Influence of Essential Inorganic Elements on Flavour Formation During Yeast Fermentation

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Abstract

The relative concentration of available inorganic elements is critical for yeast growth and metabolism and has potential to be a tool leading to directed yeast flavour formation during fermentation. This study investigates the influence of essential inorganic elements during alcoholic fermentation of brewers wort, fermented using three independent yeast strains, *Saccharomyces pastorianus* W34/70, and *Saccharomyces cerevisiae* strains M2 and NCYC2592 under a range of conditions replicated for each yeast strain. 10 treatments were applied: 1 control and 9 inorganic supplementations: standard brewers wort, ammonia-nitrogen, inorganic phosphate, potassium, magnesium, copper, zinc, iron, manganese and a composite mixture, Twenty-five chemical markers were evaluated by HPLC (ethanol, glycerol), and GC-MS (aroma). There was a significant change in volatile aroma compounds during fermentation, which was more prominent when supplemented with ammonia nitrogen, inorganic phosphate, potassium or magnesium ($P < 0.05$). Heavy metal ions mostly had a negative effect on the flavour formation.

Keywords: Brewing, *Saccharomyces*, Flavour Production, Metal ions

1. Introduction

Brewers' wort is a complex matrix in which yeast growth and fermentation occurs. Because of this, the final flavour of beer is directly influenced by the chemical composition of the wort, both directly and through the formation of flavours during fermentation (Wietstock *et al.* 2015, Bilverstone *et al.* 2015). The influence of carbohydrates and amino acid availability on yeast flavour formation is well known, however metal ions also play a vital role in the brewing process (Wietstock *et al.* 2015) as they act as enzymatic co-factors and also have major structural roles (Cyert and Philpott 2013). Fig. 1 illustrates the main glycolytic pathway and an overview of the relationship between genes, enzymes and inorganic elements involved in key fermentation reactions. Specific steps associated with glycolysis (Fig. 1A) and pyruvate catabolism (Fig. 1B) are detailed, which are important for energy production and NAD recycling respectively. The extent to which each pathway is employed is dependent on environmental conditions and nutrient availability. When conditions favour growth, pyruvate is directed away from ethanol production to acetyl-CoA via acetaldehyde/acetate and this leads to the formation of sensorial compounds and yeast-derived flavours.

As alluded to above, during fermentation yeast cells generate a number of metabolic end products, and the extent to which these are produced is intrinsically linked to yeast growth. Inorganic elements are important for enzyme activity, cell division and biomass generation, as well as carbohydrate and amino acids catabolism (Canadell *et al.* 2015, Barreto *et al.* 2012, Boubekeur *et al.* 2001). The uptake of mineral ions by yeast cells and their ultimate functions from a yeast physiological perspective are well documented (Cyert and Philpott 2013, De Freitas *et al.* 2003). However, their precise role in fermentation and flavour generation is less well understood, due primarily to the complexity of the fermentation environment. Despite this, it is clear that the mineral composition of raw materials is an important consideration when rationalising yeast behaviour in different wort source (Wietstock *et al.* 2015, Zufall and Tyrell 2008).

A number of recent studies have focused on yeast flavour formation during fermentation, including the influence of external stressors on the production of flavour compounds during wine making (Fairbairn 2012), the influence of metal ions on beer flavour stability (Zufall and Tyrell 2008), the availability of oxygen and glucose metabolism on the biosynthesis and metabolism of higher alcohols (Vidal *et al.* 2014), volatile ester synthesis (Bilverstone *et al.* 2015, Zhuang *et al.* 2015) and metabolic production of short chain fatty acids (Yu *et al.* 2016). However, none of these studies have focussed on the influence of inorganic elements on yeast flavour production across multiple yeast strains, including those belonging to the species *S. cerevisiae* and *S. pastorianus*, used for ale and lager production respectively.

This work aims to investigate the influence of a broad range of essential inorganic elements on the formation of flavour compounds during fermentation of a brewers wort, using three different commercial yeast strains. Furthermore, it is anticipated that this study will provide a more complete understanding of the fundamental role of essential inorganic elements on yeast flavour production, with the potential to provide brewers with a more complete understanding of how to control or manipulate yeast flavour production.

2. Materials and Methods

2.1. Materials

Three yeast strains were used in this study, including two *Saccharomyces cerevisiae*, ale strains (NCYC2592 and M2) and one *Saccharomyces pastorianus*, lager strain (W34/70). All strains were maintained on agar containing 10 g / L yeast extract, 20 g / L peptone, 20 g / L glucose, and 20 g / L agar (YPD agar) and grown on 10 g / L yeast extract, 20 g / L peptone, and 20 g / L glucose (YPD) in an orbital shaker (180 rpm) at 30 °C under aerobic conditions. All solutions were prepared using materials supplied by Fisher Scientific, UK.

A sweet brewers wort (SBW) with specific gravity (SG) 1.054, pH 5.42 and dissolved oxygen (DO) 12 mg / L) was manufactured at the International Centre for Brewing Science at the University of Nottingham. The basic properties of the wort were measured and are presented in Table 1 (specific gravity, pH, carbohydrates) and Table 2 (mineral composition).

