Pre-fermentation of malt whisky wort using Lactobacillus plantarum and its influence on new-make spirit character

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Struan James REID[1,2],

Robert Alexander SPEERS[1,3],

Nik WILLOUGHBY[2]

William Bain LUMSDEN[4],

Dawn Louise MASKELL[1,2†]

1. International Centre for Brewing and Distilling, School of Engineering and Physical Sciences. Heriot-Watt University, Riccarton, Edinburgh, GBR.
2. Institute of Biological Chemistry, Biophysics and Bioengineering, School of Engineering and Physical Sciences, Heriot-Watt University, Riccarton, Edinburgh, GBR
3. Canadian Institute of Fermentation Technology, Dalhousie University, Halifax, Nova Scotia, CAN.
4. The Glenmorangie Company, Leith Street, Edinburgh, GBR.

† Corresponding author

Abstract

Distillery fermentations are non-sterile, which allow bacterial communities to flourish, typically towards the end of fermentation. The effect of beginning the bacterial fermentation at the start of fermentation was investigated. Wort was treated for 48 hours using a commercial strain of *Lactobacillus plantarum* followed by fermentation using a distilling strain of *Saccharomyces cerevisiae*.

The treated wash showed a substantial increase in lactic, acetic and succinic acids Sensory analysis determined that the spirit produced with bacterial treatment were significantly different ($p<0.05$) and chemical analysis demonstrated an increase in the production of ethyl acetate.

These results show that pre-treatment using species of *Lactobacillus* could be utilised to alter the quality of new-make spirit in a distillery. By using bacterial cultures present in the surroundings or raw materials, distillers could allow naturally occurring or commercially available microflora to be added thus enhancing flavour development during fermentation and producing different spirit characters.

Words: 150 (limit 150)

Keywords

Whisky, fermentation, organic acids, bacteria, Lactobacillus plantarum

1. Introduction

During the production of malt whisky, there is no boiling stage during wort production, resulting in 'non-sterile' conditions for fermentation. Therefore, bacteria and other microflora, derived from the local environment (dust, mash tun, washbacks (fermentation vessel)) or from the raw materials (malt, cereals, yeast) (O'Sullivan, Walsh, O'Mahony, Fitzgerald, & Sinderen, 1999), can develop throughout fermentation (van Beek & Priest, 2002, 2003). These microorganisms, along with the desired strain of *Saccharomyces* yeast, influence the flavour of the fermented wort – known as wash once fermentation is complete. In a typical malt whisky distillery, this wash will be distilled twice in copper pot stills to produce new-make spirit.

Despite bacterial cultures and wild yeasts typically being associated with beer spoilage (Hammond, Brennan, & Price, 1999; Suzuki, 2015; Vriesekoop, Krahl, Hucker, & Menz, 2012), the growth of bacteria and their fermentation in Scotch whisky fermentations is often anecdotally linked with enhanced new-make spirit quality and the taxonomy of the various bacterial cultures present has been well studied in the industry (Cachat, 2005; Makanjuola & Springham, 1984; Simpson, Pettersson, & Priest, 2001; van Beek et al., 2002, 2003; Wilson, 2008). It is not common for Scottish distilleries to encourage the presence of such cultures as they are enabled through the absence of wort boiling. Also the use of wooden washbacks which harbour higher levels of bacteria are commonplace. Furthermore, distillers will often increase fermentation time to encourage a secondary, lactic, fermentation (Lee, Paterson, Piggott, & Richardson, 2001). Elsewhere in the world, more typically in the United States, a portion of spent mash (processed grain, low in carbohydrates) from the previous fermentation may be included in the fermenter, reducing the pH of the wort which will encourage the growth of lactic acid bacteria during fermentation (Buglass, 2011). Although the growth of bacteria may not be entirely eradicated, it is generally maintained at low levels so not to impact the spirit yield from a given fermentation either through competition for fermentable sugar (Barbour & Priest, 1988) or by initiating yeast flocculation (Makanjuola, Tymon, & Springham, 1992).

