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1 # Epigenetic Changes in *Saccharomyces cerevisiae* Alters the Aromatic
2 Profile in Alcoholic Fermentation

3

4 Yanzhuo Kong[1], Kenneth J. Olejar[2], Stephen L.W. On[1], Christopher Winefield[1], Philip
5 A. Wescombe[3,4], Charles S. Brennan[5], Richard N. Hider[1] and Venkata Chelikani[1*]

6

7 [1]Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln 7647, New
8 Zealand;
9 [2]Chemistry Department, Colorado State University—Pueblo, Pueblo, CO 81001, USA;
10 [3]Yili Innovation Centre Oceania, Lincoln University, Lincoln 7647, New Zealand;
11 [4]National Center of Technology Innovation for Dairy, Hohhot, Inner Mongolia, China;
12 [5]School of Sciences, RMIT University, Melbourne, Australia.

13

14 **Email addresses:** Yanzhuo.Kong@lincolnuni.ac.nz; Kenneth.Olejar@csupueblo.edu;
15 Stephen.On@lincoln.ac.nz; Christopher.Winefield@lincoln.ac.nz;
16 philip.wescombe@oceaniadairy.co.nz; charles.brennan@rmit.edu.au;
17 Richard.Hider@lincoln.ac.nz; Venkata.Chelikani@lincoln.ac.nz

18

19 [*]**Correspondence:** Venkata Chelikani;
20 **Tel:** +64 3 423 0623; **Email:** Venkata.Chelikani@lincoln.ac.nz
21 **Postal address:**
22 PO Box 85084, Lincoln University, Lincoln 7647, Christchurch, New Zealand

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23 **Abstract**

24 Epigenetic changes in genomics provide phenotypic modification without DNA
25 sequence alteration. This study shows that benzoic acid, a common food additive
26 and known histone deacetylase inhibitor (HDACi), has an epigenetic effect on
27 *Saccharomyces cerevisiae*. Benzoic acid stimulated formation of epigenetic histone
28 marks H3K4Me2, H3K27Me2, H3K18ac and H3Ser10p in *S. cerevisiae* and altered
29 their phenotypic behavior, resulting in increased production of phenylethyl alcohol
30 and ester compounds during alcoholic fermentation. Our study demonstrates the
31 HDACi activity of certain dietary compounds such as sodium butyrate, curcumin and
32 anacardic acid, suggests the potential use of these dietary compounds in altering *S.*
33 *cerevisiae* phenotypes without altering host-cell DNA. This study highlights the
34 potential to use common dietary compounds to exploit epigenetic modifications for
35 various fermentation and biotechnology applications as an alternative to genetic
36 modification. These findings indicate that benzoic acid and other food additives may
37 have potential epigenetic effects on human gut microbiota, in which several yeast
38 species are involved.

39

40 **Importance**

41 This manuscript investigates and reports for the first time utilizing microbial
42 epigentics to alter the fermentation process of Pinot noir wines. We have
43 experimentally demonstrated that certain dietary epigenetic compounds possess
44 histone deacetylase (HDAC) inhibiting activity and can alter the wine characteristics
45 by altering yeast gene expression. We have coined the term 'nutrifermentics' to

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46 represent this newly proposed field of research, which provides insights on the effect
47 of certain dietary compounds on microbial strains and their potential application in
48 fermentation process. This technological approach is a novel way to manipulate
49 microorganisms for innovative food and beverage production with quality attributes.
50

51 **Keywords:** Fermentation, Nutrifermentics, Benzoic Acid, Microbial Epigenetics and
52 Biotechnology
53

54 **1. Introduction**

55 Epigenetics is the study of phenotypic changes in organisms, which
56 predominantly result from alterations of nucleotides and histones instead of the
57 deoxyribonucleic acid (DNA) sequence; therefore epigenetic modifications are
58 considered a non-GMO approach (1, 2). Among them, DNA methylation and histone
59 acetylation are the most common and well-studied epigenetic modifications, which
60 are the processes of transferring a methyl group to adenine and cytosine, or adding
61 an acetyl group to lysine residues at the N terminus of histone (3). Several dietary
62 bioactive and phytochemicals that naturally occur in fruits and vegetables can act as
63 epigenetic modifiers and potentially can alter the target organism (4). Epigenetic
64 modifiers can be predominantly classified as DNA methyltransferase (DNMT), DNMT
65 inhibitors, histone deacetylase (HDAC), HDAC inhibitors and histone acetyl (HAT) and
66 HAT inhibitors (5, 6).

67 *S. cerevisiae* is a well-studied model system for epigenetic regulation. Since
68 DNA methylation systems are absent, histone modifications are the primary form of

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69 epigenetic regulation, making it a simple system for understanding the relationship
70 between histone modifications and epigenetic states (7, 8). Recently, the fission
71 yeast, *Schizosaccharomyces pombe* was subjected to higher thresholds of caffeine,
72 resulting in epigenetic changes producing transient epimutants with phenotypic
73 plasticity including tolerance to caffeine and cross-resistance to antifungal agents,
74 which was closely related to heterochromatin alterations and heterochromatin-
75 mediated gene silencing (9).

76 Benzoic acid is a lipophilic weak acid that occurs naturally in many fruits,
77 vegetables, nuts, and even in cultured dairy products as a microbial metabolite (10).
78 Benzoic acid and its derivatives are FDA approved food additives and known histone
79 deacetylase inhibitors (HDACi) that have been shown to stimulate a recently
80 discovered histone mark, lysine benzoylation (11, 12). HDACi compounds play an
81 important role in heterochromatin regulation and gene expression by affecting
82 histone modifications (13).