Solutions of inorganic elements for supplementation were prepared in 100 mL volumetric flasks, Inorganic elements (30 g $(NH_4)_2SO_4$ (nitrogen), 10 g KCl (potassium), 10 g $Na_2HPO_4.H_2O$ (inorganic phosphate: sodium phosphate was included as a source of phosphorous), 10 g $MgCl_2$ (magnesium), 10 g $FeSO_4.7H_2O$ (iron), 15 g $ZnSO_4.7H_2O$ (zinc), 10 g $CuSO_4.5H_2O$ (copper), 10 g $MnSO_2.4H_2O$ (manganese) were added and made up to 100 mL with distilled water. Flasks were sealed and sterilised (autoclave, 121 °C) and stored at room temperature (22 °C) until required. All chemicals used were analytical grade (> 95 % purity, Sigma-Aldrich, UK).

2.2. Fermentation procedure

Miniature fermentations were conducted using 180 mL sterile Wheaton glass serum bottles (Sigma-Aldrich, U.K.) according to the method described previously (Quain *et al*. 1985, Zhuang *et al*. 2019) using 100 mL aliquot of sweet brewers wort (SG 1.054, pH 5.42, DO 12 mg of dissolved

oxygen/L). Yeast was pitched 1.5 x 10^7 cell / mL and magnetic stirrers were used for agitation at 200 rpm. The fermentation vessels were sealed with a rubber septum and a metal crimp. A hypodermic needle was then inserted through the septum. A Bunsen valve, attached to the needle, allowed the exit of gas from the vessel. Fermentation experiments were conducted using beer sweet wort (SG 1.054 and pH 5.42) as a reference (T0) plus 9 supplementation treatments. Supplementation treatments were conducted by adding to the fermentation vessels; ammonium nitrogen (T1, 63.5 mg / L), phosphorus (T2, 19.5 mg / L), potassium (T3, 52.5 mg / L), magnesium (T4, 25.5 mg / L), copper (T5, 25.5 mg / L), zinc (T6, 34.1 mg / L), iron (T7, 20.1 mg / L), manganese (T8, 27.8 mg / L), T9 (a composite mixture of all nutrients in concentrations used in T1 to T8). Each quantity described above was achieved by adding 100 µL from the stock solution into the treatment vessel (Section 2.3). All fermentations were conducted in triplicate.

After fermentation, yeast suspensions (0.5 mL) were collected for viability assay (Section 2.5) and fermented wort samples were transferred into 50 mL falcon tubes and centrifuged at 4000 rpm for 4 min to separate the supernatant. Samples of each supernatant (25 mL) were transferred into fresh 50 mL Falcon tubes and kept frozen. These samples were defrosted for 1 h at room temperature before compositional analysis (Section 2.6).

2.3. Analysis of yeast cell viability

Viability was determined using methylene blue assessment. 100 mL of methylene blue (0.01 g / L stock solution) was dissolved in sodium citrate solution (2 % w / v) to a final concentration of 0.01 % (Pierce, 1970). Yeast suspension (0.5 mL) was mixed with methylene blue solution (0.5 mL) and incubated for 5 min at room temperature. Viability was measured using the Aber countstar instruments connected to a computer for data acquisition via countstar software.

2.4. Compositional analysis of carbohydrates, sugars, ethanol and glycerol by high performance chromatography

Maltotetraose, maltotriose, maltose, sucrose, glucose, fructose, ethanol and glycerol concentrations were quantified by high-performance liquid chromatography (HPLC) using a method developed by Wilkinson *et al.* (2014). Pre-centrifuged/filtered samples (2 mL) were pipetted into a 2 mL vials (SLS Ltd, Nottingham, UK). The HPLC system included a Jasco AS-2055 Intelligent auto sampler (Jasco, Tokyo, Japan) and a Jasco PU-1580 Intelligent pump (Jasco, Tokyo, Japan), chromatographic separation was performed on a Rezex ROA H+ organic acid column, length 300 mm, internal diameter 7.8 mm, particle size 5 µm (Phenomenex, Macclesfield, UK) at 22 ^{o}C. The mobile phase was 0.005 N H_2SO4 with a flow rate of 0.5 mL/min, the injection volume was 10 µL and analysis was completed over 28 min. For detection, a Jasco RI-2031 intelligent refractive index detector (Jasco, Tokyo, Japan) was employed. Azur (version 4.6.0.0, Datalys, St Martin d'Heres, France) was used for data acquisition and determination of peak area and concentrations determined by comparison to authentic standards. External calibration standards included maltose, glucose, and fructose in the range 0 – 8.5 g / 100 mL (0, 0.5, 2.5, 4.5, 6.5, and 8.5); sucrose and glycerol in the range 0 – 5 g / 100 mL (0, 1, 2, 3, 4, and 5 g/100 mL); ethanol in the range 0 – 9 g / 100 mL (0, 1, 3, 5, 7, and 9 g / 100 mL); and maltotetraose and maltotriose in the range 0 – 2 g / 100 mL (0, 0.4, 0.8, 1.2, 1.6, and 2 g / 100 mL). All chemicals used were analytical grade (> 95 % purity, Sigma-Aldrich, UK).