Detailed studies of bacterial communities present in distillery environments have been carried out (Simpson et al., 2001; van Beek et al., 2003), with species of *Lactobacillus*, found to be the dominating genus due to its ability to survive the high alcohol content of late whisky fermentations (7.5 – 8.5 % v/v ethanol), low pH (typically 3.5-4.0) and anaerobic conditions (Piggott, Sharp, & Duncan, 1989). Despite this field being well studied, the specific impact that these cultures have on new-make spirit is poorly understood. This could be due to several different factors. It is challenging to determine differences between spirit quality from distillery to distillery as a myriad of other factors also influence the perceived quality of new-make spirit. This includes, but is not limited to, mashing regime, yeast pitching (addition) rate and strain, fermentation volume, time and temperature as well as still design and cut points during distillation. Furthermore, researchers have established that bacterial cultures typically thrive towards the end of fermentation, when the specific gravity (SG) is low (below 1.000) (Rose, 1977; Wilson, 2014). Late in fermentation there are limited nutrients to be metabolised, these are typically derived from the autolysis of dead yeast cells. Traditionally it has been understood by distillers, and confirmed by research, that if the bacterial growth phase occurred too early in fermentation it would be detrimental to ethanol production and therefore, final spirit yield (Barbour et al., 1988; Dolan, 1976). The duration of fermentations carried out by several distillers is often linked, specifically in marketing materials, with fruity, estery and waxy character in the new-make spirit. This concept is loosely backed up by literature and argues that bacteria may increase the ester content of the distilled spirit (Geddes & Rifkin, 1989; Priest, van Beek, & Cachat, 2002). The specific influence of the bacterial species is difficult to prove both in the lab (due to a lack of indigenous microflora) or at the industrial scale (due to variances between distilleries). By moving the bacterial fermentation prior to the pitching of *S. cerevisiae* the impact of *Lactobacillus* on new-make spirit character can be determined.

Due to process intensification, the replacement of wooden washbacks with stainless steel vessels and more robust cleaning practices in many whisky distilleries for product control purposes, bacterial growth and subsequent impact on new-make spirit quality may be limited. Links between washback

89 material and spirit quality are often made, although lacking in scientific evidence, linkages have been
90 postulated between wooden washbacks and higher content of fruity esters due to the prevalence of
91 bacteria within the more highly porous wooden washbacks (Yonezawa & Stewart, 2004).

92 Strains of distillery microflora, surviving fermentation conditions, can be categorised as
93 homofermenting (*Lactococcus*, *Pediococcus* and *Streptococcus*), heterofermenting (*Leuconostoc* and
94 *Lactobacillus*) (Von Wright & Axelsson, 2004; Wilson, 2014). Homofermentative species use the
95 glycolytic (or Embden–Meyerhof–Parnas) pathway and produce 2 mol lactic acid from 1 mol glucose
96 (Burgé, Saulou-Bérion, Moussa, Allais, Athes, & Spinnler, 2015, Wilson, 2014). Facultative
97 heterofermenting strains can metabolise glucose via the 6-phosphogluconate/phosphoketolase (6-
98 PG/PK) pathway which produce either 2 mol of lactic acid or 1 mol ethanol and carbon dioxide per
99 mol of glucose depending on prevailing conditions.

100 Other metabolism pathways of *Lactobacillus* have also been shown to form succinic acid from lactic
101 or citric acid (Dudley & Steele, 2005; Zalán, Hudáček, Štětina, Chumchalová, & Halász, 2010) and also
102 play a role in the decarboxylation of cinnamic acids (van Beek & Priest, 2000) all of which may influence
103 new make sprit character

104 Production of metabolism by-products, particularly, organic acids could have a substantial impact on
105 the flavour and aroma characteristics of the distilled wash. Due to the presence of alcohols such as
106 ethanol and higher alcohols (butanol, isoamyl alcohol) and low pH conditions, condensation reactions
107 between these alcohols and acids can result in esterification (Eqn 1). This effect has been shown in
108 laboratory experiments (Swan & Burtles, 1979).

109 $$ROH + R'OOH \leftrightharpoons ROOR' + H_2O \quad [1]$$

110 Equation 1 – Generalised condensation reaction for ester production in acidic conditions

Furthermore, the volatility of esters has been shown to align well with the distillation profile of ethanol in both experimental and model systems, increasing the likelihood that these compounds will have an impact on flavour in the spirit cut of a distillation run (Ikari & Kubo, 1975).

