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Article type : JAM - Original Article

Defined co-cultures of yeast and bacteria modify the aroma, crumb and sensory properties of bread

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Running title: Mixed cultures make distinctive bread

Abstract

Aims: Yeast and bacterial communities inhabit a sourdough starter to make artisanal bread. This study shows whether the interactions of microorganism derived from Australian sourdough starters provide some of the positive flavour, and aroma properties to bread by using defined sourdough cultures as the sole leaven in bread production.

Methods and Results: A investigation of Australian sourdough starters found that they contained *Saccharomyces cerevisiae* and *Kazachstania exigua* yeasts. When these yeasts were

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/JAM.14349](https://doi.org/10.1111/JAM.14349)

inoculated alone to ferment wheat flour in an extended fermentation, the bread had a heterogeneous crumb structure, a deeper colour and a distinctive chemical aroma profile than those made with commercial baker's yeast. When bread was made combining these yeasts individually and in combinations with lactic acid bacteria also isolated from these sourdough starters, including *Lactobacillus plantarum*, *L. brevis*, *L. rossiae*, *L. casei*, the bread aroma profiles and crumb structure were more distinctive, with compounds associated with *sour* aromas produced, and preferred by sensory panels.

Conclusions: The use of defined mixed cultures as the leaven in bread-making, by exploiting the microbial diversity of artisanal Australian starters, can produce bread with distinctive and attractive aromas.

Significance and Impact of the Study: Understanding and identifying the community ecosystems found in sourdough cultures and using them as the sole leaven in bread production provide novel insights into microbial interactions and how they affect food quality by removing the effects of commercial yeast strains.

Keywords

Sourdough, leavening, starter, lactic acid bacteria, wheat

Introduction

Yeasts are an essential part of the production of food and beverages (Legras et al. 2007; Sicard & Legras 2011; Steensels & Verstrepen 2014). Yeast fermentations convert the raw materials into a wide range of compounds, including ethanol and carbon dioxide (CO₂), glycerol, acids and beyond (Fleet 2007). Beyond the dominance of *Saccharomyces cerevisiae* in the production in food, the use of non-*Saccharomyces* species is increasingly considered an important way to improve the flavour, aroma and functionality of a wide variety of foods (Carrau et al. 2015) including wine (Jolly et al. 2014), chocolate (Ho et al. 2014) and bread (Aslankoohi et al. 2016).

Sourdough is a mixture of flour and water fermented by lactic acid bacteria (LAB) and yeasts and is used as a leaven in artisanal breads. The mixture of microorganisms in the sourdough culture produce CO₂ by fermenting starch and sugars found in the flour to produce an aerated bread product (Arendt et al. 2007; De Vuyst et al. 2009). This process usually involves extended fermentation time compared to bread made with industrial methods that use a defined species of *S. cerevisiae* (Gobbetti & Gänzle 2012). The fermentation outcomes can be

variable but, in most cases, result in an attractive loaf which is increasingly favoured by consumers (Clarke et al. 2002; Corsetti et al. 2000; De Vuyst & Vancanneyt 2007; Gobbetti et al. 2005). The microbes present in sourdough starters have been catalogued and reviewed (de Vuyst et al. 2014; Ganzle & Ripari 2016; van Kerrebroeck et al. 2017). Most LAB isolated from sourdough cultures belong to the *Lactobacillus* genus (Galle & Arendt 2014; Gul et al. 2005; Vogel et al. 1999). Some studies have characterised more than 60 LAB species from mature sourdoughs and more than 30 *Lactobacillus* species alone have been isolated from traditional sourdoughs (Dertli et al. 2016; Minervini et al. 2015). Some species such as *Lactobacillus sanfranciscensis* and *Lactobacillus panis* have only been isolated from sourdough breads, while others are commonly found in other environments and foods (Catzeddu 2011). The range of yeast species in sourdough microbiota is less diverse than that of LAB. While more than 20 species of yeast have been reported to be found in sourdough, only six to seven species are commonly found (De Vuyst et al. 2009; Lattanzi et al. 2013). These species include *Kazachstania exigua* (previously known as *Saccharomyces exigua*), *Pichia kudriavzevii*, and *Torulaspora delbrueckii*, as well as *S. cerevisiae* and *Kazachstania humilis* (formerly *Candida humilis*) which are the two most common (Minervini et al. 2015). Yeast and LAB coexist in stable relationships within sourdough cultures and the nature of their interactions depends on the metabolic pathways of each microorganism (Corsetti et al. 2000; De Vuyst & Neysens 2005).

A number of quality features are found to be enhanced when sourdough is used as a leaven for bread making. This includes nutritional benefits, increased shelf-life, protection from spoilage, increased proteolysis and improved aroma, crumb and flavour profiles. Some nutritional and health benefits associated with sourdough are increased protein digestibility, total phenols, antioxidant activity and nutrient bioavailability (De Vuyst et al. 2016; Rizzello et al. 2010). Shelf-life extension is achieved through the synthesis of both antibacterial and antifungal compounds (Gobbetti et al. 2005; Rizzello et al. 2011) and by delaying staling and firmness (Arendt et al. 2007; Corsetti et al. 2000; Katina et al. 2006; Moroni et al. 2009). Sourdough LAB also produce proteolytic enzymes and increase the activity of these enzymes via acidification which causes increased gluten breakdown (Arendt et al. 2007; Rizzello et al. 2007). Research has also found that bread made with sourdough LAB has an increased number of aroma compounds (Pétel et al. 2017) and produce a wider range of aromatic acids (Hansen & Schieberle 2005). Additionally, sourdough leavens improves the crumb structure and loaf volume of bread due to acidification (Barber et al. 1992; Gobbetti & Ganzle 2012). In terms of flavour, some yeast

species have been found to produce more flavour metabolites in the presence of LAB (Maloney & Foy 2003). Bringing the benefits of microbial diversity and extended fermentation of sourdough fermentation to conventional bread making can thus be used to make bread a healthier food. Understanding the consequences of individual yeast and bacterial strains and their interactions within co-cultures during fermentation is essential for this to occur.