2.4. Compositional analysis of non-polar volatile organic compounds by gas chromatography mass spectrometry (GC-MS)

Internal standard (100 µL of 0.01 % (v / v) hexanol) was added to fermentation samples (10 mL), fermentation samples were then extracted 5 times with 2mL of diethyl ether for 1 h at room temperature (22 °C). After leaving the mixture to stand for 1 min, the higher diethyl ether layer was collected in 5 mL conic glass vials (screw top, Chromacol Ltd, Herts, UK) and mixed with 0.5 g of Na_2SO_4 to dry the organic phase; the samples were then transferred 2 mL GC vials and stored at -20

°C prior to analysis by **GC-MS**. All chemicals used were analytical grade (> 95 % purity, Sigma-Aldrich, UK).

GC-MS analysis was conducted using 2 mL of the ether layer pipetted into a 2 mL GC vial (SLS Ltd, Nottingham, UK). Sample (1.5 μL) was injected in splitless mode into the injector port of a Trace 1300 Series GC (Thermo Scientific, Massachusetts, USA) using an AS 3000 autosampler (Thermo Scientific, Massachusetts, USA). The column was ZB WAX, 30 m x 0.25 mm i.d. x 0.25 μm film thickness (Phenomenex, Macclesfield, UK). Column temperature was held initially at 40 °C for 2 min, increased by 8 °C / min to 250 °C held for 2 min. Samples were injected in triplicate and analytes detected using an ISQ mass spectrometer (Thermo Scientific, Massachusetts, USA) operating in full scan mode from 35 to 300 m/z at 1.8 scans/s. Method was based in part on Yang, *et al.* (2016). Analytes were identified by comparing the formula, mass weight and retention time to authentic standards, peak areas were used to quantify target compounds.

The compounds analysed by GC-MS were acetic acid, *n*-propanol, isobutanol, 2-methyl butanol, isoamyl alcohol, 2-phenyl-ethanol, isobutanoic acid, butanoic acid, isovaleric acid, hexanoic acid, octanoic acid, decanoic acid, isoamyl acetate, isobutyl acetate, 2-phenylethyl acetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl butanol, ethyl hexanol, acetoin and 2,3-butanediol.

2.5. Multi-element analysis by inductively coupled plasma-mass spectrometry (ICP-MS)

Sample aliquots (1 mL) were taken and added to 4 mL of HNO_3. Samples were allowed to react for 24 h at 22 °C (room temperature). Samples then were exposed to microwave heat (Multiwave PRO, Anton Paar) using the following temperature program: Power (1,500 W), heat to 140 °C over 10 min, hold at 140 °C for 30 min, and cool for 30 min. Digested samples were transferred into fresh tubes and adjusted up to 20 mL using milliQ water. Diluted samples (1 mL) were transferred into ICPMS tubes and adjusted up to 10 mL using milliQ water.

Multi-element analysis of diluted solutions was undertaken by ICP-MS (Thermo-Fisher Scientific iCAP-Q, following in part Khokhar *et al.* (2018) Thermo Fisher Scientific, Bremen, Germany) using the following parameters: plasma power, 1,550 W; cool gas (14 L / min), sample flow rate, 4.0 mL / min, argon gas flow rates: auxiliary 0.8 L / min, nebulizer 0.4 L / min;. The instrument was run employing three operational modes, including (i) a collision-cell (Q cell) using He with kinetic energy discrimination (He-cell) to remove polyatomic interferences, (ii) standard mode (STD) in which the collision cell is evacuated and (iii) hydrogen mode (H_2-cell) in which H_2 gas is used as the cell gas. Samples were introduced from an autosampler (Cetac ASX-520) incorporating an ASXpress™ rapid uptake module through a PEEK nebulizer (Burgener Mira Mist). Internal standards were introduced to the sample stream on a separate line via the ASXpress unit and included Ge (10 µg / L, Rh (10 µg / L) and Ir (5 µg / L) in 2 % trace analysis grade (Fisher Scientific, UK) HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA) included Ca, Cu, Fe, K, Mg, Mn, P, and Zn, in the range 0 – 100 µg / L (0, 20, 40, 100 µg / L). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, France) was used to create Ca, Mg, and K standards in the range 0-30 mg / L. Phosphorus calibration utilized in-house standard solutions (KH_2PO_4). In sample switching was used to measure B and P in STD mode, Se in H_2 cell mode and all other elements in He cell mode. Sample processing was undertaken using Qtegra™ software (Thermo-Fisher Scientific) utilizing external cross-calibration between pulse-counting and analogue detector modes when required.

2.7. Data Analysis

Results were analysed by ANOVA with Tukey's post hoc test to identify significant differences ($p < 0.05$) using XLSTAT and Microsoft excel®. Ethyl butanol, ethyl hexanol were found below brewing industry relevant thresholds and were therefore not included in the ANOVA analysis.

Standardization value (SV) was calculated using the equation, $SV = (T_n - T_0)$, which considers the comparison of compound concentration between inorganic elements treatments and sweet brewers wort (reference), (T_n = Supplementation treatments; T_0 = Reference/control).

Principal Component Analysis (PCA) was used to reduce the dimensionality of the complex multivariate data sets and to ease interpretability and aid in identifying trends / correlations among treatments and samples. PCA was conducted for ethanol, glycerol and 23 aroma compounds, in total there were 90 fermentations across the 3 strains tested (10 treatments and triplicate replication), PCA analysis was carried out using XLSTAT and Microsoft excel®. Kaiser Criteria (eigenvalue > 1) and cumulative variance (> 60 %) was used to describe / select data, which is summarised in the PCA bi-plot.

