noir grapes from the 2018 harvest from across the Martinborough, Marlborough and
183 Central Otago regions. Fruit was thawed, hand-destemmed, macerated and mixed to create a
184 standardised homogenised must. A 20% v/v solution of dimethyl dicarbonate (DMDC) in ethanol was
185 applied at a rate of 300 µL/L to sterilise the grape must (Daudt and Ough 1980; Delfini *et al.* 2002;
186 Costa *et al.* 2008) for 8 hours at 22 ± 1 °C. Aliquots of 100 mL of juice were transferred along with 25
187 mL of grape skins and seeds into sterilised tubes and stored at -80 °C until required.
188

189 The day before inoculation, the frozen must aliquots were thawed and an additional DMDC treatment
190 was employed at a rate of 200 µL/L overnight at 22 ± 1 °C. The following morning the must was
191 placed in a cold room. Two hours before inoculation the must was put at room temperature to warm.
192

193 *Yeast community preparation*

194 Isolates were revived from frozen 96-well culture plates by transferring via a flame sterilised, 96-well
195 pin microplate replicator to another 96-well culture plate containing liquid YPD which was then
196 incubated at 28 °C for 72 hours. This extended period for time allowed each isolate to grow in isolation
197 to maximum cell density. Immediately prior to inoculation, all 96 isolates representing a vineyard
198 community were mixed. Since the yeast were isolated in a random manor from the original ferment
199 sample, the 96 yeast represent not only the species diversity of the most abundant yeast at the time of
200 sampling, but also the proportion in which they existed in the original ferment community. By growing
201 the communities in isolation to maximum cell density first, then mixing immediately prior to
202 inoculation, the original species composition and relative abundances of each isolate can be replicated
203 for these mixed yeast community inoculations. The yeast mixed for each vineyard was then centrifuged
204 at 3000 *g* for 5 minutes to pellet the cells which were resuspended in 5 mL of sterile water ready for
205 inoculation.
206

207 *Inoculation and fermentation*

208 Zip® 350 mL coffee plungers (French presses) were autoclaved and used as fermentation vessels to
209 mimic commercial red wine production methods (Sparrow and Smart 2015). Prepared must was
210 thawed and added to each plunger to provide a total ferment volume of 250 mL. Specific gravity (Brix)
211 and temperature were recorded. A 100 µL sample was taken from the negative control, serially diluted,
212 and plated on YPD agar to quantify any ambient yeast present in the must after all sterilisation steps
213 had been completed. Triplicate OD measurements of the yeast community inoculums from each
214 vineyard site were taken at 600 nm to estimate cfu/mL of each inoculum from comparisons to OD
215 standard curves, where 0.26 nm = 2.51E+08 cfu/mL = 1.992 mL inoculation volume. (Supplemental
216 Methodology, Supplemental Table 5, and Supplemental Figures 4 - 6). Each representative yeast
217 community was inoculated into the homogenised must with approximately 2.5×10^6 cfu/mL and placed
218 in a 28 ± 1 °C room to ferment. This entire process from growing the yeast inoculums, mixing the yeast
219 isolates from each site together to create mixed yeast community inoculums and inoculating the
220 communities into the Pinot Noir must for fermentation was repeated three times (i.e. three batches).
221 Each vineyard site was represented once in each batch to control for any batch variability. Therefore,
222 there were a total of three replicate ferments per vineyard site for analysis. Malolactic fermentation
223 was neither induced nor suppressed, and metabolites from this process were not measured or analysed.
224

225 *Fermentation monitoring and wine sample collection*

226 Ferments were plunged three successive times daily to submerge the cap and mimic commercial
227 winemaking conditions. Fermentation progress was monitored daily via weight loss (El Haloui, Picque
228 and Corrieu 1988). Fermentation was considered complete when ferments had lost a total of more than
229 5% of their starting weight (El Haloui, Picque and Corrieu 1988), or after 10 days of fermentation
230 (whichever came first).

231

232 Upon completion, the vessels were plunged to their maximum to press the solids and the liquid was
233 poured into sterile flasks. The flasks sat overnight at 4 °C to settle the heavier solids. The wine was
234 then decanted into polypropylene Thermo Scientific Nalgene centrifuge tubes and centrifuged at 6000
235 *g* in Thermo Scientific Sorvall Lynx4000 Superspeed Centrifuge for 10 minutes to pellet any remaining
236 solids and yeast cells. The supernatant (wine) was transferred to sample containers and stored at -80 °C
237 until chemical analysis.