Esters typically have a lower vapour pressure than their corresponding alcohols and acids and therefore, these products will be more readily distilled, thus driving the equilibrium of this reaction to the right by Le Chatelier's principle. Esters have a large impact on new-make spirit quality due to their low volatility and low aroma thresholds contributing fruity, sometimes floral aromas to new-make spirit (Buxton & Hughes, 2013; Christoph & Bauer-Christoph, 2007). Thus, an understanding of the production of such congeners could lead to a greater understanding of how the behaviour of bacteria species in a malt whisky distillery affect new-make spirit character.

Organic acids may also be produced from the tricarboxylic acid (TCA) cycle. Typically, this occurs early through the action of yeast during the lag phase of fermentation. Changes in the organic content of a rice and malted barley based beer have been studied, showing that acetic, lactic and succinic acids all increase largely during fermentation (Li & Liu, 2015). Generally, the release of organic acids only occurs when the TCA cycle is not completed and therefore, could also serve as an indicator of poor cell growth or heightened yeast stress. The organic acid content of beers has been studied and shown that levels of acetic, lactic and succinic acids all increase during fermentation with malic and fumaric acids remaining stable however, there is limited information on the organic acids formed during malt whisky fermentations (Li et al., 2015).

As detailed in the next section of this paper, a high-performance liquid chromatography (HPLC) method was developed to investigate the concentration of organic acids in fermenting wort. Initially, a commercial strain of *Lactobacillus plantarum* was inoculated into distillers' wort. Following three bacterial treatments, a control (CP), where no bacterial treatment occurred, a "sterile" treatment (SS) where bacteria was pitched into boiled wort and "non-sterile" treatment (US) where bacteria was pitched into unboiled wort. A commercially available distilling strain of *Saccharomyces cerevisiae* was

136 then pitched into the wort. Fermentation data was collected and modelled using a 4-parameter
137 logistic equation to determine any differences in fermentation profiles. The fermented wash was then
138 first distilled to produce low-wines and then subsequently to produce a new-make spirit which was
139 subjected to chemical and sensory analysis.

2. Materials and Methods

All specific gravity readings were measured by filtering and degassing the sample followed by measurement using an Anton-Paar DMA 35 (Anton-Paar, St Albans, GBR). The pH was recorded using a VWR pH110 meter (VWR, Lutterworth, GBR) and all fermentation samples (pre-distillation aliquots) were frozen prior to analysis.

2.1 Experimental Plan

An outline of the experimental procedure is shown in figure 1.

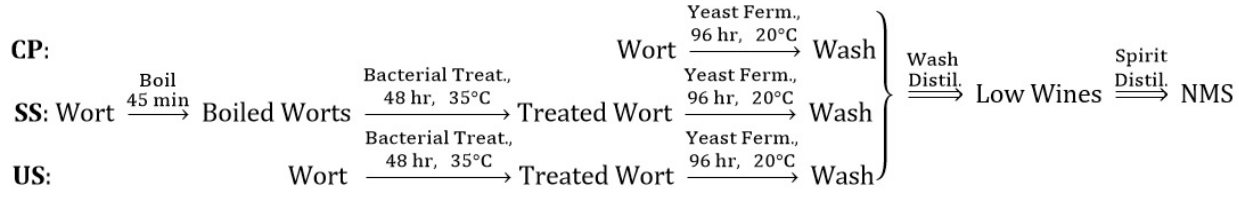


Figure 1 – Experimental outline, showing the three spirit producing regimes employed. All wash treatments were subjected to the same experimental procedures to produce low wines and new make spirit (NMS).

2.2 Wort Production

Wort (200 L) of specific gravity 1.066 was mashed using 55 kg distillers malted Concerto barley (Simpsons Malt, Berwick-upon-Tweed, GBR) in the International Centre for Brewing and Distilling (ICBD) pilot brewery using a two-roller mill (Fraser Agricultural, Inverurie, GBR). The grain was milled and then mashed with 150 L of water at 64 °C for 1 hour prior to separation using a lauter tun. The grain bed was then sparged continuously with 81 °C water to obtain the desired volume and gravity. The produced wort was frozen in 20 L aliquots for future use. All wort was thawed prior to use.

2.3 Wort Treatment

Two types of treated wort were produced in triplicate – a "sterile sour" wort (SS), produced by boiling the mixture for 45 min and siphoned into a fermentation vessel to remove any hot break formed. An "unsterile sour" wort (US) which was not boiled prior to pitching the bacteria. The bacterial culture was a commercially available freeze-dried strain of *Lactobacillus plantarum* (Lallemand Inc., Felixstowe, GBR). Bacteria were pitched according to the manufacturer's instructions at a rate of 1 g hL^{-1} and wort was soured for 48 hrs at 35 °C. The control wort used (CP) was treated without bacteria and was not boiled prior to use.