The origins and composition of yeasts and bacteria in a sourdough starter reflect the grain, and flour used, and the bakery environment (Alfonzo et al. 2017; Ercolini et al. 2013; Minervini et al. 2015; Rizzello et al. 2015; Vrancken et al. 2010). A recent report of the yeast and bacteria found on insect frass in a grain silo and their transfer into the flour provides an insight into the transfer of these organisms into the sourdough (Boiocchi et al. 2017). These reports paint an important picture to the influence of the immediate environment on the establishment of the sourdough ecosystem, and recent comprehensive surveys have uncovered previously undescribed yeasts and bacteria in sourdoughs produced in France (Jacques et al. 2016; Lhomme et al. 2016). The importance of place is clearly necessary for the introduction and establishment of the microbes present in sourdough, but as yet the surveys of yeasts and bacteria have all taken place in the northern hemisphere.

This paper describes the microbial diversity of yeasts and bacteria in sourdoughs originating from Australia. Furthermore, it outlines the fermentation characteristics of wheat doughs inoculated with single yeasts and defined mixed cultures with selected LAB. This study aims to observe the effects of sourdough derived microorganisms in bread making by using defined co-cultures to produce leavens and comparing these breads with those made with commercial yeast strains. Our methodology systematically understands these interactions through analysis of aroma, crumb colour and structure, and sensory panel feedback it is suggested that the combination of LAB and yeast results in breads with increased complexity and attractive aromas and flavours. Our paper demonstrates that the consequences of yeast and bacterial diversity in traditional artisan bread production fundamentally changes the final bread to distinguish it from bread made with a commercial strain of *S. cerevisiae*. Understanding these interactions will enable food producers to rationally apply fermentation protocols to make bread with defined flavour, aroma and structural characteristics.

121 Materials and Methods

122 *Isolation of yeasts and bacteria from sourdough starters*

123 Eleven sourdough starters were donated to this project from various bakeries within
124 Victoria, Australia. The original names, identifiers, and origins of each are listed in Table 1. For
125 each starter, 1.0 g was diluted with 0.1% peptone water and serially diluted and plated onto de
126 MAN Rogosa and Sharpe (MRS), yeast extract peptone dextrose (YPD) and Wallerstein Nutrient
127 (WLN) agar media using the spread plate method. Sourdough starters SNO, PHI, TRS, TVR, RAW,
128 and RAG required supplementation with antibiotic compounds to control fungal over-growth by
129 adding 50 mg mL⁻¹ cycloheximide to WLN media. To control yeast growth without bacteria, YPD
130 media was supplemented with 34 mg mL⁻¹ chloramphenicol and 25 mg mL⁻¹ ampicillin. All plates
131 were incubated at 27°C for 2 - 3 days in aerobic conditions and single colonies were taken and
132 re-plated onto fresh media to obtain pure colonies, which were stored in 60% glycerol at -70°C.

133 Gram-staining, catalase and oxidase tests broadly characterised LAB isolates by defining
134 their biochemical properties (De Vuyst & Vancanneyt 2007; Mattarelli et al. 2014). For yeasts,
135 plate morphology was observed and recorded, as well as cell morphology under a light
136 microscope.

137 Taxonomic assignments of bacteria were carried out with the 16S rRNA gene using a
138 commercial service provided by the Australian Genome Research Facility (AGRF). For yeast
139 identification, deoxyribonucleic acid (DNA) was recovered from pure colonies using the
140 MasterPure™ Yeast DNA purification kit (Epicentre, Madison, WI, USA) following the
141 manufacturer's instructions. Primers ITS1 and ITS4 were used to amplify the ITS and 5.8S rRNA
142 DNA regions using Kapa HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems, Wilmington, MA,
143 USA) as described previously (Lam & Howell 2015). The 26S rDNA region was amplified using
144 primers NL1 and NL4 as described by Kurtzman and Robnett (1998) for sequencing by AGRF
145 (Melbourne, Australia). Returned sequences were trimmed, aligned and the consensus
146 sequences checked for similarity using the BLAST algorithm and the nucleotide databases (NCBI)
147 using Geneious R.10.2.2 (BioMatters, Auckland, New Zealand).

148 *Gas Production of Small-Scale Fermentations*

149 Gas production of the different cultures was measured in small dough fermentations
150 with an ANKOM Gas Production system (ANKOM Technology, Macedon, NY, USA). Dough
151 containing 25 g of wheat flour (Laucke T55, Laucke, Australia) and 50 g of distilled water was

152 inoculated with a cellular suspension containing 10^6 CFU mL⁻¹ (0.153 g) of yeast and 10^7 CFU mL⁻¹
153 (0.042 g) of bacteria according to composition given in Table 2. The dough was then placed into
154 the sealed bottles and in a water bath maintained at 30°C for 24 hours. Measurements of
155 pressure were taken every 0.25 minutes.