3. Results

3.1. Effect of essential inorganic elements on yeast viability

Yeast viability is an important indicator of fermentation quality/performance, the overall impact of essential inorganic elements on yeast viability is shown in Fig. 2.

NCYC2592 strain had 88.6 % viability after fermentation in SBW (control); however, its viability decreased in fermentation supplemented with ammonia-nitrogen (66.8 %), potassium (66.1 %), iron (70.5 %), magnesium (73.5%), manganese (63.2 %) or CMN (82.9 %). W34/70 had 84.7% of viability following fermentation in SBW; however, its viability significantly decreased when inoculated in fermentation media supplemented with ammonia-nitrogen (78.9 %), copper (59.4 %) or CMN (61.9 %). M2 strain had 73.1 % of viability when used to ferment in SBW; however, its viability decreased when used to ferment a wort supplemented with ammonia-nitrogen (66.5 %), copper (52.0 %) and CMN (70.7 %).

NCYC2595 viability increased when the fermentation was supplemented with copper (89.8 %), W34/70 and M2 viability increased when inoculated in fermentation supplemented with magnesium. Furthermore, M2 increased its viability when inoculated in fermentation supplemented with inorganic phosphate (75.2 %) and manganese (77.3 %). All other treatments did not show a significant change in viability.

Overall ammonia-nitrogen decreased the viability of all three strains; Inorganic phosphate increased the viability of M2 ($p < 0.05$); Potassium decreased the viability of NCYC2592 ($p < 0.05$), magnesium decreased NCYC2592 viability ($p < 0.05$) and increased W34/70 and M2 viability ($p < 0.05$); copper increased NCYC2592 viability ($p < 0.05$) and decreased W34/70 and M2 viability ($p < 0.05$); zinc had no effect ($p > 0.05$); iron decreased NCYC2592 viability ($p < 0.05$); manganese decreased NCYC2592 viability ($p < 0.05$) and increased M2 viability ($p < 0.05$); CMN decreased NCYC2592, W34/70 and M2 viability ($p < 0.05$).

3.2. Effect of essential inorganic elements on ethanol and glycerol formation

Fig. 3A and 3B illustrate the impact of essential inorganic elements on ethanol/glycerol formation. All treatments were compared to SBW. When fermentations using NCYC2592, M2 or W34/70 strains were supplemented with ammonia-nitrogen, inorganic phosphate, magnesium, zinc or iron, the production of ethanol and/or glycerol was not affected

When the W34 fermentation was supplemented with copper or CMN, ethanol formation was significantly reduced (Fig. 3 A) from 76.3 g/L (control) to 30.7 g / L and 36.2 g / L, respectively. In contrast, the ethanol formation by the M2 fermentations were not significantly impacted by the supplementation used. When the NCYC2592 fermentation was supplemented with potassium, ethanol formation increased (Fig. 3A) from 37.6 g / L to 66.1 g / L.

There was no impact of supplementation on glycerol formation in the NCYC2592 fermentation, however glycerol formation was negatively influenced in the W34/70 fermentations by copper (W34/70), manganese (W43/70); and for the M2 fermentations by CMN supplementation (M2).

3.3. Effect of essential inorganic elements on acetic acid formation

Fig. 3C illustrates the influence of essential inorganic elements on acetic acid formation. NCYC2592 fermentations had a reduced acetic acid formation (control, 19.8 mg / L) when supplemented with ammonia-nitrogen (8.5 mg / L), inorganic phosphate (6.9 mg / L), potassium (13.1 mg / L), magnesium (8.3 mg / L), copper (12.2 mg / L)), zinc (7.7 mg / L) or manganese (11.7 mg / L). W34/70 and M2 strains increased acetic acid formation when fermentations were supplemented with copper or CNM. For copper supplementation acetic acid formation increased from 2.5 mg / L and 1.4 mg / L (control) to 13.8 mg / L and 13.7 mg / L, for W34/70 and M2 respectively. For CNM supplementation, acetic acid formation increased from 2.5 mg / L and 1.4 mg / L to 24.3 mg / L and 31.5 mg / L, for W34/70 and M2 respectively.

246 Whilst in most supplementations, NCYC2592 decreased acetic acid accumulation when compared
247 to the control, it should be noted that W34/70 and M2 produced relatively low concentrations of
248 acetic acid compared to NCYC2592, and only copper and CNM treatments produced more acetic
249 acid for W34/70 and M2.

250 **3.4. Effect of essential inorganic elements on higher alcohol formation**

251 Fig. 3D summarizes the influence of essential inorganic elements on higher alcohol formation.
252 NCYC2592 produced 210.1 mg of total higher alcohols/L when inoculated SBW (control) and wort
253 supplemented with inorganic phosphate or magnesium increased the concentration of higher
254 alcohols to 318.5 mg / L and 319.8 mg / L, respectively. Supplementation treatments with
255 ammonia-nitrogen, potassium, iron or manganese increased higher alcohols formation for strain
256 NCYC2592. In contrast, wort supplemented with CNM (composite mixture of all nutrients)
257 decreased the higher alcohols concentration to 155.8 mg/L.