2.4 Fermentation

A commercially available strain of dried *Saccharomyces cerevisiae* (N379) distiller's yeast (Lallemand Inc., Felixstowe, GBR) was pitched directly into the untreated control ("control pitch", CP) and two treated worts (i.e., an unsterile (US) and sterile (SS). All fermentations were pitched with yeast at a rate of 1 g L^{-1} (approximately 2 x10^7 cells mL^{-1}). The yeast was pitched directly into the wort at 20 °C and was allowed to ferment for 96 hours. This temperature inhibited further bacterial fermentation and encouraged the domination of *S. cerevisiae*. The fermented wash was filtered and analysed using an Anton-Paar DMA 4500 coupled to an Alcolyzer ME module (Anton-Paar, St Albans, GBR) to determine final gravity and alcohol by volume (%v/v ethanol).

2.5 Distillation

Fermented wash was distilled on ICBD's pilot distillery. During all distillation runs the % v/v ethanol of the distillate was recorded to ensure consistent profiles were achieved to avoid alteration of flavour. Stills were charged with 20 L (or 18 L with SS boiled wort) wash. Wash was heated using a steam coil and a steady run off of low-wines was achieved. The wash distillation was ceased when the ethanol level of the distillate reached 1 %v/v (an economic cut point).

Spirit distillation was carried out in 5 L copper Al-Ambiq stills (Al-Ambiq, Gandra, PRT) using a worm-tub style condenser and heated using a 5 L isomantle (Electrothermal, Staffordshire, GBR) at a

constant heat setting. At the start of this second distillation foreshots (50 ml) were collected and then the spirit cut was collected by allowing the %v/v ethanol of the distillate to fall by 12 % before halting the collection of new make spirit.

2.6 Chemicals

All chemicals were supplied at purity ≥ 98 % (Sigma-Aldrich, Dorset, GBR). HPLC eluent was produced using distilled water which was further purified and degassed by vacuum filtration (0.45 µm).

2.7 Organic Acids Analysis

Methods were adapted for wort and wash analysis from those found in the literature (Li & Liu, 2015; Lopez & Gomez, 1996; Park, Shin, Lee, & Lee, 2017). Organic acids were analysed using a Shimadzu LC-20AD (Shimdazu, Tokyo, JPN) chromatogram fitted with SIL-20A HT autosampler and a CTO-10AS column oven coupled to a SPD-M20A diode array detector. Two Phenomenex Kinetex® (Phenomenx, Macclesfield, GBR) 150 x 4.6 mm C18 columns were used as the stationary phase and a H_3PO_4 (5 mM, pH 2.1) solution was used as the mobile phase. A flow rate of 0.65 ml min^{-1} was achieved with a column oven temperature of 60 °C. A 10 µL sample was injected and the run time was 30 minutes. All compounds were detected at wavelength of 210 nm.

Formic, malic, lactic, acetic, fumaric and succinic acid were used as external standards. Gallic acid (10 g L^{-1}) was used as an internal standard by addition of 10 µL to 1 ml of sample, giving a working concentration of 100 mg L^{-1} and was undetectable in representative samples of wort and wash. However, the compound may be found in low quantities (far below the instruments limit of detection) in beer, potentially due to the addition of stabilisers which help to precipitate polyphenols and proteins (Gerhäuser, 2005; Leiper, Stewart, McKeown, Nock, & Thompson, 2005). Standards were made in ranges specific to their typical concentration found in wort and wash and were reflective of the amount found in beer samples based on peer-review reports (Li & Liu, 2015). All standard curves

had a coefficient of determination (r^2) ≥ 0.999 and repeatability in wort, wash and beer of relative standard deviation <10 %.

Samples were centrifuged at 4500 rpm for 15 min and 1 ml of the centrifuged media was transferred into a sample vial containing 100 µl of gallic acid internal standard.