156 *Breadmaking*

157 Wheat flour (Bilby Type 55, Laucke, VIC, Australia), tap water and cooking salt (Black &
158 Gold, NSW, Australia) were used to produce the leavens and duplicates of bread dough. The
159 inoculum amounts used for the experimental treatments were 10^6 cells g⁻¹ sourdough of yeast
160 and 10^7 cells g⁻¹ sourdough for LAB (Katina et al. 2006). The leaven was prepared by mixing 50 g
161 flour and 50 g water and fermented for 20h at room temperature (approx. 25 °C) . This leaven
162 was mixed with 325 g of water and 500 g of flour in a BM2500 Compact Bakehouse (Sunbeam,
163 NSW, Australia) for 5 min. After 30 minutes, salt (10g) and water (25mL) were added and
164 fermented for 3 hours. The dough was divided into three pieces of 300 g, shaped and put into
165 loaf tins (14.6 x 7.62 cm). The loaves were proofed for two hours in a commercial oven
166 (Convotherm 4 EasyDial 10.10, Moffat, VIC, Australia) at 30°C (Combi-steam setting: fan 2,
167 HumidityPro 2). The loaves were baked for 20 min at 200°C, cooled for 90 min and then sliced
168 transversely into 2 cm thick slices. The baker's yeast bread used the same protocol as the
169 sourdough, but the second proof was shortened to one hour.

170 *Analysis of aroma of baked bread by HS-SPME-GC-MS*

171 The aroma profile of bread was analysed using headspace solid phase micro-extraction
172 gas chromatography mass spectrometry (HS-SPME-GC-MS), the method was adapted from
173 Aslankoochi et al. (2016). Briefly, after the baked bread had cooled, 2 g sample was taken from
174 the middle of the loaf. Three samples were taken from each loaf to give a total of eighteen
175 samples for each treatment. An internal standard comprising 20 µL 4-octanol (Sigma-Aldrich,
176 internal standard, 0.01%) was added and agitated for 15 min at 40°C before being sampled by a
177 fused silica SPME fibre (polydimethylsiloxane/divinylbenzene; Supelco Co, Bellefonte, PA, USA)
178 for 30 minutes. The compounds were desorbed for 10 min at the injection port at 250°C in
179 splitless mode. Agilent Technologies 6850 Series II gas chromatograph connected to an Agilent
180 Technologies 5973 Network mass spectrometer coupled with PAL multipurpose auto-sampling
181 system was used to achieve the sample analysis (Agilent Technologies, Santa Clara, CA, USA).
182 Data was collected in Agilent G1701EA MSD software. The gas chromatograph was fitted with

183 Agilent J&W DB-Wax Ultra Inert GC column (30 m, 0.25 mm i.d. and 0.25 μ m). The carrier gas
184 was helium with a flow rate of 1.4 mL min⁻¹. The temperature was initially held at 36°C for 10
185 min and then rose to 220°C at a rate of 6°C min⁻¹, where it was held for a further 5 min. The
186 mass spectrometer was operated in scan mode (35-600 amu). MS transfer line, ion source and
187 quadrupole temperature were set at 250°C, 230°C and 150°C, respectively. C7-C30 alkane
188 standard were analysed to establish retention index. Identification and quantification of volatile
189 compounds were achieved by comparing retention index and MS spectrum with NIST spectral
190 library database ver. 2011 (NIST, Maryland, USA). The internal standard was used to semi-
191 quantify volatile compounds based on peak area ratio.

192 *Crumb analysis*

193 Three images of loaf cross sections were taken in a light box (Photosimile 50, Ortery,
194 California, USA) using an iPhone SE (Apple Inc, Cupertino. CA. USA) using the locked manual
195 functions for focus and exposure from iOS 10. A total of eighteen images were taken from the
196 six loaves produced in duplicated bakes for each treatment. Images were cropped to the largest
197 rectangle possible containing only crumb. For the crumb colour analysis these cropped images
198 were analysed using an algorithm in Matlab® R2018b (Mathworks Inc., Matick, MA. USA) to
199 output L*a*b colour values for each image using a modified code from Gonzalez Viejo et al.
200 (2016).

201 For the crumb structure analysis, these images were input into a customised code
202 developed in Matlab® R2018b which converts the image to grayscale and then segments it using
203 a locally adaptive threshold method using a sensitivity factor of 0.68. This image was inverted
204 such that a value of 0 (black) meant cell wall material and a value of 1 (white) meant cells. Small
205 objects were cleaned out by an opening operation (erosion and dilation) using a square 3x3 pixel
206 structuring element. Any holes in the resultant binary image were also filled using an algorithm
207 based on morphological reconstruction (Soille 2004). Cell detection from the binary image was
208 based on an algorithm that defines cells to be any 8-connected region of pixels with value 1
209 (MathWorks 2016). The crumb grain features were extracted from the resulting binary image
210 and included the void fraction (VF, cell area/total area*100), mean cell area (MCA, mm²), small
211 to large cell ratio (SLR), and cell area range (R, mm²).

212 *Sensory evaluation*

213 An untrained sensory consumer panel was conducted. A total of 40 participants aged
214 between 18 and 25 (32.5% males, 67.5% females) attended two sensory panels held on different
215 days; the bread treatments were baked the day prior to the panels and the loaves were cut into
216 1cm thick slices on the day. Each participant was presented with eight slices of bread, given a
217 three-digit random code. Participants were asked to score the treatments based on aroma and
218 flavour. Questions related to the intensity of aroma (not intense to very intense), the complexity
219 of the aroma (simple to complex), colour (light to dark), uniformity (irregular to uniform),
220 porosity (dense to porous), overall appearance (dislike to like), texture (dislike to like) and
221 overall preference (dislike to like) using a 7-point semi-structured scale, in which the extremes
222 were described (Granato et al. 2010; Hayes et al. 2013). Approval for this panel was given by the
223 Faculty of Veterinary and Agricultural Sciences Human Research Ethics Committee (HREC
224 1545786.2).

225 *Statistics*

226 Statistical tests were conducted by Minitab 17 (Minitab Pty Ltd, Sydney, Australia) and
227 MetaboAnalyst 3.0 (McGill University, Montreal, Canada). Minitab was used to perform analysis
228 of variance (ANOVA) on gas production, crumb grain features and sensory results. Where an
229 ANOVA found a significant difference ($p < 0.05$), Tukey post-hoc analysis was conducted to
230 determine which treatments were significantly different from each other. MetaboAnalyst was
231 used to conduct statistical similarity and graphical tests on the GC data set. The principal
232 component analysis was run on the CVA and aroma data using Matlab® R2018b.