238

239 *Fermentation analysis*

240 Maximum rate of fermentation was determined by taking the derivative of CO_2, as determined via
241 weight loss, with respect to time dCO_2/dt (El Haloui *et al.* 1989). To further examine fermentation
242 kinetics, ethanol by volume (ABV) was measured directly in the final wines using an Anton Parr
243 Alcolyzer Wine M (Supplemental Table 4). Residual sugar was measured via the Megazyme D-
244 Fructose and D-Glucose enzymatic assay (Megazyme) (Supplemental Table 4). The conversion
245 efficiency of sugar into ethanol was determined via calculation, with an ideal fermentation converting
246 sugar in the following manner: $1\ X\ Sugar\ (Glucose\ and\ Fructose) \rightarrow 2\ \text{X Ethanol} +$
247 $2\ X\ Carbon\ Dioxide$. Prior to fermentation trials, the sterilised must was 22.75° Brix (same across all
248 trials). Therefore, an ideal trial ferment would lose roughly 22 g to CO_2 production.

249

250 ***Wine chemical analysis***

251 A total of 28 yeast-derived aroma compounds (esters, higher alcohols, terpenes, C6 alcohols, and fatty
252 acids) were measured using Headspace Solid-Phase Microextraction Coupled with Gas
253 Chromatography-Mass Spectrometry (HS-SPME GC-MS) (Malherbe *et al.* 2009; Herbst-Johnstone *et*
254 *al.* 2013; Pinu *et al.* 2014; Parish *et al.* 2016). Each sample was incubated for 10 minutes in the Gerstel
255 MultiPurpose Sampler VT32-20 and agitated at 500 rpm prior to extraction. A 2 cm, 23-guage, 50/30
256 µm, DVB/CAR/PDMS fibre was exposed to the sample for 60 minutes at 45°C. After extraction, the
257 fibre was transferred to the rear injection port of an Agilent 7890A GC system coupled to a mass
258 selective detector model 5975C inert XL. Helium was used as the carrier gas at a low rate of 1 mL/min.
259 Volatile compounds were separated on a tandem column composed of an Agilent HP-1ms and an
260 Agilent HP-INNOWax. Agilent MassHunter Quantitative Analysis software was used to quantify the
261 resulting peaks via integration. The integration values were compared to standards to determine the
262 concentration of volatile compounds (µg/L) in each sample.

263

264 ***Statistical analysis***

265 Contingency tables to investigate if the yeast community composition of the sites differed by region
266 were analysed with chi-square tests using Chi-Square Test Calculator (Stangroom 2018) where any
267 zero counts were replaced with 1 to allow the analyses to be conducted; all other analyses were
268 conducted with R via RStudio 3.4.2 (R Studio Team 2020).

269

270 To categorise the community composition for each site as a factor for statistical tests against the wine's
271 chemical composition, a presence/absence method was utilised to form discrete groups based on the
272 yeast species detected. The species present in each community were assigned letters and each
273 community was then given a letter for each member present (Supplemental Table 1).

274

275 To confirm there were no batch differences between the experimental ferments, the sugar to ethanol
276 conversion efficiency and maximum rate of fermentation were tested using ANOVA (Chambers,
277 Hastie and Pregibon 1990). The factors of region and community composition were also tested in these
278 analyses.

279

280 Because some ferments were incomplete and this may have consequences for the chemical
281 composition of the resulting wines (Conner *et al.* 1998; Robinson *et al.* 2009; Mestre *et al.* 2019),
282 ANOVA was used to test whether the residual sugar concentration varied between regions.

283

284 PermANOVA analyses as implemented in the "vegan" package were performed to test the effect of
285 yeast region of origin and community composition on the wines chemical composition and the strata
286 function was implemented to constrain permutations within replicates where applicable (Anderson
287 2001; Legendre and Legendre 2012; Mcardle and Anderson 2018; Oksanen *et al.* 2019; R Studio Team
288 2020). Whether individual aroma compounds varied by yeast region of origin and community
289 composition was analysed with ANOVA and P values were adjusted for multiple tests using the
290 Benjamini & Hochberg method (Benjamini and Hochberg 1995). Constrained Correspondence
291 Analysis (CCA) was used visualise the data (Legendre and Legendre 2012; Oksanen *et al.* 2019).