258 W34/70 and M2 strains produced the highest concentration of higher alcohols when inoculated into
259 SBW (control) and generated, respectively, 348.2 mg / L and 453.7 mg / L. W34/70 and M2 higher
260 alcohol formation was decreased when inoculated in wort treatments supplemented with ammonia-
261 nitrogen, copper, zinc, manganese or CNM (Fig. 3D). Compounds such as 2-methyl alcohol,
262 isoamyl alcohol and 2-phenyl-ethanol were the most affected by wort supplementation. These
263 compounds increased with ammonia nitrogen, inorganic phosphate, potassium or magnesium
264 supplementation under NCYC2592 fermentation, however there was no effect with W34/70 or M2.
265 Furthermore, supplementation with heavy metals (copper, manganese or CMN) reduced the
266 production of higher alcohols (e.g. 2-methyl alcohol, isoalmyl alcohol or 2-phenyl-ethanol) for
267 W34/70 and M2 (Table S1.3).

268 The lowest concentration under supplementation conditions of isoamyl alcohol observed when wort
269 was supplemented with CMN, which had 35.9 mg / L, 27.1 mg / L and 57.6 mg / L, respectively,

for NCYC2592, W34/70 and M2 (Table S1.3). Similarly, the lowest concentration of 2-phenyl-ethanol was observed when wort was supplemented with CMN, which had 98.2 mg / L, 72.6 mg / L and 152.4 mg / L for NCYC2592, W34/70 and M2, respectively (Table S1.3).

3.5. Effect of essential inorganic elements on acetate ester formation

Fig. 3E illustrates the influence of essential inorganic elements on acetate ester formation. NCYC2592 produced a low concentration of acetate esters (1.35 mg / L, control); however, in all NCYC2592 supplementation treatments the formation of acetate esters increased (Fig. 3E). Furthermore, when inoculated in wort supplemented with ammonia nitrogen (12.8 mg / L) and inorganic phosphate (14.1 mg / L), NCYC2592 produced the highest concentration of total acetate esters ($P < 0.05$).

3.6. Effect of essential inorganic elements on fatty acid formation

Fig. 3F shows the influence of essential inorganic elements on fatty acid formation. NCYC2592 produced a low concentration of acetate esters without supplementation (1.35 mg / L, SBW control). In all NCYC2592 supplementation treatments fatty acid formation increased (Fig. 3F). Furthermore, when inoculated in wort supplemented with ammonia nitrogen (12.8 mg / L) and inorganic phosphate (14.1 mg / L), NCYC2592 produced the highest concentration of total fatty acids. When inoculated in SBW (control), NCYC2592 and M2 produced lower concentrations of fatty acid (3.1 mg / L and 5.0 mg / L, respectively) than W34/70 (7.6 mg / L) (Fig. 3F). W34/70 decreased its fatty acid formation when supplemented with potassium, copper, zinc, manganese and CMN (Fig. 3F). M2 showed an increasing in the amount of fatty acid produced when inoculated in wort supplemented with inorganic phosphate, potassium, magnesium, iron, manganese or CMN (Fig. 3F).

3.7. Effect of essential inorganic elements on fatty acid ester formation

Fig. 3G illustrates the influence of essential inorganic elements in fatty acid ester formation. NCYC2592 produced a low concentration of fatty acid esters in SBW (8.8 mg / L, control); however, in all supplementation treatments it was observed that NCYC2592 increased fatty acid ester formation, except CMN treatment (Fig. 3G). Furthermore, when inoculated in wort supplemented with magnesium, NCYC2592 produced the highest concentration of total fatty acid esters (116.6 mg / L).

W34/70 produced 91.8 mg of total fatty acid esters/L, when inoculated in SBW (control), which was the highest concentration produced among all treatments (Fig. 3G/Table S1.4). There was no impact of inorganic phosphate, zinc or iron, in all other supplementations fatty acid ester production was negatively affected. When fermentations were supplemented with CNM (composite mixture of all nutrients), W34/70 produced the lowest concentration of fatty acid esters (14.6 mg / L) (Fig. 3G). Fatty acid ester produced using M2 were not affected by any supplementation treatment.

When considering individual fatty acid esters, NCYC2592 inoculated in SBW (control) produced ethyl hexanoate (0.34 mg / L), ethyl octanoate (0.40 mg / L) and ethyl decanoate (7.9 mg / L), respectively. Whilst most supplementation of NCYC2592 increased the formation of ethyl hexanoate, ethyl octanoate and ethyl decanoate, this was most significant when inoculated in wort supplemented with iron, ammonia-nitrogen and magnesium (ethyl hexanoate, 2.6 mg / L; ethyl octanoate, 4.6 mg / L and ethyl decanoate, 111.5 mg / L respectively) (Fig. 3G), there was also a positive effect of ammonia-nitrogen, inorganic phosphate and magnesium.

Fatty acid esters produced from W34/70 or M2 metabolism were in general negatively impacted by supplementation, except for potassium, zinc and iron, which had similar concentration to the control (Fig. 3G).