233 *Results*

234 *Microbial composition of the sourdough starters*

235 The identification of yeasts and bacteria found in the sourdough starters revealed a
236 diversity of species. All isolated colonies were examined morphologically, with some simple
237 biochemical tests and cellular morphology, with the results given in Table 1. Confirmation of the
238 species identity was performed by DNA sequencing, which targeted the 16S rRNA gene for
239 bacteria and the D1/D2 region of the 26S rDNA gene for yeasts. All DNA level identifications
240 confirmed the taxonomy at genera level suggested by colony and cell morphologies (data not
241 shown). For the bacteria, percent identity for hits by a Blast database search ranged from 90 to
242 100%, and query cover percentage was relatively high for all yeast BLAST hits, with a range of 95

to 99%. All starters contained at least one isolated yeast species, and between 1 and 5 bacteria partners. Four different yeast species were isolated from the sourdough starters, these were *Saccharomyces cerevisiae*, two from the *Kazachstania* genus and one from the *Pichia* genus. Furthermore, a total of six *Lactobacillus* species were isolated, two *Acetobacter* species and one *Kocuria*.

Gas production of small-scale fermentations

Gas production of different strain combinations (Table 2) were investigated through mini-fermentations in an ANKOM gas production system over 12 hours. Comparison of total gas production at 12 hours of fermentation (Fig. 1) found that cultures containing *S. cerevisiae* (M7-M11, BY) produced significantly greater amounts of gas than cultures contain *K. bulderi* (M1-M6, Y1). Maximum gas production was observed in the culture containing *L. casei* and *S. cerevisiae* (M10). *L. casei* and *L. plantarum* were the only two bacteria that produced an increase in gas produced when combined with *S. cerevisiae* (M7, M10) compared to the yeast alone (BY). When *K. bulderi* was combined with bacteria, the mixed cultures either reduced gas production (M2, M4, M6) or were not significantly different (M1, M3, M5) to the yeasts fermentative capacity alone (Y1). Acidity (titratable acidity g/100g of dough) was measured and found to be slightly increased when bacteria were included in the co-cultures (data not shown).

Small-scale breadmaking

Different combinations of yeast and bacteria were selected for small scale bread making. Since few small-scale bread making experiments have considered the biochemical activity of *K. bulderi*, this yeast was combined with various bacteria to unique combinations (Table 2). Optimising small scale bread making produced 150 g loaves for analysis, as detailed below.

Aroma analysis

Fifteen volatile compounds were identified from the HS-SPME-GC-MS analysis, including a mix of alcohols, aldehydes and ketones. Ten of these compounds were present in all the bread treatments (Table S1) with distinctive aromas (Table S2). The pairing of *K. bulderi* and *L. plantarum* (M2) had the highest ratios for most volatiles. When *L. rossiae* was paired with *L. plantarum* and *K. bulderi* (M4), it lowered the amounts of volatiles, suggesting possible competition or a pathway divergence with this combination. *L. casei* did not perform well with *K. bulderi* (M3) in terms of aroma as it had lower ratios of most compounds compared to the other sourdoughs containing one bacterium. However, the combination of *L. rossiae* and *L. casei* with

274 *K. bulderi* (M5) produced the most volatile compounds with two lactic acid bacteria which
275 indicates a beneficial relationship between these two bacteria.

276 Based on the similarity of relative amounts of the volatiles in each treatment, the volatile
277 and bread treatments were clustered and analysed (Fig. 2). There were two distinct groups in
278 the treatments; one contained BY and Y1 and the other containing the sourdough treatments.
279 From the cluster heatmap, a clear separation was distinguished between the yeast-leavened and
280 sourdough breads. These compounds (heptanal, 1,2,4,5-tetramethylbenzene and 2-pentylfuran)
281 had higher ratios in the yeast-leavened breads, followed by the sourdoughs with one bacterium
282 and the lowest amounts in the sourdoughs with two bacteria (Fig. 2). The addition of bacteria
283 tended to lower the amounts volatiles compared with the control Y1.

284 *Computer Vision Analysis (CVA) of crumb structure*

285 The structure and colour of the crumb were assessed using computer vision analysis
286 (CVA) of sliced bread using a custom code developed on Matlab® R2018b. The crumb colour
287 results are summarised in Figure 3. L* values suggest that both control treatments (BY and Y1)
288 are significantly lighter than the sourdough leavens containing paired bacteria (M4, M5, M6). No
289 significant differences were observed between the different single bacteria leavens (M1, M2,
290 M3). Loaves made with yeast alone had more negative a* values than the loaves made with
291 sourdough leavens. There was no significant difference in b* value between the yeast species or
292 between the sourdough treatments and the controls. Results of the four crumb structure
293 features analysed through CVA are shown in Figure 3. The VF and MCA values are analogous to
294 crumb porosity. The control loaves (BY, Y1) were not found to be significantly different in terms
295 of VF which suggests that the species of yeast does not affect this parameter. Only sourdough
296 treatments containing *L. rossiae* (M1, M4, M5) had a significantly greater VF than the control
297 treatments. The MCA of the sourdough treatments was found to be significantly greater than
298 the controls. The greatest MCA value was found in the *L. plantarum* treatment (M2), suggesting
299 this bacterium has the greatest ability to produce larger cell sizes. The addition of other LAB
300 species seemed to reduce the MCA since both mixed treatments containing *L. plantarum* (M4,
301 M6) had significantly lower MCA values than the single bacteria treatment (M2). The SLR and R
302 measurements are analogous to the cell size uniformity within the crumb. The control loaves
303 (BY, Y1) displayed the highest SLR values, meaning they possess few large cells and are thus the
304 most uniform. All the sourdough treatments had significantly lower SLR values than the Y1
305 control treatment. Overall, loaves made with *L. plantarum* had the lowest SLR and were thus the

least uniform. Furthermore, most of the sourdough treatments (M1, M2, M3, M4, M6) had a significantly lower R value compared to both controls (BY, Y1).