2.8 GC-FID Analysis

Higher alcohols were analysed using an Agilent 7820 A operating in liquid injection mode (Agilent, California, USA). Separation was achieved using an Agilent DB-WAX 30 m x 0.25 mm x 0.25 µm column. The oven started at 35 °C with a 6 min hold, temperature was ramped up to 60 °C at a rate of 5 °C min^{-1} with a 2 min hold followed by a ramp to 210 °C at a rate of 20 °C min^{-1} and a final ramp to 250 °C at a rate of 70 °C min^{-1} with a hold of 1 min. Compounds were detected using an FID detector at 270 °C.

A 1 ml sample aliquot was added to a vial, followed by 100 µl of pentan-1-ol to give a final concentration in vial of 500 mg L^{-1}. From this vial, 1 µl was injected on to the GC column. Congeners analysed were acetaldehyde, ethyl acetate, acetal, methanol, n-propanol, isobutanol, isoamyl acetate, n-butanol, isoamyl alcohol and furfural. Calibration ranges were in the range of 5 – 1250 mg L^{-1} with the exception of isoamyl alcohol which was measured in a range of 10 – 2500 mg L^{-1}. All calibrations had $r^2 > 0.99$.

2.9 GC-MS Analysis

Ester analysis was conducted on a Shimdadzu QP2010 Ultra GC/MS with AOC 5000 autosampler (Shimdazu, Tokyo, JPN) using a 65 µm PDMS/DVB solid phase microextraction (SPME) fibre (Supleco, Pennsylvania, USA). A sample (1 ml) was added to a headspace flask and mixed with distilled water (5 ml). Methyl heptanoate (10 µL, 25 mg L^{-1}) was used as an internal standard. The sample was incubated for 5 min, followed by 5 min of adsorption of analyte by exposing the fibre to the headspace of the sample vial. Sample was then desorbed onto a GC column for 1 min at 200 °C.

Separation was achieved on a DB-WAX 30 m x 0.25 mm x 0.25 µm column (Agilent, California, USA) the oven program ran 40 °C for 3 min, followed by an initial ramp to 100 °C at 10 °C min^{-1} and a second ramp to 160 °C at 4 °C min^{-1}. The final ramp raised the oven temperature to 220 °C at a rate of 10 °C min^{-1} and held for 10 min. The mass spectrometer was operated in both SIM and SCAN mode. SIM ions used were m/z 45, 55, 74, 88, 101, 104, 129. The SCAN mode ranged from m/z 42 – 400.

2.10 Sensory Analysis

An untrained panel (n=12) was used to evaluate the olfactory aspects of the spirit samples produced. All samples were presented blind to the panellists after dilution to 20 % v/v ethanol.

A triangle test was used to determine if a significant statistical difference existed between the produced spirits. Three different sets consisting of various combinations of the new-make spirit samples were presented to the panellists. Each set contained two spirits – two of the same new-make sample and one different. Candidates were asked to choose which sample they believed to be different. The test was carried out as stated in the guidelines of BS EN ISO 4120 (British Standard, 2004). From the results, the statistical confidence interval was determined to prove that a difference between samples could be determined by the panel.

Consumer panel testing was then carried out using the same sensory analysts. Ten sensory characteristics were selected using the revised Scotch whisky flavour wheel that were deemed appropriate for new-make spirit and recognised by the Scotch Whisky Research Institute for both trained panel testing and consumer trials (Lee et al., 2001). Candidates were asked to rank each flavour component on a scale from 0 to 5, where 0 was undetectable and 5 was strongly detectable. Finally, candidates were presented with three anonymous samples and asked to rank their preference.

2.11 Data Analysis

To enhance understanding of the fermentations produced, the specific gravity (SG) was modelled in RStudio (RStudio, Boston, USA) using the 4-parameter logistic equation (Eqn 2) as this has been shown to effectively model fermentations in beer to prevent premature yeast flocculation (ASBC, 2012; MacIntosh, 2015; MacIntosh, Adler, Eck, Speers, & Speers, 2012; MacIntosh, Josey, & Speers, 2016; Speers, Rogers, & Smith, 2003).

$$P_{(t)} = P_e + \frac{P_i - P_e}{1 + \exp(-B(t-M))} \quad [2]$$

Equation 2 – The 4-parameter logistic equations used to model the specific gravity decline during fermentation

Where: $P_{(t)}$ is the specific gravity at given time *t,*

P_i is the initial asymptotic gravity,

P_e is the final asymptotic gravity (FG),

B is a function of the gradient at the point of inflection,

M is the point of inflection with respect to time.