292

293 **Results**

294 ***Yeast isolation and identification***

295 Yeast community samples were obtained from uninoculated ferments deriving from 17 vineyards when
296 2° Brix were lost. One sample from Central Otago (CFRP) had lost more than 2° Brix prior to sampling
297 and was subsequently discarded from all further analyses. A total of 1495 isolates were obtained with
298 432 from Central Otago, 552 from Martinborough, and 511 from Marlborough. In total, 1440 isolates
299 were successfully RFLP profiled and clustered into 13 cohorts. Sanger sequencing indicated these
300 belonged to five taxonomic groups: *Saccharomyces cerevisiae, Hanseniaspora sp., Metschnikowia sp.,*
301 *Candida zemplinina*, and *Saccharomyces uvarum*. The *Hanseniaspora species* group contains DNA
302 sequences matching to *H. valbyensis* and *H. uvarum*, and the *Metschnikowia species* group includes *M.*

303 *pulcherrima* and another *Metschnikowia* sp. not identified to species level (Table 1 and Supplemental
304 Table 2). Contingency table analyses revealed that the yeast community composition (the numbers of
305 different taxa) significantly differed between the three regions (chi-sq = 346.55, $P < 1.0\times10^{-05}$),
306 confirming the representative yeast communities used to inoculate the lab-scale ferments are regionally
307 distinct.
308
309 ***Fermentation***
310 Despite repeated treatments with DMDC, the negative control samples reported the innate yeast
311 community remained viable in the starting juice at approximately 10^3 cfu/mL: this is 1,000 times lower
312 than the 2.5 x 10^6 cfu/mL inoculation rate of the yeast communities. Weight loss of the control
313 fermentation was an average of 2-times slower than the inoculated fermentations, but 2 of the 51
314 inoculated ferments had rate losses slower than the controls (Supplemental Figure 1-3). However, since
315 the same batch of must was used for all experimental ferments, including the controls, any effect of the
316 background community is consistent among all ferments and thus unlikely accounts for any differences
317 between ferments. Fermentation batch had no significant effect on conversion efficiency (ANOVA,
318 $F_{2,39} = 0.112$, $P = 0.895$), maximum rate of fermentation (ANOVA, $F_{2,39} = 0.173$, $P = 0.842$), or
319 residual sugar (ANOVA, $F_{2,42} = 0.61$, $P = 0.85$ and Supplementary Figure 17).
320
321 Yeast community region of origin had a significant impact on conversion efficiency of sugar to ethanol
322 (ANOVA, $F_{2,39} = 3.74$, $P = 0.032$), and the concentration of residual sugar in the wine (ANOVA, $F_{2,42}$
323 $= 3.65$, $P = 0.035$); however, it had no significant effect on the maximum rate of fermentation
324 (ANOVA, $F_{2,39} = 1.15$, $P = 0.128$). Community composition was found to have a significant impact on
325 conversion efficiency (ANOVA, $F_{6,35} = 4.87$, $P = 6.88\text{x}10^{-04}$), maximum rate of fermentation
326 (ANOVA, $F_{6,35} = 24.83$, $P = 1.26\text{x}10^{-12}$), and the concentration of residual sugar in the wine (ANOVA,
327 $F_{6,30} = 4.518$, $P = 0.002$).
328
329 ***Wine chemical analysis***
330 There was a significant effect of yeast community region of origin on wine chemical profiles, with
331 10% of the variation in wine chemical profiles attributed to yeast community region of origin
332 (PermANOVA, $F_{2,41} = 3.98$, $R^2 = 0.106$, $P = 0.0029$, Figure 2-A; Supplemental Table 3). Analyses
333 (with error correction incorporated) of each of the 28 compounds showed that 11 significantly differed
334 due to yeast community region of origin (P_{adj} range 0.03-1.5×10^{-7}, Table 2 and Figure 2-B). ANOVA
335 and CCA analyses (Table 2 and Figure 2-C) revealed that β-damascenone, α-terpineol, ethyl
336 isovalerate, isovaleric acid and linalool differ the most by yeast community region of origin. Tukey
337 HSD reveals these compounds significantly differ between all regions for β-damascenone, linalool and
338 α-terpineol with concentrations being highest in Martinborough (Supplemental Figures 7-9). For ethyl
339 isovalerate and isovaleric acid concentrations were not significantly different between Central Otago
340 and Marlborough but were significantly higher in these two regions compared with Martinborough
341 (Supplemental Figures 10 and 11).