Effect of fermentation mode on the aroma, crumb structure and colour of bread

A principal component analysis of the aroma, crumb structure features and colour obtained from CVA. Figure 4 shows the score plot for the first two principal components grouped according to the leaven treatment. The clustering of the two yeast controls towards the bottom left corner of the plot suggest that the use of bacteria in sourdough cultures does alter the crumb and aroma of bread produced, particularly in terms of lightness (L) and SLR. The first principal component (PC1) was found to explain 37.61 % of the variance. The largest coefficients in PC1 corresponded to benzaldehyde, 1-octen-3-ol and 1-pentanol. The second principal component (PC2) was found to explain 22.05% of the variance. The largest coefficients in PC2 corresponded to a*, 1,2,3,4-tetramethylbenzene and 2-pentylfuran.

Sensory evaluation

A consumer panel was performed on the bread leavened with the different sourdough treatments. Figure 5 gives the mean scores for each treatment based on the questions from the questionnaire. No significant differences were found for the questions relating to the overall crumb appearance, texture, colour, overall aroma or overall preference but significant differences were found for sour aroma intensity, fruity aroma intensity, aroma complexity, uniformity and porosity. The three sourdough treatments containing *L. plantarum* (M6, M2, M4) were scored higher than the control treatments for sour aroma intensity while loaves leavened with *K. bulderi* alone (Y1) were scored the lowest. For intensity of fruity aroma, the loaves made with *S. cerevisiae* (BY) scored the highest while the sourdough containing *L. rossiae* (M1) scored the lowest. Treatments that scored highest for aroma complexity were the *S. cerevisiae* control (BY) and the sourdough treatments containing *L. plantarum* (M2, M4, M6). Overall the aroma sensory results suggest that *L. plantarum* produces loaves with a perceptible sour aroma and high complexity while the control containing *S. cerevisiae* generally scored higher for positive quality features and appeared to be preferred by consumers. The two control treatments containing yeast alone (BY, Y1) were not significantly different in terms of uniformity or porosity but some sourdough treatments were. This indicates the use of bacteria in sourdough cultures to leaven bread influences consumer scoring of crumb uniformity and porosity. Comparing individual bacterial species, *L. rossiae* (M1) scored significantly higher in uniformity than *L.*

337 *plantarum* (M2). In terms of porosity, only treatments containing *L. plantarum* (M2, M4, M6)
338 scored significantly higher compared to the controls (BY, Y1).

339 Discussion

340 Our study isolated and identified yeasts and bacteria present in the breadmaking
341 starter of Australian sourdough producers. By producing bread with these yeasts and bacteria,
342 in isolation or combination, the impact on bread is apparent. Inclusion of bacteria with yeasts
343 to ferment wheat dough affects the aroma, crust colour, crumb structure and sensory
344 attributes of the final bread.

345 More than 70 species of LAB and 25 species of yeast have been identified in wheat
346 sourdoughs (Moroni et al. 2009; Nionelli & Rizzello 2016). Minervini et al. (2015) found that the
347 two most dominant yeast species in sourdough were *S. cerevisiae* and *K. humilis*. This is
348 consistent with the findings of this study as yeasts of genus *Saccharomyces* or *Kazachstania*
349 were found in nine of the ten starters investigated. While *Kazachstania* yeast species have often
350 been described in sourdoughs, Vrancken et al. (2010) suggested that it is not as commonly found
351 as *Saccharomyces* species. The starters in this study were sourced from artisan bakers who
352 restrict the yeast in the bakery to naturally derived leaven, and do not generally use commercial
353 preparations of *S. cerevisiae*. Most bacteria identified from the sourdough starters were lactic
354 acid bacteria of the *Lactobacillus* genus which is consistent with previous research (De Vuyst &
355 Vancanneyt 2007; Galle & Arendt 2014; Vogel et al. 1999). Three of the identified bacterial
356 species are commonly found in sourdough. These include *Lactobacillus rossiae*, *Lactobacillus*
357 *plantarum* and *Lactobacillus brevis* (Corsetti & Settanni 2007; Scheirlinck et al. 2008; Vogel et al.
358 1999). Less commonly identified were the two *Acetobacter* species, which has been found to be
359 the predominant bacteria in sourdough (Li et al. 2017; Scheirlinck et al. 2008). *L. casei* was
360 identified in two of the starters. While this bacterium is regularly used in the dairy industry,
361 especially for milk and cheese fermentation (Reale et al. 2016), it is not commonly found in the
362 sourdough environment. Notably, *L. sanfranciscensis* was not isolated in this study. It is a
363 common partner in sourdough fermentations around the world (de Vuyst & Neysens 2005). It
364 may not be a common partner in the Australian context, but further comprehensive surveys
365 shed light onto its role in breadmaking in Australia.

366 The sourdough yeast species *K. bulderi* has previously been found to have a lower
367 fermentative capacity than commercial *S. cerevisiae* strains (Gobbetti et al. 1994). This is

consistent with our findings as *K. bulderi* produced about half the amount of gas compared to *S. cerevisiae* during the 12-hour model fermentation (Figure 1). Strains of *S. cerevisiae* are highly specialized in the rapid production of CO₂ and ethanol from sugars (Maloney & Foy 2003). Co-cultures of *S. cerevisiae* and certain species of LAB increase yeast CO₂ production compared to *S. cerevisiae* alone. *L. plantarum*, particularly, has been found to improve fermentation and gas producing ability of *S. cerevisiae* (Gobbetti 1998; Gobbetti et al. 1995; Martinez-Anaya et al. 1990). Similarly, in this study *L. plantarum* increased the gas producing ability of *S. cerevisiae*, but not *K. bulderi*. The increase in the fermentative capacity of *S. cerevisiae* in the presence of LAB may be due to LAB proteases generating peptides to support the growth of yeast cells (Maloney & Foy 2003). Alternatively, the decreased leavening capacity observed in certain bacteria-yeast combinations may be associated with nutrient depletion and inhibition of yeast growth by acid production (Valmorri et al. 2008).