Data analysis was carried out in Microsoft Excel (Microsoft, Redmond, WA, USA) and OriginPro (Originlab, MA, USA). All significance tested was conducted in RStudio (RStudio, Boston, MA, USA, 2019) with significance determined at the 95 % confidence interval.

3. Results and Discussion

3.1 Fermentation Data

The fermentation profiles are shown in Figure 2, and the coefficients to the applied logistic functions (Supplementary 2) demonstrate that each fermentation followed a consistent sigmoidal pattern. Fermentation of the unsterile wort appeared to have a shortened lag phase, with very little effect seen with respect to the change in specific gravity upon pitching *S. cerevisiae*, however, the overall rate of the exponential phase was the lowest (B = 0.049 ± 0.005). Both treated worts did not fully attenuate (Figure 2a), with the sterile treated wash fermenting 84.4 ± 2.0 % of the gravity (carbohydrates), compared with the control wort and the unsterile treated fermenting to 94.4 ± 2.3 % of the control wort. Part of this loss will be through the formation of biomass and consumption of carbohydrates by the bacterial population which was to be expected.

Limited utilisation of all the nutrients in the wort is likely to be due to the prevailing conditions at the end of fermentation. The average final pH measured in SS and US was 3.35 ± 0.22 and 3.34 ± 0.13 respectively. It is commonplace for distillery yeast strains to have a pH tolerance down to *ca.* 3.5 and so it is likely that the pH limit of the yeast has been reached. Although treatment brought the pH below this value for much of fermentation, it is likely that a loss of cell viability, caused by the combination of increasing levels of alcohol and low pH, causes the yeast to cease activity. Higher ethanol concentrations will increase the re-entry of protons into the cell membrane, reducing the pH within the cytoplasm and inhibiting membrane function (Walker, 1999). It has been reported that *S. cerevisiae* has various mechanisms to adapt to organic acid stress, however, it is likely that the accumulation of such acids has exceeded the tolerance of this strain (Sugiyama, Sasano, & Harashima, 2015). A drop in yeast viability is commonly observed and may also be due to a rise in bacterial number towards the end of fermentation such as *Lactobacillus delbreuckii* and *Lactobacillus paracasei* (Wilson, 2014).

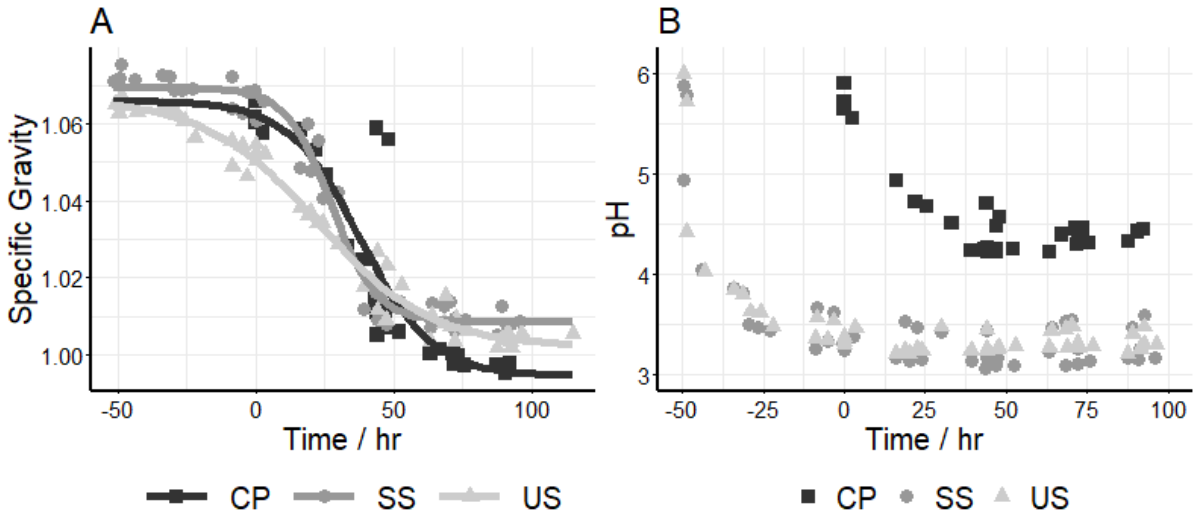


Figure 2a & 2b – 2). a. (left) Trend in specific gravity during fermentation modelled by the 4-parameter logistic function (coefficients in Supplementary 2). b. (Right) Trend in pH during fermentation.