342
343 There is a significant effect of community composition on wine chemical profiles, explaining 50% of
344 the total variation, five-fold greater than yeast community region of origin (PermANOVA, $F_{6,41} = 6.31$,
345 $R^2 = 0.505$, $P = 9.9\times10^{-5}$, Figure 2-B, Supplemental Table 3). Figure 2 and Table 2 show the
346 compounds that differed due to yeast community composition, and as well as ethyl isovalerate,
347 isovaleric acid which also differed by yeast community region of origin, isoamyl alcohol, methionol
348 and octanoic acid differed the most between ferments with different yeast community compositions.
349 Tukey HSD reveals these compounds significantly differ between communities with *Saccharomyces*
350 species present and those without *Saccharomyces* species present. For all these compounds,
351 concentrations were higher if *Saccharomyces cerevisiae* was present in the yeast community
352 (Supplemental Figures 12, 13, 14, 15, and 16). Fermentation batch, and various interactions between
353 factors had no significant effect on wine chemical profiles (Supplemental Table 3).
354
355 **Discussion**
356 The data and analyses presented here provides evidence that region-specific mixed yeast communities
357 contribute to the regional distinctiveness of a wine's volatile composition, providing the first objective
358 evidence that microbial communities, beyond the fermenting yeast *Saccharomyces cerevisiae*, have the
359 potential to contribute to a regional wine distinctiveness, or *terroir*. In fact, the 10% difference in wine
360 chemistry due to regional yeast communities observed here is consistent with that reported for the
361 effect of regionally genetically distinct populations of *S. cerevisiae* on Sauvignon Blanc (Knight *et al.*
362 2015). Additionally, a novel method to prepare and inoculate mixed-yeast communities for
363 fermentation trials in a controlled, replicable manner is detailed.
364
365 As first formulated, the hypothesis concerning whether there is a microbial aspect to *terroir* did not
366 claim that microbes played a dominate role in regional wine differentiation (Gayevskiy and Goddard
367 2012), but simply tested whether microbes may play any role. The data are converging to suggest that
368 microbes do play a role, but that this is small and just one part of the many other factors that drive wine
369 regional distinctness, which makes intuitive sense. The salient point is that regionally differentiated
370 microbes do play a part in the complex drivers of wine regionality. As seen in *S. cerevisiae*, it is
371 possible different non-*Saccharomyces* species of yeast have genetically distinct regional sub-
372 populations (Knight and Goddard 2015; Alexandre 2020), which could potentially be contributing to
373 the regional wine differences observed here. This highlights the importance of understanding yeast
374 community differentiation at a finer scale of strain distinctiveness when considering how these mixed
375 communities contribute to regional wine characteristics. Further investigation into the strain differences
376 of the isolates used in this study is required to verify if this is the source of the variation observed here.
377
378 Wines from yeast communities isolated from Central Otago and Martinborough have greater separation
379 between them with Marlborough resting in the middle (Figure 2A). This is consistent with patterns in
380 wine chemistry reported for *S. cerevisiae* (Knight *et al.* 2015) and with differentiation in microbial