3.8. Summary of all fermented samples

PCA was applied as a multivariate technique to summarise all linear correlations into a Bi-Plot (Fig. 4). PC1 and PC2 accounted for 34 %, 18 % of the variance, respectively, which totals 52 % of total variability. Overall, ethanol and glycerol were positively correlated with PC1 and acetic acid formation was negatively correlated with PC1. Increased aroma formation was correlated with PC1. The relative profile of aroma compounds formed varied by species and this is separated on PC2. The PCA shows that the three yeasts grown under different supplementation conditions, produced different flavour profiles when compared to standard brewers wort, shown as reference 1 (NCYC2592), reference 2 (W34/70) and reference 3 (M2) (Fig. 4).

The first component (PC1) separates the reference 1 (NCYC2592) from references 2 and 3 (W34/70, M2). The second component separates references 1 and 3 (NCYC2592, M2) from reference 2 (W34/70). Of the yeasts that were selected for this study there are two *Saccharomyces cerevisiae*, ale strains (1) NCYC2592 and (3) M2 and one *Saccharomyces pastorianus*, lager strain (2) W34/70. PC1 therefore illustrates the differences between the two ale strains (1) NCYC2592 and (3) M2 and PC2 illustrates the differences between the ale strains (1) NCYC2592 and (3) M2 and the lager strain (2) W34/70 (Fig. 4).

Although single component chemical analysis of NCYC2592 and W34/70 strains did not produce any initial direct similarities, the PCA illustrates that supplementation of NCYC2592 with ammonia-nitrogen, inorganic phosphate, potassium, magnesium and iron would trend towards an increased production in flavour compounds and move the flavour profile of NCYC2592 to be more similar to W34/70 (Fig. 4). Likewise, fermentations of W34/70 supplemented with copper, manganese and a composite mixture of all nutrients drove the metabolism of W34/70 to produce less aroma compound and to have a similar flavour profile to un-supplemented NCYC2592 (Fig. 4). Therefore, the identified supplementations using NCYC2592 strain produced more aroma compounds and therefore would be classed as being positive; on the other hand, supplementations using W34/70 strain in many cases had a negative impact on total flavour formation.

M2 strain presented a particular behaviour when applied in all supplementation treatments (Fig. 4). Fermentations supplemented with ammonia-nitrogen, zinc and a composite mixture of all nutrients showed limited differences on the flavour profile. However, when M2 strain supplemented with inorganic phosphate, potassium, magnesium and iron resulted in a positive increase in flavour. M2 Fermentations supplemented with copper and manganese had a reduced flavour and presented more similarities to NCYC2592 fermentation. However CMN was less negative for M2 compared to the other yeasts.

Fatty acid esters such as ethyl hexanoate and ethyl octanoate are positively correlated with their respective medium chain fatty acid (hexanoic acid and octanoic acid) and were correlated with PC2 (Fig. 4). Decanoic acid and ethyl decanoate did not follow a similar trend. Higher alcohol formation and acetate ester synthesis were also highly correlated. These include isoamyl alcohol and 2-phenyl-ethanol and their respective acetate esters (isoamyl acetate and 2-phenylethyl acetate) and were correlated with PC1.

4. Discussion

The relationship between oxygen, yeast growth and flavour development is well known. However, in this study we demonstrate that there is also an intricate and complex interaction between yeast growth, nutrients and flavour formation, independent of oxygen. Despite this, it should be stressed that oxygen has a key role since it allows activation of the Ehrlich pathway for higher alcohol biosynthesis and triggers yeast growth through the generation of sterols, fatty acids and the biosynthesis of lipids (Yu *et al.* 2016, Verbelen *et al.* 2009). In addition, the formation of yeast flavour can be related to process temperature, and this, along with an understanding of yeast growth and viability is key to understanding the overall generation of yeast flavour compounds (Layfield and Sheppard 2015, Luarasi *et al.* 2016, Marechal and Gervais 1994). In order to allow focus on inorganic ions, the concentration of oxygen (12 ppm) and temperature (22 °C) were fixed, so that

the direct impact of mineral composition on yeast growth/viability and yeast flavour could be evaluated.

Inorganic phosphate, potassium and magnesium are involved in enzymatic reactions in several major pathways (including glycolysis, ethanol formation and acetyl-CoA production) (Walker *et al.* 1996, Maguire and Cowan 2002, Boubekeur *et al.* 2001). These ions also had an impact on yeast viability, most likely because they are important factors required for growth, viability and yeast homeostasis through internal pH regulation and exclusion of hydrogen ions (Cyert and Philpott 2013, De Freitas *et al.* 2003, Boubekeur *et al.* 2001). Heavy metals mostly had a negative impact on yeast viability, notably copper, which is related to electro-negativity and toxicity of heavy metal (transition metals) (De Freitas *et al.* 2003). Furthermore, all heavy metals are involved in ensuring metabolic activity of yeast during growth and fermentation (Wietstock *et al.* 2015, Cyert and Philpott 2013); however, many can negatively influence yeast viability due to toxicity at elevated concentrations.