Most aroma compounds are formed during the baking of bread (Corsetti & Settanni 2007; Gobbetti 1998), however, sourdough volatiles are also created during fermentation (Pétel et al. 2017). While 14 major volatile compounds were identified here, previous research on sourdough and other wheat breads have found more volatiles (Paucean et al. 2013; Pétel et al. 2017; Quílez et al. 2006). The time of fermentation here was a relatively short 5 - 6 hours (Katina et al. 2006) compared to general sourdough fermentation times of up to 6 days (Gänzle 2014). The aroma analysis clearly distinguished the yeast versus sourdough leavened breads. Generally, the mixed cultures containing both bacteria and yeast tended to be lower in the amounts of some volatiles produced. This could be explained by the interaction of yeast and LAB, which could affect the use of carbohydrates by LAB during sourdough fermentation (Paucean et al. 2013), and therefore production of metabolites. The highest relative production for most volatiles was found for the yeast and LAB combination of *K. bulderi* and *L. plantarum*, which suggests a potential beneficial interaction between the two microbes. The production of acetoin may be manipulated by the genetic potential of certain bacteria and it has been shown that *L. plantarum* and *L. casei* may activate relevant pathways (Zotta et al. 2017) which could explain the presence of acetoin in only certain sourdough treatments. Also, Pétel et al. (2017) reported that 2-heptenal was only produced by *L. plantarum*, but the current study suggested that *L. casei* may also be capable to produce this compound. Further research is required to profile bread volatiles on a more detailed level, to investigate the odor activity value in the

399 bread matrix, and therefore better illustrate the importance of microbial species on volatile
400 production.

401 Colour analysis from baked bread crumb suggested that interactions between LAB and
402 yeast decrease lightness, increase redness and have no effect on yellowness. This is consistent
403 with previous research which found that the colour of loaves fermented with LAB were
404 significantly different from the controls and were not influenced by the LAB strains used
405 (Settanni et al. 2013). Research conducted by Bartkiene et al. (2017), Settanni et al. (2013) and
406 Rizzello et al. (2012) found that the measure of redness (a^*) for the crumb of sourdough loaves
407 increased and yellowness was lower in sourdough while Rizzello et al. (2012) found them to be
408 higher in control loaves. This difference in colour may be due to a change in the concentration of
409 Maillard reaction products. Since sourdough fermentation results in increased proteolysis
410 (Arendt et al. 2007; Gobbetti et al. 2005; Thiele et al. 2002) and carbohydrate metabolism to
411 organic acids (Ganzle et al. 2007) this method is likely to alter the amount of amino acids and
412 sugars available for Maillard reactions.

413 Porosity was measured through void fraction (VF) and mean cell area (MCA) using CVA.
414 Both of these measurements suggested that sourdough bread made with a mixture of yeast and
415 LAB was more porous than loaves made with yeast only. Particularly, *L. rossiae* produced loaves
416 with a greater void fraction, even when paired with other bacteria. Rizzello et al. (2012) found
417 that the VF was significantly higher for sourdough breads made with *L. sanfranciscensis* and *L.*
418 *plantarum* compared to bread made with baker's yeast alone but Settanni et al. (2013) found
419 that the VF of sourdough containing *L. plantarum* was not significantly different from the control
420 which is more consistent with our experimental results. In the same study, other LAB species
421 were found to produce significant differences in VF. MCA resulted suggested that sourdough
422 leavens have the ability to increase the mean cell area of crumb cells, and hence the perceived
423 porosity, compared to leavens containing yeast alone. Settanni et al. (2013) also found that the
424 MCA of sourdough made with *L. plantarum* was significantly lower but only when made with
425 sterile flour. The reasons for a lower MCA may be due to a crumb with more gas cells or smaller
426 gas cell size, with the coalescence of close cells leading to the formation of larger gas cells
427 (Jahromi et al. 2014). Crumb uniformity was measured through the small to large cell ratio (SLR)
428 and cell size range (R). CVA results of cell size range suggests that the sourdough had a greater
429 cell size range and were thus less uniform than the control loaves leavened with yeast alone. SLR
430 results also suggested that the addition of bacteria produces less uniform loaves with a greater

number of large cells compared to loaves leavened with yeast alone. In contrast to our results, Clarke et al. (2002) found sourdough style loaves were more uniform than control loaves as they had a greater SLR. It may be that the choice of bacteria can significantly affect all these measures in yeast and bacterial fermentations in breadmaking.