381 communities associated with vines and wines in New Zealand generally (Taylor *et al.* 2014; Knight
382 and Goddard 2015; Morrison-Whittle and Goddard 2015). Marlborough is a major hub for the New
383 Zealand wine industry, accounting for 71% of New Zealand's wine producing area compared to 3% for
384 Martinborough and 5% for Central Otago (New Zealand Wine Growers 2022b). This increased
385 industry activity and transportation of fruit from smaller regions into Marlborough for fermentation can
386 facilitate yeast dispersal amongst geographic locations via human assisted migration (Liti *et al.* 2009;
387 Goddard *et al.* 2010; Knight *et al.* 2015; Liti 2015). Therefore, it is plausible that the overlap observed
388 between Marlborough and the other regions could be explained, in part, by human assisted migration,
389 but further investigation is required. This pattern also mirrors that of geographic space, with
390 Marlborough physically located between Martinborough and Central Otago. It may be that the yeast
391 communities become more dissimilar with increasing geographic distance, and this is then reflected in
392 the chemical differentiation in the wines. Previous research in New Zealand Sauvignon Blanc
393 vineyards found that the geographic distance separating microbial communities explained 6.1% of the
394 variance in community composition observed (Morrison-Whittle and Goddard 2015). A Chilean study
395 also found that dissimilarities amongst leaf and berry fungal communities increased with geographic
396 distance (Miura *et al.* 2017). More extensive sampling of additional regions would be required to
397 objectively test this for Pinot Noir in New Zealand.
398
399 Ethyl octanoate, isoamyl acetate, isoamyl alcohol, methionol, linalool, β-damascenone, ethyl
400 isobutyrate, ethyl isovalerate, ethyl-2-methyl butanoate, isovaleric acid, and isobutyric acid have been
401 reported as being significant to Pinot Noir aroma around the world (Brander, Kepner and Webb 1980;
402 Miranda-Lopez *et al.* 1992; Fang and Qian 2006; Rutan *et al.* 2014). This study adds α-terpineol, 1-
403 butanol, and ethyl butanoate as being important to regional distinctiveness of New Zealand Pinot Noir.
404 Exactly how the chemical composition of red wines contribute to the sensory perception of different
405 characteristics is complex and poorly understood in red wines; however, these compounds of
406 significance are reported to contribute to Pinot Noir sensory properties in a variety of ways. Esters
407 contribute fruity aromas to wine with ethyl isobutyrate attributed to strawberry, ethyl isovalerate to
408 cherry, ethyl-2-methyl butanoate to fruit and resin, isoamyl acetate and ethyl butanoate both to fruit,
409 and ethyl octanoate to baked fruit aromas in Pinot Noir (Fang and Qian 2006; Rutan *et al.* 2014). Other
410 studies suggest that ethyl octanoate increases the perception of cherry aroma in Pinot Noir, but when in
411 combination with 2-phenyl ethanol it increased the violet aroma (Tomasino *et al.* 2015). Savoury
412 aromas can be attributed to alcohols with methionol responsible for vegetable and potato and isoamyl
413 alcohol for cheese and overripe banana aromas in Pinot Noir (Rutan *et al.* 2014). Terpenes and
414 norisoprenoids, such as linalool, α-terpineol, and β-damascenone have floral and fruity aromas with
415 linalool contributing floral, α-terpineol sweet floral, and β-damascenone tea, floral, fruity and honey
416 aromas (Fang and Qian 2006; Rutan *et al.* 2014). Monoterpenes have also been suggested to have an
417 indirect effect by enhancing or suppressing Pinot Noir wine attributes, rather than contributing directly
418 to them (Longo *et al.* 2021). Isovaleric acid and isobutyric acid are fatty acids that both have cheese
419 aromas (Rutan *et al.* 2014). The complexity of how compounds may be perceived in wine means we

420 can only speculate on the differences these compounds contribute to regional Pinot Noir aroma and
421 flavour and controlled sensory trials are required to confirm any differences in perception.
422
423 While this research adds to our understanding of the contribution of mixed yeast communities to
424 regional wine attributes, the ability of microbes to contribute to regional wine attributes is possibly
425 larger than that reported here. Firstly, the diversity of microorganism in wine fermentations is larger
426 than just yeasts (Povhe Jemec *et al.* 2001; Selli *et al.* 2005; Goddard 2008; Zott *et al.* 2008; Šuranská,
427 Vránová and Omelková 2016; Bagheri, Bauer and Setati 2017). As few as five different species of
428 yeast are reported here, while there are numerous other species of yeast known to contribute to wine
429 fermentation. The low diversity of species found in this study could be a result of the limited number of
430 isolates we could manage while using a culturing approach; however, this approach was necessary to
431 be able to replicate and test the mixed yeast communities in experimental ferments. Despite this, grape
432 juice is a hostile environment with low pH and a high osmotic pressure, and previous studies have also
433 reported low yeast diversity during fermentation (e.g. Goddard 2008). Additionally, bacteria may also
434 influence regional wine characteristics (Bokulich *et al.* 2016); however this has not yet been tested in a
435 controlled environment or with mixed bacterial communities. Similar methodologies to those used in
436 this study could be utilised to explore whether bacterial communities also contribute to regional
437 character and could potentially shed light on how these natural isolates impact both alcoholic
438 fermentation and malolactic fermentation in red and white wines. Secondly, this study does not
439 consider how microbial communities present in the vineyard may influence grape production and
440 quality throughout the growing season. Different geographic regions experience different microbial
441 disease pressures, but how fungi and bacteria affect fruit development in other ways is not well
442 understood. For example, *Botrytis cinerea* and other grapevine pathogens have long-lasting effects on
443 grape development in the vineyard, which impacts wine quality (Barata, Malfeito-Ferreira and Loureiro
444 2012; Blanco-Ulate *et al.* 2017; Griggs *et al.* 2021). This additional information would give a more
445 complete picture of how microbial communities (inclusive of yeast and bacteria) contribute to regional
446 character and warrants further investigation to test such hypotheses.
447
448 There are two main caveats to this study: Firstly, the Pinot Noir juice used for the experimental
449 ferments could not be completely sterilised prior to inoculation. As such, the negative controls did
450 eventually ferment during the trials. Statistical analyses of these control samples compared with our
451 experimental ferments shows they fermented slower, indicating our inoculated yeast communities were
452 active and outgrowing any ambient microbial communities in the must. Since the same batch of must
453 was used for all experimental ferments, including the controls, any effect of the background
454 community is consistent among all ferments and thus unlikely accounts for any differences between
455 treatments (i.e. this is not confounded to one region) and thus the regional distinctions we detected
456 were indeed due to differences in the regional yeast communities inoculated. Secondly, not all ferments
457 finished, and some had high levels of residual sugar. Statistical analyses report the residual sugar in the
458 wines varied with yeast community region of origin, potentially confounding the results of regional