To understand yeast flavour formation it is also important to evaluate alternative end-products of metabolism such as ethanol, glycerol and acetic acid formation. Ethanol and glycerol play an important role in yeast redox balance (Zhang *et al.* 2013), while glycerol also acts as a compatible solute in osmoprotection (Zhang *et al.* 2011). In this study, both compounds were produced proportionally and when fermentations using M2 or W34/70 were supplemented with ammonia nitrogen, inorganic phosphate, magnesium, zinc or iron, the production of ethanol and glycerol was not affected. However, when fermentations were supplemented with manganese, strain M2 showed a decrease in glycerol formation, and for CMN (a composite mixture of all nutrients), W34/70 significantly decreased ethanol formation (Fig. 3 A). In contrast, when fermentations were supplemented with potassium using strain NCYC2592, ethanol and glycerol increased (Fig. 3A/B). The increase in ethanol was perhaps surprising given that it has previously been reported that maltose transport is inhibited by KCl (Serrano, 1977), however it is possible that this ion plays a

role in maintaining intercellular electrical balance during proton-mediated active transport (Loureiro-Dias and Peinado 1984). Related to this, enhanced glycerol production could be a response to the increase osmolality associated with ethanol formation (Zhang *et al.* 2013, Zhang *et al.* 2011), however further work would be required to determine these precise relationships.

Acetic acid (acetate) is a precursor of cytosolic acetyl-CoA (Chen et al 2013, Takahashi *et al.* 2006) and is important for maintaining yeast metabolic activity via acetyl-CoA formation (Pietrocola *et al.* 2015, Galdieri *et al.* 2014). As a consequence it can regulate flavour generation including higher alcohols, short chain fatty acids, esters and fatty acids esters (Fig. 3). Formation of acetic acid was reduced when NCYC2592 fermentations were supplemented with ammonia-nitrogen, inorganic phosphate, potassium, magnesium, copper, zinc or manganese (Fig. 3C). However, W34/70 and M2 strains showed an increase in acetic acid formation when fermentations were supplemented with copper or CNM (composite mixture of all nutrients). Furthermore, acetic acid accumulation occurred mostly when yeast viability decreased (Fig. 2/Fig. 3). Since acetic acid is an intermediary compound it is likely that this was released from lysed cells and is not a direct link to the experimental conditions applied (Fig. 2/Fig. 3); conversely a reduction in acetic acid is likely to indicate efficient metabolism and carbon flux from acetaldehyde through to acetyl-CoA.

Acetate ester formation is dependent on the reaction between an alcohol and a coenzyme (typically acetyl-CoA) as well the activity of key enzymes, notably ATF1 and ATF2 (Knight *et al.* 2014, Saerens *et al.* 2010, Saerens *et al.* 2008). NCYC2592 increased acetate ester formation when inoculated into all evaluated supplementations, likely related to cellular growth. Conversely, acetate ester formation decreased when W34/70 fermentations were supplemented with copper, manganese or CNM (composite mixture of all nutrients). Fermentations using the M2 strain showed an increase in acetate ester formation when fermentations were supplemented with inorganic phosphate, potassium, magnesium, iron or CNM. In contrast, M2 decreased acetate ester formation when fermentations were supplemented with copper or manganese (Fig 3E). In general, acetate ester

formation was inversely proportional to acetic acid formation; this is likely related to an insufficient conversion of acetyl-CoA via acetic acid/acetate due to low yeast viability as discussed previously (Fig. 2).

Therefore, observing yeast viability, amino acid consumption may be affected through supplementations such as inorganic phosphate, potassium or magnesium, which are involved in the enzymatic synthesis of acetate esters. Firstly, acetaldehyde is converted to acetate via acetaldehyde dehydrogenases (ACDH), which requires potassium (K-ACDH) and/or magnesium presence (Mg-ACDH) using as coenzymes NAD1 and NADP1, which is related to redox balance (Boubekeur *et al.* 2001). Second, acetate is converted to acetyl-CoA by phosphorus and magnesium (Mg-ATP complex), which consumes ATP and magnesium (Maguire and Cowan 2002). Therefore, acetyl-CoA is involved in all metabolic activity necessary for yeast growth/survival and ester formation.

Higher alcohol production occurs because of amino acid consumption via Ehrlich pathway (Dack *et al.* 2017, Hazelwood *et al.*, 2008, Hazelwood *et al.* 2006). Higher alcohols are immediately secreted into the wort or esterified with acetyl-CoA to synthesize their respective esters because they cannot be used as a carbon source for central metabolism (Vidal *et al.* 2014, Hazelwood *et al.* 2008). NCYC2592 increased higher alcohol formation when fermentations were supplemented with ammonia-nitrogen, inorganic phosphate, potassium, magnesium, iron, manganese or CNM (composite mixture of all nutrients). W34/70 and M2 strains decreased higher alcohol formation when fermentations were supplemented with ammonia-nitrogen, copper, zinc, manganese or CNM (Fig. 4). Higher alcohols formation by alcohol dehydrogenase and zinc-dependence are well known. However, while the function of zinc in ADH1, ADH3 and ADH5 is not fully understood, this and other divalent cations including magnesium and potassium may play a similar role (Walker *et al.* 1996, Mahler and Nudel, 2000).

Fatty acid ester (FAE) formation is dependent on the production of short and medium chain fatty acids, acetyl-CoA and related enzyme activity (Saerens *et al.* 2010) which leads to desirable fruit-

type aromas (Knight *et al.* 2014). NCYC2592 increased fatty acid ester formation when fermentations were supplemented with ammonia-nitrogen, inorganic phosphate, potassium, magnesium, copper, zinc, iron or manganese. W34/70 strain decreased fatty acid ester formation when fermentations were supplemented with ammonia-nitrogen, inorganic phosphate, magnesium, copper, manganese or CNM (Fig. 4).