83 Here, we investigated the possibility of developing *S. cerevisiae* strains with
84 desirable characteristics for alcoholic fermentation by treating them with the
85 epigenetic modifier, benzoic acid. Benzoic acid was selected due its known capacity
86 to modify histone proteins, its cost-effectiveness and solubility in the aqueous
87 system. We also demonstrate that genes responsible for aroma compounds were
88 upregulated in epimutants compared to the original *S. cerevisiae* strain. The effect of
89 benzoic acid on *S. cerevisiae* H3 histone marks, as benzoic acid is a known HDACi,
90 was also investigated. The results showed that there is several other dietary

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91 compounds that could be used to epigenetically alter microbial phenotypes to
92 produce fermented products with desirable characteristics (14, 15).
93 Wine plays an important role among alcoholic drinks and therefore is
94 representative as a model fermentation system. The wine industry is a competitive
95 industry and developing novel wines is necessary to maintain a competitive
96 advantage in the global market. (16). Compared to some existing approaches, such
97 as grapevine breeding and isolation of wild yeast, epigenetic modification of wine
98 yeast is time and cost effective.
99 Our study demonstrates the exciting possibility of using dietary epigenetic
100 compounds to develop non-GMO microbial strains with desirable characteristics for
101 fermented products and biotechnology applications.
102
103 **2. Materials and methods**
104 **2.1 *S. cerevisiae* starter preparation**
105 A commercial wine yeast *S. cerevisiae* EC-1118 was used as fermentation
106 starters in this study. Three types of starters were involved and applied, including
107 wild type (500 h growth in regular YPD broth, 20 h/sub-culture), epimutant 1 (500 h
108 growth in YPD broth containing 10 mM benzoic acid, 20 h/sub-culture) and
109 epimutant 2 (500 h growth in YPD broth containing 10 mM benzoic acid, followed by
110 20 h growth in regular YPD broth w/o stress, 20 h/sub-culture). 0.5 mL of cultured
111 broth was transferred for each subculture.
112 **2.2 Histone H3 modification multiplex assay**

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113 The 21 histone H3 modification patterns of 5 mM benzoic acid treated *S.*
114 *cerevisiae* compared to untreated wild type strain were measured using EpiQuik™
115 Histone H3 modification multiplex assay kit (Colorimetric; EpiGentek, NY, USA)
116 following manufacturer's instructions. Absorbance was measured using FLUOstar
117 Omega microplate reader (BMG LABTECH, Ortenberg, Germany) at 450 nm with a
118 reference wavelength of 655 nm.

119 **2.3 RNA purification and gene expression analysis**

120 *S. cerevisiae* under different treatments were harvested after 12 h growth to
121 reach a sample size of 1×10^8 cells for RNA purification. Total RNA from *S. cerevisiae*
122 was isolated using RiboPure™ RNA Purification Kit (Invitrogen, MA, USA), following
123 manufacturer's instructions. RNA purity was measured by DeNovix DS-11
124 Spectrophotometer (DeNovix Inc., DE, USA). The RNA expression was measured
125 using nCounter technology (NanoString Technologies, Inc., WA, USA). RNA samples
126 were posted to The University of Auckland, where all the preparation and
127 measurement were completed. Assay was carried out on 12 samples/24 genes
128 (including 5 housekeeping genes), the RNA input amount was 300 ng for each
129 sample. Expression counts were normalized and analyzed using the nSolver 4.0
130 software (NanoString Technologies, Inc., WA, USA).

131 **2.4 DAPI staining**

132 *S. cerevisiae* strains were cultured overnight to an OD_{600} = 1.0 ± 0.2, followed
133 by being treated with 2 volumes of 100% Ethanol for 45 min at room temperature.
134 The mixture was centrifuged at 2500 rpm for 1 min, 1 mL $1 \times$ PBS was used to wash
135 the cells, followed by another centrifugation at 2500 rpm for 1 min. The pellet was

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136 resuspended in 200 µL of 1 × PBS/1:2000 dilution DAPI mixture, and was observed
137 under Nikon Eclipse 50i fluorescence microscope (Nikon, Tokyo, Japan) after 45 min.

138 **2.5 Yeast morphology**

139 *S. cerevisiae* starters were transferred from YPD broth onto corresponding
140 YPD agar plates. Cultured media were serially diluted to OD_{600} = 0.1, 5 µL strain
141 solution was spotted onto corresponding YPD agar plates after an additional 10
142 times dilution being applied, 1 × PBS was used for dilution. The growth temperature
143 was set at 32 ^{o}C.

144 **2.6 GC-MS and chemical analysis of wine samples**

145 The alcohol and ester aroma compounds analysis was conducted using
146 headspace-solid phase microextraction (HS-SPME) and Shimadzu QP-2010 GC-MS
147 (Shimadzu, Kyoto, Japan). The methodology was adopted from previous published
148 articles, with slight modification regarding the diluent and sample matrix used with
149 the standards (17, 18). Detailly, 0.9 mL of sample was pipetted into a 20 mL amber
150 SPME vial and diluted with 8.06 mL of 5 g/L tartaric acid buffer (pH 3.5), 40 µL of
151 composite internal standard was added followed by 4.5 g of sodium chloride before
152 the vial was immediately capped.

153 For the preparation of the highest standard of the calibration curve, the
154 composite standard was diluted in 136 mL sample matrix which was rotary
155 evaporated at 36 ^{o}C for 40 min to remove volatile background, and reconstituted
156 with 14.2% Ethanol as well as 40 µL of 5 M sodium hydroxide which returned the pH
157 back to 3.15. It was then serially diluted in the provided matrix to ensure each vial
158 had a maximum volume of 0.9 mL of matrix present. Each vial was then diluted

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159 further with 8.06 mL of tartaric acid buffer as in the samples with 40 µL of composite
160 internal standard being added, followed by 4.5g of sodium chloride before the vials
161 were immediately capped.