Sensory Evaluation

The sensory data revealed some significant differences regarding sour and fruity aroma intensities and aroma complexities between treatments, but there were no differences in the overall liking of the aroma nor general preference. Sourdoughs containing *L. plantarum* scored highest for sour aroma which suggests that it can produce compounds that contribute to sourness being detected by panellists. Paterson and Piggott (2006) found that bread made with sourdough containing *L. plantarum* was preferred in terms of taste and aroma compared to the yeast-leavened loaf. Additionally, yeast-leavened bread samples had the lowest scores for sour aroma. This is as expected since LAB are responsible for acid production which are usually linked with sour odour and taste due to their pungent nature (Makhoul et al. 2015). Fruity aroma is often associated with volatile esters and these have been reported in higher amounts when non-conventional yeast species are used (Aslankoohi et al. 2016), such as the *Kazachstania* yeast. Surprisingly, the samples made with baker's yeast scored the highest for fruity aromas. It is possible that the untrained sensory panel used in this study were not able to detect subtle differences in aroma. In terms of crumb colour and structure, sensory results either showed no significant differences between the sourdough treatments and the controls, or that the sourdough was scored less uniform and more porous for some treatments. In general, previous research has found that sourdough loaves have improved sensory properties compared to doughs fermented with either yeast or LAB alone (Edeghor et al. 2016). Breads made with LAB have been found to score higher for texture, crumb colour (Hadaegh et al. 2017; Rizzello et al. 2012; Saeed et al. 2016), overall acceptability (Hadaegh et al. 2017; Saeed et al. 2016), appearance and taste (Hadaegh et al. 2017) compared to bread made with baker's yeast alone. Our results confirm these findings, and show that the chemical and computer vision measures applied in this paper are valuable as a sensory panel is able to distinguish our breads.

Specific metabolic interactions between yeasts and bacteria involved in sourdough bread production are likely to result in the formation of desirable breads. As shown in our study, the fermentation rate is affected by inclusion of bacteria in the fermentation, but the metabolic interactions of these microbes are likely to dramatically affect the aroma compounds produced.

Cross-feeding of essential compounds is likely to play an important part in metabolic output. For example, maltose utilisation is likely to be a major factor in enhancing growth of both yeast and bacteria in a co-culture. Here, maltose-positive *L. sanfranciscensis* can release glucose for maltose-negative *K. humilis* to utilise (recently reviewed by Carbonetto et al. 2018). Additionally, release of amino acids by yeasts can maintain and enhance growth of lactic acid bacteria (see for example Ponomarova et al 2017). We do not know if these factors are at play to maintain the co-culture in our system, but further studies will allow us to understand these metabolic interactions. However, it is clear that metabolic interactions between microorganisms in wine can significantly impact the production of fermentation aroma (Howell et al 2005) and this was shown in this study. We suggest that rational inclusion of lactic acid bacteria with chosen biochemical attributes to enhance and complement the growth of yeasts will enable the baking industry to produce distinctive breads.

The research carried out in this paper investigated the diversity of microbes present in sourdough cultures, which is the first of its kind in Australia. Using deliberate additions of these yeasts and bacteria as the main leaven in bread production we have directly observed how these microorganisms affected the gas production, aroma, and crumb features of bread. Our results show that combining yeast and bacteria can alter the composition, structure, quality and perception by consumers of the final loaf. Depending on consumer preferences and desires, these findings may thus be used by bread producers to alter their products to suit a particular market. This method of bread making reflects the production of traditional artisan bread. The use of *S. cerevisiae* yeast may hide the subtleties of the more interesting and less noticeable aspects of the sourdough ecosystem. The mechanisms of microorganisms interacting within these sourdough cultures and affect aroma, flavour, structure and fermentation, and can be used as a method to create bread with distinctive features.

Acknowledgements

The bakers of Victoria who generously gave their sourdough starters to our project are gratefully acknowledged. In particular, Kim Hol from the Bakehouse in Portland, Michael James from Tivoli Road Bakery in South Yarra, Cameron Russell from Sparrow's in Beaufort, Phillippa Grogan from Phillippa's in Collingwood and Stephen Noonan from Ox the Baker in the Mornington Peninsula gave their starters and knowledge very generously. Dr Damir Torrico assisted in planning the sensory trials and his expertise is gratefully acknowledged.

494 Conflict of Interest

495 The authors declare no conflict of interest

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Figure legends

Fig 1. Gas production of small-scale fermentations using the ANKOM gas production system with standard error in the mean shown as error bars.

Fig 2. Aroma compounds in mixed culture bread. Treatments included BY (■), M1 (■), M2 (■), M3 (■), M4 (■), M5 (■), M6 (■) and Y1 (■). The volatile compounds identified for each bread treatment were clustered based on similarity using Euclidean distance and ratios have been auto-scaled. Volatile compounds are listed by the number on the right-hand side. 1: Ethanol, 2: Hexanal, 3: Heptanal, 4: 2-pentylfuran, 5: 1-pentanol, 6: Acetoin, 7: 2-heptenal, 8: 1-hexanol, 9: 3-ethyl-2-methyl-1,3-hexadiene, 10: 1,2,4,5-tetramethylbenzene, 11: 1-octen-3-ol, 12: 1-heptanol, 13: Benzaldehyde, 14: 2-phenylethanol.

Fig 3. Bread crumb colour and structure analysis of the small-scale loaves in the mixed culture fermentations by digital imaging. (a) Means of L*, a* and b* values with the standard error in the mean shown as error bars. (b) Means of Void Fraction, Mean Cell Area (MCA), Small to Large Cell Ratio (SLR), and Cell Size Range (R) with the standard error in the mean shown as error bars.

Fig 4: Principal component analysis of the bread crumb structure and colour from CVA (Void fraction, Mean Cell Area, SLR, L*, a* and b*) and aroma compounds from GC-MS analysis (Ethanol, Hexanal, Heptanal, 2-pentylfuran, 1-pentanol, Acetoin, 2-heptenal, 1-hexanol, 3-ethyl-2-methyl-1,3-hexadiene, 1,2,4,5-tetramethylbenzene, 1-octen-3-ol, 1-heptanol, Benzaldehyde and 2-phenylethanol). Treatments included BY (◆), M1 (✱), M2 (★), M3 (●), M4 (✕), M5 (▼), M6 (■) and Y1 (▲).