In the study conditions, supplementation of NCYC2592 and M2 fermentations with inorganic phosphate and magnesium increased the formation of ethyl hexanoate, ethyl octanoate and ethyl decanoate. On the other hand, W34/70 produced the highest concentration of all fatty acid esters when inoculated in SBW (control) and when the fermentations were supplemented with inorganic phosphate, potassium and magnesium, W34/70 strain produced a similar concentration of FAEs comparing to the SBW. FAEs are important active flavour, but they are generated from fatty acids (off-flavour); therefore, to fully understand the influence of essential inorganic elements on fatty acid esters it is vital to evaluate fatty acid formation.

Yeasts can produce short- and medium-chain fatty acids (SMFAs) (Yu *et al.* 2016), most of which are considered undesirable off-flavours (Boulton and Quain, 2001). NCYC2592 increased fatty acid formation when fermentations were supplemented with ammonia-nitrogen, inorganic phosphate, potassium, magnesium, zinc, iron, manganese or CMN (composite mixture of nutrients). M2 strains increased fatty acid formation when fermentations were supplemented with inorganic phosphate, potassium, magnesium, copper, iron, manganese or CNM. W34/70 strain decreased fatty acid formation when fermentations were supplemented with potassium, copper, zinc, manganese or CNM. Although essential inorganic elements can increase the formation of fatty acids, none of them were found above threshold values (Xu *et al.* 2017, ASBC Methods of Analysis: Beer Flavour Database 2011). Therefore, in all treatments, it is likely that fatty acids were produced as precursor of fatty acid esters.

Fermentations supplemented with inorganic phosphate, potassium and magnesium generally had a positive influence on flavour production in all strains, primarily because of the role of these inorganic elements in cell growth and biomass synthesis. Firstly, inorganic phosphate is required for nucleic acid, nucleoprotein and phospholipid production, as well as for ATP generation and metabolic pathways (Canadell *et al.* 2015). Secondly, potassium is involved in the export of H^+ (internal pH control), Na^+ and toxic cations like lithium and phosphorus uptake (Canadell *et al.* 2015, Barreto *et al.* 2012). Third, magnesium is vital for yeast division/growth, metabolic activities, respiro-fermentative metabolism, mitochondrial structure/function, response to environmental stress, fermentation performance and ethanol production (Udeh and Kgatla 2013, Udeh *et al.* 2014). Finally, magnesium can be transported into cells via potassium and phosphorus transport systems, two plasma membrane transporters, ALR1 and ALR2 (Knoop *et al.* 2005) and Mg-ATP complex, which consumes ATP and magnesium acts as an enzymatic co-factor (Pisat *et al.* 2009, Maguire and Cowan 2002, Conway and Beary 1962).

From the alcoholic beverages' industry perspective, several variables can influence the wort composition such as raw material's type, production location, environmental conditions, soil characteristics and also the brewing practice (Palmer 2018, Wietstock *et al.* 2015, Vaculova *et al.* 2010). Breweries, wineries, and distilleries consume a large diversity of raw material that can generate a large variety of worts for several purposes. Wort can vary from its specific gravity, the quantity of dissolved oxygen, and the concentration of nutrients such as carbohydrates, amino acids, lipids, vitamins, and minerals (Palmer 2018, Wietstock *et al*. 2015). Also, raw material produced in different locations differs its composition of starch, fats, fibres, amino acids and minerals (Vaculova *et al*. 2010). Barley malt and wheat malt do not differ significantly in magnesium concentration; however, during the boiling process they react differently, with increased adsorption of magnesium and manganese in wheat (Poreda *et al.* 2015). For brewers, raw materials and brewing practices generate different wort composition including mineral composition and as a consequence, yeast flavour formation and beer quality will be affected. As a consequence, it would appear that an

obvious relevance of this work is to optimize the level of anions and cations for the yeast nutrition given what would be expected to be a somewhat variable levels of key inorganic nutrients from the wort. A brewer who optimised the level of inorganic nutrients for their yeast strain would therefore be expected to achieve more consistent beer flavour.

This study shows a new understanding of the role of the essential inorganic elements and their effect on yeast flavour formation during supplemented fermentations. Results show that inorganic phosphate, potassium and magnesium are the most important metal ions to increase yeast-flavour formation, which creates a paradigm for future studies of the activation of enzymes in yeast and the influence of essential inorganic elements for yeast flavour formation via metabolic activities. Moreover, the influence of metal ions on ATP and acetyl-CoA generation and gene activation could be very useful for a more complete understanding of metal ions' role on flavour formation during fermentation.

5. Conclusion

This study illustrated for the first time how flavour profiles were impacted when singular or a complex mix of eight different essential inorganic elements were added during fermentation of three physiologically diverse yeasts. Ammonia-nitrogen, inorganic phosphate, potassium and magnesium significantly increased the production of desirable compounds (ethanol, glycerol, higher alcohols and esters); these treatments decreased acetic acid accumulation and off-flavour formation. Copper, manganese or a composite mixture of all nutrient supplementations influenced negatively flavour formation. In general, zinc and iron had less impact on flavour formation.

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Conflict of interest

The authors declare that there is no conflict of interest.

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