162 Ethanol content was analyzed by GC-FID, which was carried out on a
163 Shimadzu GC-2010 gas chromatograph-flame ionization detector equipped with an
164 AOC-20i autoinjector and AOC-20s autosampler. The chromatography was
165 performed using an 19091N-133 HP-Innowax GC column (Polyethylene Glycol -
166 Agilent Technologies, CA, USA). Residual sugars including glucose and fructose were
167 measured using Vintessential enzymatic test kit (Vintessential Laboratories –
168 Tasmania, TAS, Australia), and glycerol content was measured using Megazyme
169 glycerol assay kit (Megazyme, Wicklow, Ireland).

170 **2.7 HDAC inhibition assay**

171 The HDAC inhibition capacity of candidate epigenetic modifiers was
172 measured by a fluorometric HDAC assay kit (Active Motif, Inc., CA, USA), following
173 the manufacturer's instructions with slight modification to suit the objectives of this
174 research. HeLa nuclear extract was used as the HDAC source, with an input volume
175 of 5 µL. Candidate epigenetic modifiers/HDAC inhibitors, including the positive
176 control Trichostatin A (TSA), were added at the volume of 10 µL. The volume of
177 HDAC assay buffer was adjusted to reach a total volume of 50 µL in each well.
178 Fluorescence was measured using FLUOstar Omega microplate reader (BMG
179 LABTECH, Ortenberg, Germany) with excitation wavelength at 360 nm and emission
180 wavelength at 460 nm.

181 **2.8 Statistical analysis**

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182 Results were gathered from three independent biological replicates unless
183 otherwise stated. Data were analyzed using analysis of variance (ANOVA) with a
184 generalized linear model, followed by *post-hoc* Tukey's mean comparison test, using
185 Minitab 20 (Minitab, LLC, PA, USA). PCA and AHC were analyzed using XLSTAT
186 Statistical Software 2016 (Addinsoft, Paris, France). A confidence level of 95% was
187 applied to the statistical analysis and data are presented as mean ± SD.
188

189 **3. Results and discussion**

190 **3.1 Project scope: the practice of altering fermentation by epigenetics**

191 The schematic diagram put forward to fit the entire scope of the project is
192 shown in Figure 1 and demonstrates the proposed innovation to food fermentation
193 by impacting gene expression levels of microbial starters using diet-derived
194 epigenetic modifiers. There are a range of diet-derived epigenetic modifiers
195 including bioactive compounds and phytochemicals, which are of health benefits to
196 humans. For example, diet derived short-chain fatty acids are a group of HDAC
197 inhibitors which are known to play a key role in epithelial hemostasis and repair
198 process (14). The research investigating the effect of food and food components on
199 gene expression, and its role involved in the interaction between host/microbes and
200 the nutritional environment is a well-established research field called
201 "nutrigenomics" (Figure 1A). In this study, we have shown that we can use these
202 dietary epigenetic compounds such as dietary HDACi to alter the microbial
203 phenotypes used in the fermentation process. We coined the word
204 "nutrifermentics" (Figure 1B) to represent this new field of research. These dietary

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205 HDACi could also provide health benefits to consumers, in addition to improving
206 starter microbial cultures in the fermentation process.

207 **3.2 Influence of benzoic acid on *S. cerevisiae* histone H3**

208 Benzoic acid and its derivatives are known HDACi (11) and recent study
209 revealed that sodium benzoate can stimulate a new histone mark, lysine
210 benzoylation with significant physiological relevance (12). Figure 2A shows the
211 percentage of relative changes in 21 distinct histone H3 modification patterns in *S.*
212 *cerevisia,e* which was treated with 5 mM benzoic acid, in comparison with untreated
213 wild type strain. Specific antibodies, including 15 for methylation, 4 for acetylation
214 and 2 for phosphorylation were utilized to measure the 21 patterns. Most
215 modification patterns between treated and untreated strains were around 100%
216 when taking the variation into consideration. However, both stimulation and
217 inhibition in histone marks were seen with exposure to 5 mM benzoic acid.
218 H3K4me2, H3K9me3, H3K27me2, H3K9ac, H3K18ac and H3ser10p were stimulated
219 more than four-fold in treated strains, whereas few methylation patterns including
220 H3K4me3, H3K9me2 and H3K27me3 were about half compared to the untreated
221 strain. Histone modifications are directly relevant to gene expression levels in the
222 organism

223 **3.3 Gene expression analysis by NanoString**

224 NanoString transcription analysis revealed the expression of 24 genes
225 including five housekeeping genes. Figure 3 shows the gene expression levels of *S.*
226 *cerevisiae* under different treatments, TSA treatments including first-time exposure,
227 500 h treatment (20 h/sub-culture) and 1 generation w/o treatment after 500 h

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228 exposure were included as the HDACi controls, which were in accordance with
229 benzoic acid treatments (first-time exposure to 5 mM benzoic acid, 500 h treatment
230 (20 h/sub-culture) at 10mM benzoic acid /epimutant 1 and 1 generation w/o
231 treatment after 500h exposure/epimutant 2). The untreated wild type strain was
232 included as a negative control along with 0.9% sodium chloride and with a dietary
233 polyphenol epigallocatechin gallate (EGCG) that has been reported to inhibit DNMTs
234 (4).

235 Results are presented as a heat map graph after Z-score transformation,
236 ranging from -3 to 3, blue (downregulation) to orange (upregulation), Figure 3. As
237 shown in Figure 3, RNA samples with different treatments were clustered after data
238 normalization, in which benzoic acid supplementation became a distinct influencing
239 factor.