459 differentiation in wine chemistry. However, the competency of regional yeast communities to complete
460 fermentation is a function of the species composition of those ferments; and given the yeast
461 communities are regionally distinct, it could be argued that residual sugar (and by proxy ferment
462 completeness) is a function of the microbial community, which is what we aimed to test. Furthermore,
463 yeast-derived aroma compounds are generated throughout all stages of fermentation (Swiegers and
464 Pretorius 2005; Hall *et al.* 2017), such as thiols, which are primarily generated during early stages of
465 fermentation by non-*S. cerevisiae* yeast (Zott *et al.* 2011).

466

467 The use of regionally distinct 'native' microbes in fermentation is of increasing interest to the wine
468 industry and in other fermentation products. Currently, this is only possible via spontaneous
469 fermentation, which carries risks of spoilage and incomplete fermentation. This work describes a
470 method of creating and using representative region-specific synthetic yeast communities for wine
471 fermentation that is reproducible and effective. If scaled-up, this method provides significant potential
472 to produce tools for winemakers to safely use the region-specific natural microbial biodiversity
473 inherent to their sites to add distinctness and value to products. Furthermore, the approach used in this
474 study could be leveraged to experimentally test similar mixed microbial ecologies beyond those found
475 during wine fermentations. Overall, this work highlights the importance, both economically and
476 ecologically, of better understanding the origins and maintenance of microbial diversity to promote
477 sustainable management practices that protect and potentially enhance these local communities.

478

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490 **Conflicts of interest**

491 The authors declare no conflict of interest.

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664

665 **Figure 1:** Location of the New Zealand Pinot Noir growing regions tested in this study and the site
666 codes of the respective vineyard sites, where fruit was collected from to isolate the regional yeast
667 communities with experimental design. *While all 18 vineyard sites are shown here, the yeast
668 community was unable to be isolated from site CFRP in Central Otago, resulting in 17 yeast
669 communities for the subsequent fermentation trial, as described in the methods.
670

671 **Figure 2**: CCA analyses of the experimental ferments. A) Region of origin impact on aroma
672 compounds coloured by region of origin and depicts 50% ellipses. B) Community composition impact
673 on aroma compounds coloured by yeast community composition, where A = *S. cerevisiae*, B =
674 *Hanseniaspora species*, C = *Candida zemplinina*, D = *Metschnikowia species*, and E = *S. uvarum*. C)
675 Region of Origin and Community Composition Loadings for Aroma Compounds According to CCA
676 Species Score. Vectors representing statistically significant aroma compounds are labelled. Vectors and
677 compounds in black are of significance to Region of Origin, vectors and compounds in blue to
678 Community Composition, and vectors in grey are compounds that overlapped those of significance to
679 Community and Region of Origin.
680

681 **Table 1:** Yeast Species Distribution by Region
682

683 **Table 2:** Volatile Aroma Compounds Measured via HS-SPDE GC-MS. The ions and retention time
684 used to identify each compound are listed along with their statistical significance to Community
685 Composition and Region of Origin as determined by ANOVAs run for each compound. All p-values
686 have been adjusted using the Benjamini & Hochberg (1995) method. Bold, underlined, and italicised

687 indicate Aroma Compounds of Significance to Community Composition or Region of Origin as
688 determined by P_{adj} value < 0.05.