Fig 5: Sensory analysis of baked loaves. Bar Charts of mean sensory panel scores of different bread treatments with error bars represented by the standard error. A. Colour (P-value 0.139), B. Uniformity (P-value = 0.00), C. Porosity (P-value = 0.000), D. Overall Visual (P-value = 0.165), E. Texture (P-value = 0.733), F. Sour/Vinegar Aroma (P-value = 0.001), G. Fruity Aroma (P-value = 0.029), H. Aroma Complexity (P-value = 0.003), I. Overall Aroma (P-value = 0.359) and J. Overall Liking (P-value = 0.977).

Supplementary table legends

Supplementary Table 1 Presence or absence of identified volatile compounds of different bread treatments from GC-MS analysis

Supplementary Table 2: Odour description of the volatile aroma compounds identified in bread-making from GC-MS analysis. Source: Leffingwell and Associates (2015)

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Tables

Table 1. Origin of leavens and the yeast and bacteria identified in each sourdough

Leaven	Origin	Code	Species
POR	The Bakehouse, Portland VIC	Y01	<i>Kazachstania bulderi</i>
		B02	<i>Lactobacillus plantarum</i>
SNO	Snow Gum Road, Beaufort VIC	SNOSY1	<i>Kazachstania humilis</i>
		SNOSB1	<i>Lactobacillus plantarum</i>
		SNOSB2	<i>Acetobacter cerevisiae</i>
RAG	Ragland Road, Beaufort VIC	RAGSY1	<i>Saccharomyces cerevisiae</i>
		RAGSB1	<i>Lactobacillus casei</i>
TRS	Tivoli Road Bakery, South Yarra VIC	TRSSY3	<i>Saccharomyces cerevisiae</i>
		TRSSB1	<i>Acetobacter cerevisiae</i>
TVR	Tivoli Road Bakery, South Yarra VIC	TVRSY1	<i>Pichia membranefaciens</i>
		TVRSB1	<i>Lactobacillus hammesii</i>
TAM	Natimuk VIC	TAMSY1	<i>Kazachstania humilis</i>
		TAMSB2	<i>Kocuria rhizophilia</i>
		TAMSB5	<i>Lactobacillus plantarum</i>
RAW	Natimuk VIC	RAWSY2	<i>Saccharomyces cerevisiae</i>
		RAWSB1	<i>Lactobacillus brevis</i>
		RAWSB2	<i>Lactobacillus plantarum</i>
PHI	Phillippa's Bakery, Richmond VIC	PHISY1	<i>Kazachstania humilis</i>

		PHISB1	<i>Lactobacillus paralimentarius</i>
ALA	Ox the Baker, Mornington Peninsula VIC	AlaMT1	<i>Saccharomyces cerevisiae</i>
		AlaT2	<i>Lactobacillus rossiae</i>
		AlaMT1	<i>Lactobacillus plantarum</i>
		CO1 T2	<i>Lactobacillus brevis</i>
		AlaT5	<i>Acetobacter malorum</i>
BLBN	Ox the Baker, Mornington Peninsula VIC	BLBNDT1	<i>Saccharomyces cerevisiae</i>
		BLBNDT2	<i>Lactobacillus casei</i>

Table 2. Composition of the mixed cultures used in cultured experiments in this paper.

Abbreviation	Species present	Description	Source
BY	<i>S. cerevisiae</i>	Baker's yeast (commercial <i>Saccharomcyes cerevisiae</i>)	Anchor Yeast (South Africa)
Y1	Y01	<i>Kazachstania bulderi</i>	This paper
Lbp	B02	<i>Lactobacillus plantarum</i>	This paper
Lbp2	AlaMT1	<i>Lactobacillus plantarum</i>	This paper
Lbr	AlaT2	<i>Lactobacillus rossiae</i>	This paper
Lbc	BLBNDT2	<i>Lactobacillus casei</i>	This paper
Lbb	CO1 T2	<i>Lactobacillus brevis</i>	This paper
Am	AlaT5	<i>A. malorum</i>	This paper
M1	<i>K. bulderi</i> Y01 <i>Lb. rossiae</i> AlaT2		
M2	<i>K. bulderi</i> Y01 <i>Lb. plantarum</i> B02	This is the reconstructed ecology of the POR starter culture in Table 1.	
M3	<i>K. bulderi</i> Y01 <i>Lb. casei</i> BLBNDT2		

M4	<i>K. bulderi</i> Y01 <i>Lb. rossiae</i> AlaT2 <i>Lb. plantarum</i> B02	POR starter culture with <i>Lb. rossiae</i>
M5	<i>K. bulderi</i> Y01 <i>Lb. rossiae</i> AlaT2 <i>Lb. casei</i> BLBNDT2	
M6	<i>K. bulderi</i> Y01 <i>Lb. plantarum</i> B02 <i>Lb. casei</i> BLBNDT2	POR starter culture with <i>Lb. casei</i>
M7	<i>S. cerevisiae</i> AlaMT1 <i>Lb. plantarum</i> AlaMT1	This is the reconstructed ecology of the Ala starter culture in Table 1.
M8	<i>S. cerevisiae</i> AlaMT1 <i>Lb. brevis</i> CO1 T2	
M9	<i>S. cerevisiae</i> AlaMT1 <i>Lb. rossiae</i> AlaT2	
M10	<i>S. cerevisiae</i> AlaMT1 <i>Lb. casei</i> BLBNDT2	
M11	<i>S. cerevisiae</i> AlaMT1 <i>A. malorum</i> AlaT5

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Title:

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Date:

2019-09-01

Citation:

Winters, M., Panayotides, D., Bayrak, M., Remont, G., Viejo, C. G., Liu, D., Le, B., Liu, Y., Luo, J., Zhang, P. & Howell, K. (2019). Defined co-cultures of yeast and bacteria modify the aroma, crumb and sensory properties of bread. *Journal of Applied Microbiology*, 127 (3), pp.778-793. <https://doi.org/10.1111/jam.14349>.

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