240 Genes responsible for overproducing phenylethyl alcohol (ARO4 and TYR1)
241 (19), fusel alcohol and ester synthesis (EEB1) (20), biosynthesis of higher alcohols
242 (BAT1) (21) were all upregulated. There was no significant change in the expression
243 levels of another ester synthase gene (EHT1) (20) between samples. Several genes
244 responsible for stress tolerance and cell cycle were analyzed finding the histone
245 deacetylase gene (RPD3) (22) expression was downregulated while the histone
246 acetyltransferase gene (GCN5) (23) was upregulated in both 5mM benzoic acid-
247 treated strain and epimutant 1, clearly supporting the role of benzoic acid as an
248 HDACi. Kurat et al. (23), previously reported that upregulation of the GCN5
249 expression led to histone acetylation and global transcriptional activation. The
250 variation observed could potentially indicate alternative acetylation mechanisms in

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251 *S. cerevisiae* resulted from different concentrations and exposure time of certain
252 epigenetic compounds such as benzoic acid. With respect to the clusters, RNA from
253 *S. cerevisiae* epimutant 1 (500 h growth in YPD broth containing 10 mM benzoic acid,
254 20 h/sub-culture) showed quite similar expression patterns to 5 mM benzoic acid
255 treatment (first time exposure). Moreover, the epimutant 2 (500 h growth in YPD
256 broth containing 10 mM benzoic acid, followed by 20 h growth in regular YPD broth
257 w/o stress, 20 h/sub-culture) exhibited significantly different RNA expression
258 patterns compared with the benzoic acid treatment group. The epimutant 2 tended
259 to be more relevant to wild type and other *S. cerevisiae* treatments. This observation
260 suggests that the alteration of gene expression caused by dietary epigenetic
261 compounds, which is revealed by direct counts of RNA transcripts, is transient and
262 tends to ease out once the stress inducer is eliminated from the environment.

263 **3.4 Influence of benzoic acid on *S. cerevisiae* nucleus**

264 As shown in Figure 4A, DAPI (4', 6-diamidino-2-phenylindole) staining was
265 applied to wild type and epimutant 1 to visualize their nuclei in terms of any size
266 changes (expansion) that may have resulted from benzoic acid treatment. As DAPI
267 stoichiometrically binds to DNA, which enables the detection and comparison of
268 DNA content variation by fluorescence microscopy (24). The corrected total cell
269 fluorescence (CTCF) was calculated based on the integrated density in the nucleus
270 region. The mean comparison results indicate that there is a significant difference
271 between two samples ($p < 0.05$), which suggests an expansion to the nucleus region
272 in *S. cerevisiae* when they are exposed to 10 mM benzoic acid. A. D. Walters et al.
273 (25) suggested that the expansion of nuclear envelope in budding yeast is

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274 independent from cell growth, but potentially related to nucleoplasmic factors, such
275 as one or more nucleoplasmic proteins that are synthesized or imported into the
276 nucleus. As HDAC and HDACi have been well researched being involved in multiple
277 cell processes, such as cytokinesis and apoptosis (26, 27). Therefore benzoic acid
278 induced, HDACi related modifications could have occurred in nucleoplasm, resulting
279 in expanded nucleus or relaxed genome state, which led to more fluorescence in this
280 study.

281 **3.5 Yeast morphology in relation to epigenetic alteration**

282 The cellular morphology of benzoic acid-treated *S. cerevisiae* was recorded to
283 depict phenotypic differences compared to the wild type (Figure 4B). Epimutant 1
284 was more tolerant to benzoic acid treatment and showed visible alteration in colony
285 morphology. Observed tolerance and adaptation to benzoic acid faded soon after
286 the stress was eliminated from the environment, demonstrating the transient nature
287 of the treatment and possibly epigenetic change. The observation is supported by S.
288 Torres-Garcia et al. (9); phenotypic plasticity can be promoted by epigenetic
289 processes that let the wild type cells adapt to certain unfavorable environments
290 without altering genetic information, although these alterations are generally
291 unstable and will be gradually lost without the stress. This observation is in line with
292 NanoString assay, where *S. cerevisiae* epimutant 1 showed very similar expression
293 patterns to 5 mM benzoic acid-treated strain (first time exposure). However,
294 epimutant 2 exhibited significantly different gene expression patterns when
295 compared within the benzoic acid treatment group. Epimutant 2 gene expression
296 patterns were more similar to wild type than other treatments. This suggests that

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297 the alteration of gene expression caused by dietary epigenetic compounds is
298 transient and tends to fade once the compound is eliminated from the environment.
299 Overall, the robustness of *S. cerevisiae* epimutants and their adaption to stressed
300 environment were improved by continuously treating the strain with the threshold
301 levels of benzoic acid. However, epigenetic plasticity could be an issue in retaining
302 the robust characteristics for future generations once the epigenetic modifiers is
303 removed from the environment. However, commercial yeast starter culture
304 producer could potentially prefer single/double use strains similar to commercial
305 seed companies.

306 **3.6 Wine characteristics changes due to epigenetic alteration**

307 To test the impact of benzoic acid-stimulated epigenetic changes on
308 fermentation characteristics of *S. cerevisiae*, treated cultures were used to ferment
309 wine samples. Wines were fermented using three *S. cerevisiae* starters, including
310 wild type, epimutant 1 (500 h growth in YPD broth containing 10 mM benzoic acid,
311 20 h/sub-culture) and epimutant 2 (epimutant 1 followed by 20 h growth in regular
312 YPD broth without stress). Principal component analysis (PCA) of aromatic attributes
313 of wine samples and agglomerative hierarchical clustering (AHC) were utilized for
314 classification of fermented wine samples (Figure 5A). In addition, GC-MS analysis was
315 carried out on wine samples, distinct results are shown in Figure 5B, with full analysis
316 of 18 compounds listed in Table 1. The three starters resulted in three wine
317 categories, each with distinct aromatic profiles (Figure 5A & 5B). The positive
318 correlation between epimutant 1 and *cis*-3-hexen-1-ol may indicate a kiwifruit and
319 leaf-like aroma is potentially associated with wine produced by epimutant 1 (28).

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320 Since *cis*-3-hexen-1-ol is an important aroma compound in many white wines, it
321 might confer a complex aromatic profile on the altered wine, by adding partial
322 aromatic features of white wine. As shown in Figure 5B, the content of five ester and
323 higher alcohol compounds is listed as potential indication of wine aroma alterations
324 resulting from epimutation of the starters. Wine fermented by epimutant 1
325 possessed significantly increased phenylethyl alcohol (rose scent), ethyl lactate
326 (butter aroma), *cis*-3-hexen-1-ol (leaf alcohol confers grassy-green odor) and ethyl
327 pentanoate (fruity aroma), whereas the content of ethyl octanoate was reduced
328 (soapy, floral aroma) ($p < 0.05$) (29). This GC-MS analysis clearly supports the gene
329 expression observed using Nanostring assay. This is a significant advancement in the
330 fermentation field considering the treatments lead to over expression of genes
331 associated with favorable aromas such as Phenyl ethyl alcohol and overproduction of
332 these compounds are confirmed by GC-MS analysis. In addition to aroma alterations,
333 major chemical composition changes due to epigenetic alterations were also
334 investigated, including residual sugars (glucose and fructose), glycerol and ethanol
335 (Supplementary Dataset 1). Generally, epimutant 1 tended to increase the fructose
336 content in wine ($p < 0.05$), whereas not significantly affect the content of other
337 chemicals analyzed ($p > 0.05$). The study detected a few more distinct aromatic
338 compounds by qualitative GC-MS in these wine samples and is listed in
339 Supplementary Dataset 2.

340 **3.7 HDAC inhibition capacity**

341 As in previous NanoString assay, significantly different patterns of transcribed
342 genes by other treatments compared to benzoic acid group, suggesting that HDACi

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343 and compounds capable of modifying epigenetic states have different effects on
344 histone proteins and gene expression patterns, potentially indicating wide range of
345 application for these HDACi (Figure 3). This led us to consider the HDACi activity of
346 dietary epigenetic compounds. To investigate this we applied a well-established
347 HDAC assay using HeLa cell lines (14, 15). The dietary compounds tested were
348 sodium butyrate, quercetin, genistein, anacardic acid, curcumin and EGCG (Figure
349 2B). Untreated HeLa nuclear extracts were used as the negative control. 5-Aza-2'-
350 deoxycytidine, which is a well-recognised DNMT inhibitor but not HDAC inhibitor
351 (30), and glucose were also included as negative controls for assay calibration. A
352 well-known HDACi, namely TSA, was used as a generic inhibitor of histone
353 acetylation to test the impact on gene transcription (31). Relevant half-maximal
354 inhibitory concentrations (IC_{50}) were referred for determining the testing
355 concentrations of candidate chemicals, except for benzoic acid since an IC_{50} was not
356 determined. In general, most of the tested dietary compounds exhibited equivalent
357 or better HDACi capacity compared to TSA, the positive control, suggesting their
358 potential application in the food industry, particularly the food fermentation field.
359

360 **4. Conclusions**

361 This study showed the potential applications of dietary epigenetic
362 compounds in food research. As a proof of concept, it has been shown that
363 epigenetic changes in yeast *S. cerevisiae* can be induced using dietary compounds
364 resulting in different aromatic profiles in alcoholic fermentation. This opens the
365 exciting possibility of using a non-GMO approach to obtain microbial strains with

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366 desirable characteristics for fermented food products. Interestingly, it was observed
367 that the downregulation of H3K27me3 histone mark and upregulation of GCN4 gene,
368 which are associated with life span extension in *C.elgans* and *S. cerevisiae,*
369 respectively (32-34). Understanding the role of these dietary epigenetic compounds
370 on cell ageing is potentially an interesting future research.

371

372 **Author Contributions: Yanzhuo Kong:** Investigation, Validation, Formal Analysis,
373 Writing – Original Draft & Writing – Review & Editing; **Kenneth J. Olejar:**
374 Investigation, Validation & Writing – Review & Editing; **Stephen L.W. On:** Validation
375 & Writing – Review & Editing; **Christopher Winefield:** Validation & Writing – Review
376 & Editing; **Philip A. Wescombe:** Validation & Writing – Review & Editing; **Charles S.**
377 **Brennan:** Writing – Review & Editing; **Richard N. Hider:** Investigation & Validation;
378 **Venkata Chelikani:** Conceptualization, Writing – Review & Editing, Resources,
379 Supervision & Funding Acquisition.

380

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384

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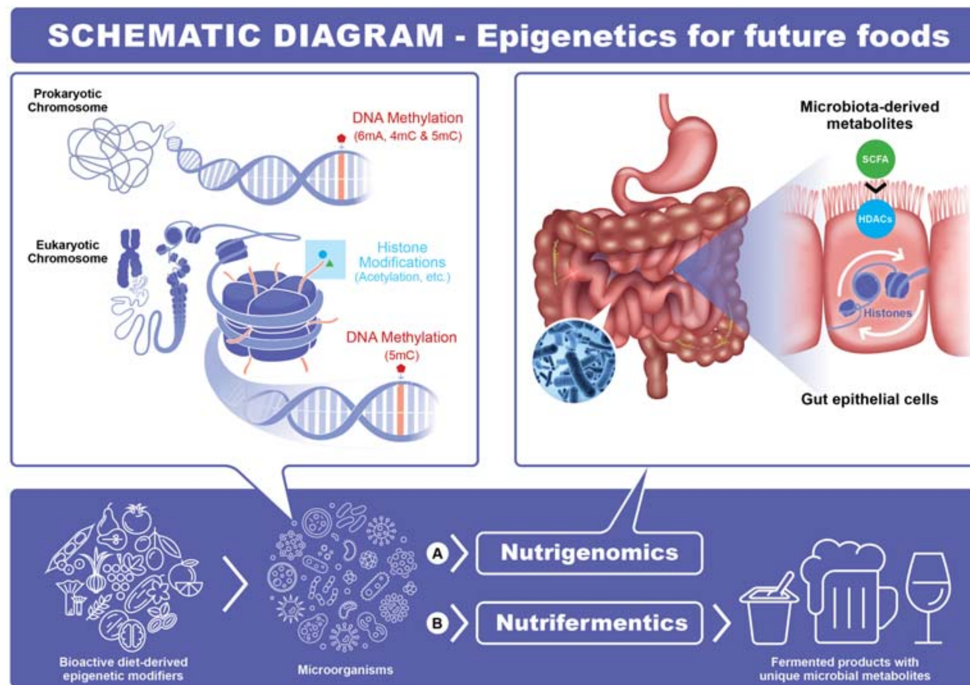
505 **Figures and Tables:**



506
507 **Figure 1.** Schematic diagram: An innovation to food fermentation by impacting gene
508 expression levels of microbial starters using diet-derived epigenetic modifiers.

509 A) **Nutrigenomics:** A well-established field of research investigating the effect of
510 food and food components on gene expression, and its role involved in the
511 interaction between host/microbes and the nutritional environment;

512 B) **Nutrifermentics:** A new research direction firstly proposed in this study,
513 which provides insights regarding the effect of food and food components on
514 microbial starters and its potential application in fermentation.

515 **Abbreviations: SCFA:** short-chain fatty acids; **HDACs:** histone deacetylases.

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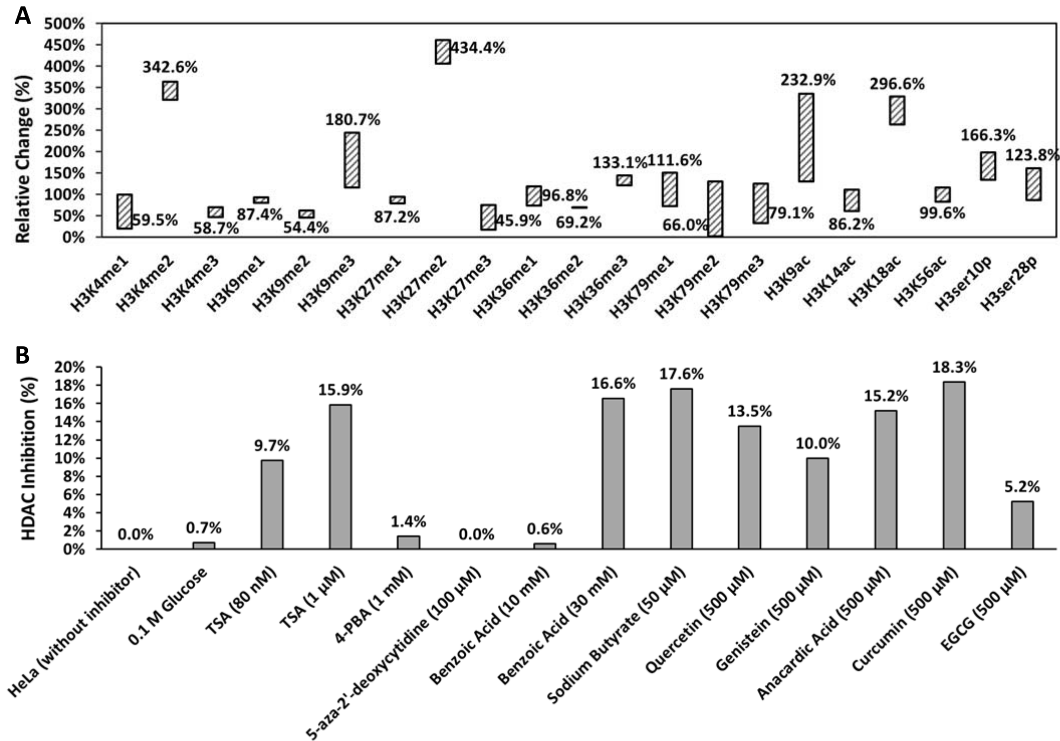

516
517 **Figure 2. A.** Relative changes of 21 histone H3 modification patterns between *S.*
518 *cerevisiae* wild type and strains treated with 5 mM benzoic acid. **B.** Histone
519 deacetylase (HDAC) inhibition capacity of candidate epigenetic modifiers at different
520 concentrations, in comparison with HeLa cells without inhibitors.

521 **Abbreviations: TSA:** trichostatin A; **4-PBA:** 4-phenylbutyric acid; **EGCG:** epigallocatechin
522 gallate.

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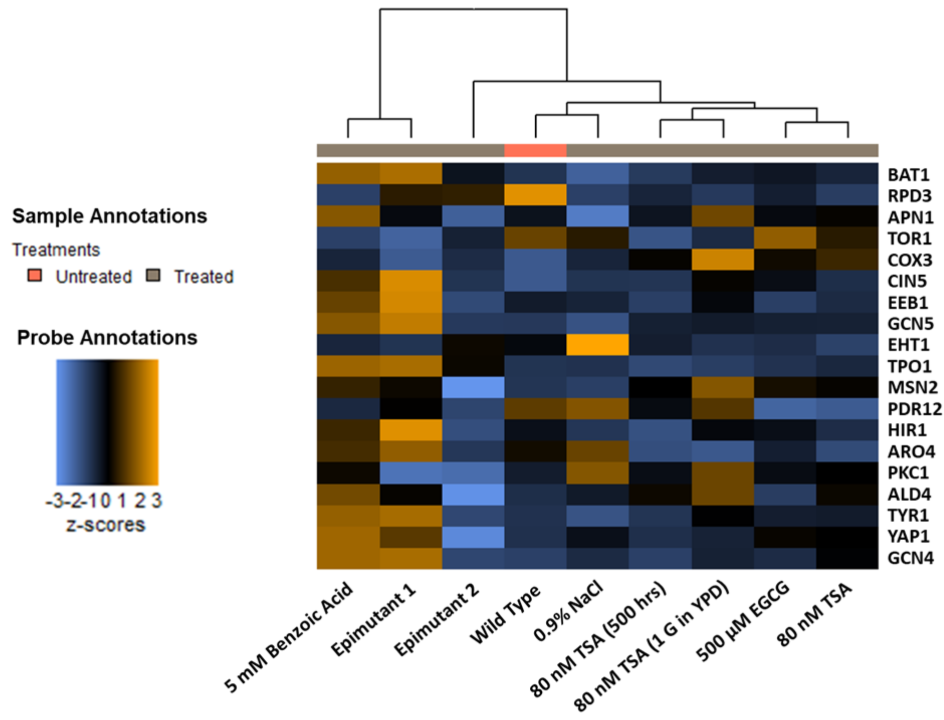

523

524 **Figure 3.** Heat map showing unsupervised hierarchical clustering of 9 S. cerevisiae
525 samples under different treatments, based on their expression levels of 19 selected
526 genes.

527 **Abbreviations: TSA:** trichostatin A; **EGCG:** epigallocatechin gallate.

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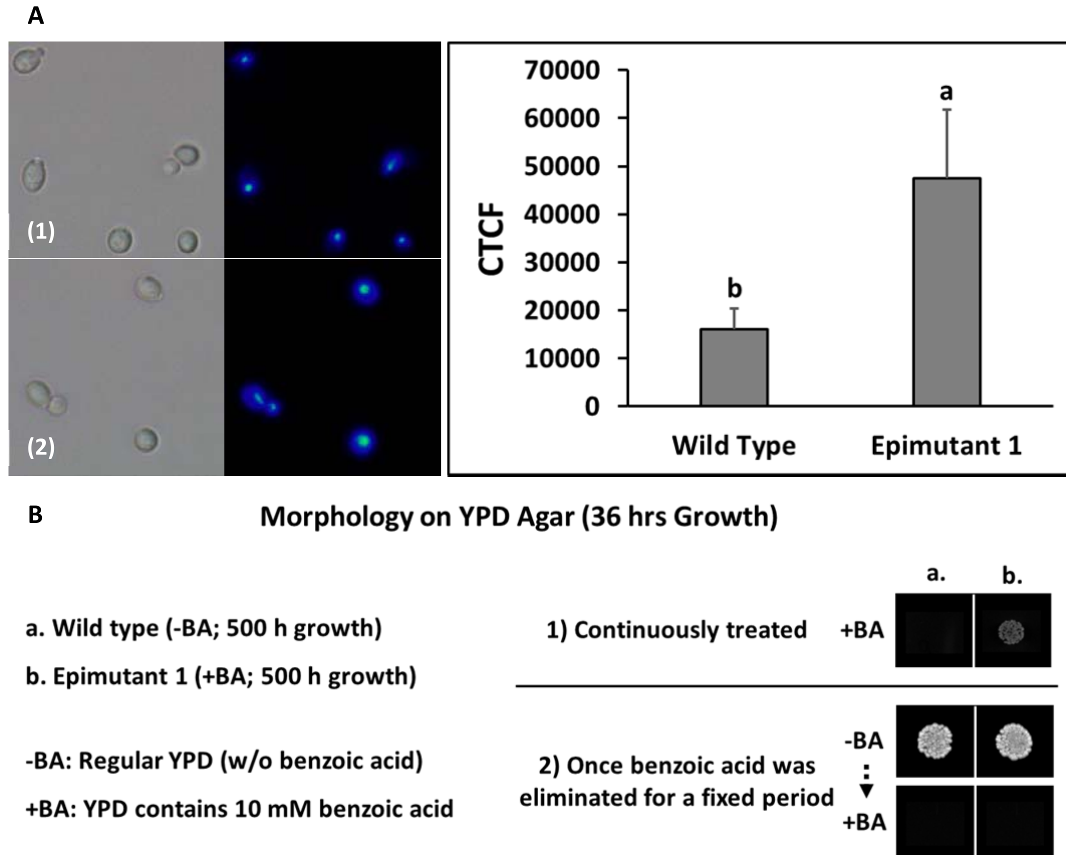

528

529 **Figure 4**. **A.** DAPI staining (bright-field/fluorescent, at 100X) and corrected total cell
530 fluorescence levels of benzoic acid-treated *S. cerevisiae* in comparison with wild
531 type. **B.** Phenotypic plasticity of benzoic acid-treated *S. cerevisiae* epimutant.
532 **(1):** Wild Type; **(2):** Epimutant 1; **CTCF:** corrected total cell fluorescence; **a-b:**
533 different letters indicate significant difference based on Tukey pairwise mean
534 comparison results ($p < 0.05$).

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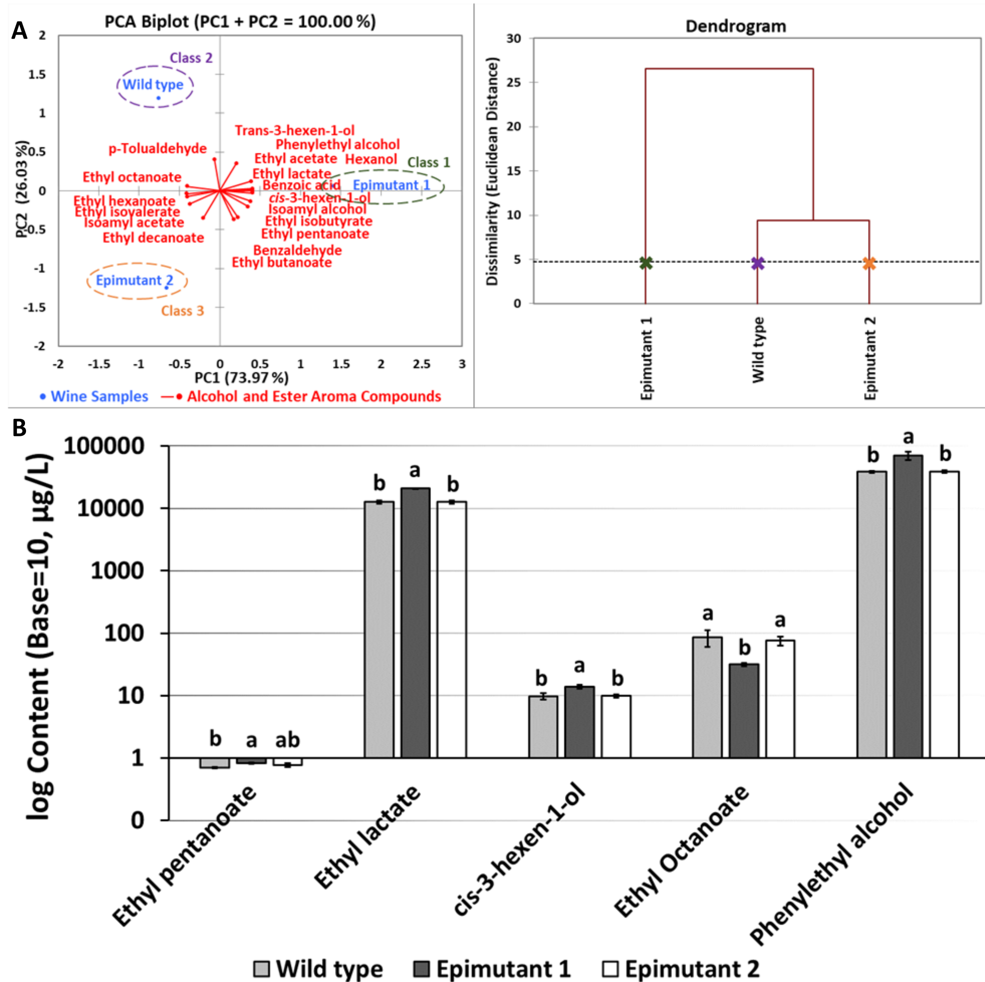

535

536 **Figure 5. A. Left:** Principal component analysis (PCA) bi-plot illustrating the
537 relationship between wine samples fermented under different conditions and the
538 variance of alcohol and ester aroma compounds; **Right:** Wine samples grouped using
539 agglomerative hierarchical clustering (HCA) according to dissimilarity levels based on
540 GC-MS analysis. **B.** The content of ester and higher alcohol compounds potentially
541 contributing to distinct aromatic profiles of wine samples fermented by epimutated
542 *S. cerevisiae*.

543 **a-b:** different letters indicate significant difference based on Tukey pairwise mean
544 comparison results ($p < 0.05$).

Table 1. Analysis of alcohol and ester aroma compounds detected in Pinot Noir wine.

Wine Samples*	Ethyl Acetate (mg/L)	Ethyl Isobutyrate (µg/L)	Ethyl butanoate (µg/L)	Ethyl Isovalerate (µg/L)	Isoamyl Acetate (µg/L)	Ethyl pentanoate (µg/L)	Isoamyl alcohol (mg/L)	Ethyl hexanoate (µg/L)	Ethyl lactate (mg/L)
Wild Type	27.5 ± 0.5^{ab}	43.8 ± 4.4^{a}	64.5 ± 9.0^{a}	4.1 ± 0.5^{a}	165.7 ± 24.9^{a}	0.7 ± 0.0^{b}	143.7 ± 6.1^{a}	75.0 ± 16.4^{a}	12.6 ± 0.6^{b}
Epimutant 1	30.9 ± 1.9^{a}	51.6 ± 4.0^{a}	66.9 ± 4.9^{a}	3.3 ± 0.7^{a}	151.3 ± 20.7^{a}	0.8 ± 0.0^{a}	175.2 ± 23.7^{a}	54.0 ± 9.5^{a}	20.8 ± 0.3^{a}
Epimutant 2	26.2 ± 2.1^{b}	46.8 ± 2.5^{a}	67.3 ± 5.6^{a}	4.3 ± 0.3^{a}	174.5 ± 19.1^{a}	0.8 ± 0.1^{ab}	147.6 ± 4.4^{a}	76.2 ± 2.5^{a}	12.6 ± 0.7^{b}

Wine Samples*	Hexanol (µg/L)	Trans-3-hexen-1-ol (µg/L)	Cis-3-hexen-1-ol (µg/L)	Ethyl Octanoate (µg/L)	Benzaldehyde (µg/L)	Ethyl decanoate (µg/L)	Phenylethyl alcohol (mg/L)	p-Tolualdehyde (µg/L)	Benzoic Acid (mg/L)
Wild Type	605.7 ± 14.1^{a}	54.4 ± 10.4^{a}	9.7 ± 1.2^{b}	86.1 ± 25.6^{a}	35.5 ± 11.3^{a}	20.2 ± 4.0^{a}	38.4 ± 1.4^{b}	159.6 ± 21.1^{a}	2.4 ± 0.1^{b}
Epimutant 1	694.1 ± 24.8^{a}	56.4 ± 7.8^{a}	13.8 ± 0.9^{a}	31.6 ± 1.7^{b}	45.3 ± 11.7^{a}	20.0 ± 2.2^{a}	69.9 ± 10.6^{a}	146.1 ± 13.6^{a}	3.3 ± 0.1^{a}
Epimutant 2	608.1 ± 65.1^{a}	21.3 ± 11.0^{b}	9.9 ± 0.6^{b}	75.4 ± 12.1^{a}	45.2 ± 12.2^{a}	24.4 ± 6.0^{a}	38.8 ± 1.9^{b}	137.1 ± 13.0^{a}	2.3 ± 0.1^{b}

Note: Results are presented as mean ± SD (standard deviation, N = 3); $^{a-b}$ Results with different superscripts within the same column indicate significant differences between three types of wines (Tukey's HSD test, $p < 0.05$); *Wine samples were fermented using three types of the starters: **Wild Type:** 500 h growth in regular YPD broth, 20 h/sub-culture; **Epimutant 1:** 500 h growth in YPD broth containing 10 mM benzoic acid, 20 h/sub-culture; **Epimutant 2:** 500 h growth in YPD broth containing 10 mM benzoic acid, followed by 20 h growth in regular YPD broth w/o stress, 20 h/sub-culture.

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