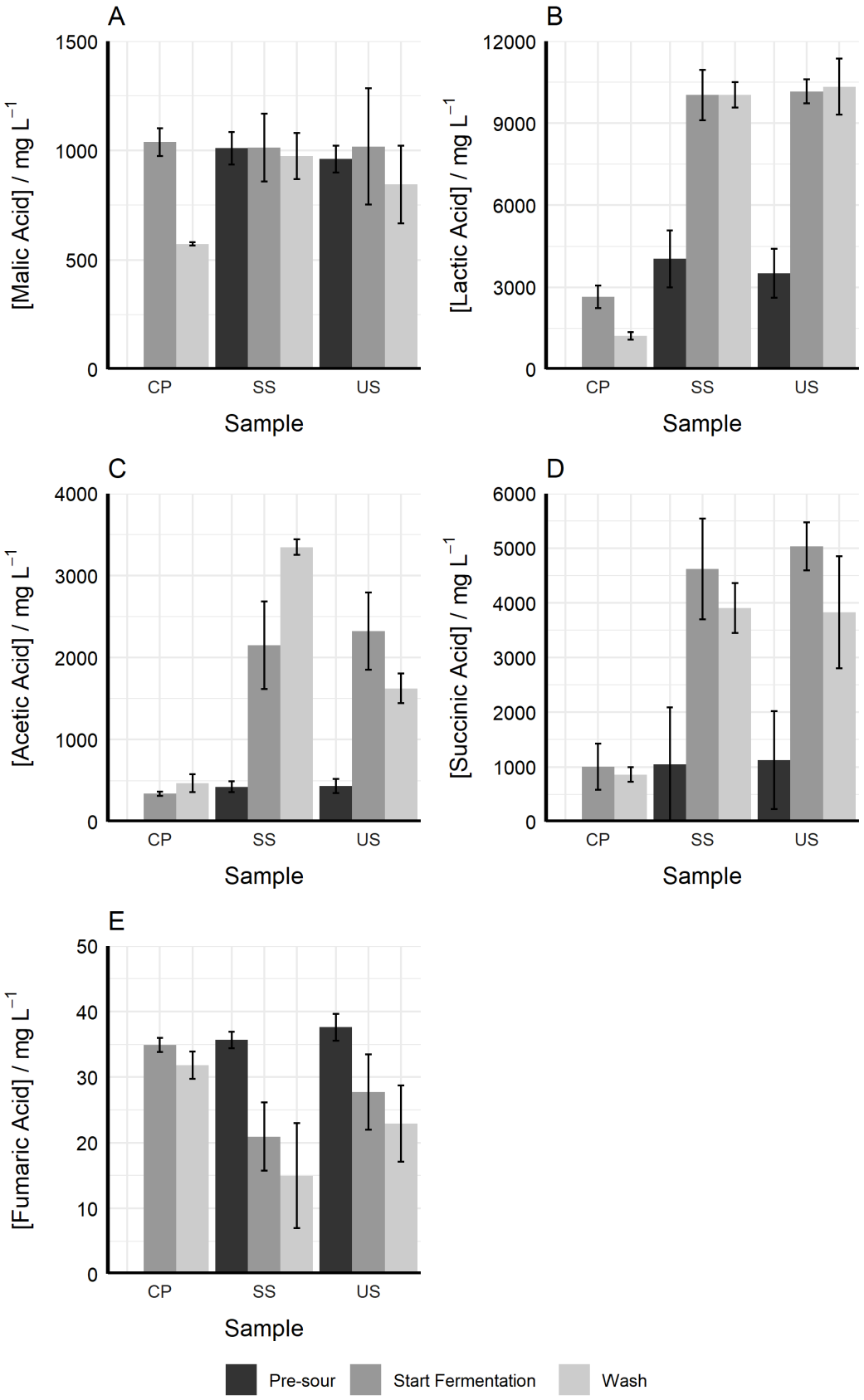
The presence of bacteria will also reduce the opportunity for yeast cell growth at the start of fermentation through the consumption of both dissolved O_2 and nutrients in the wort prior to yeast addition. As dried yeast was used, the impact of nutrient depletion will be less, however, as the yeast will have reduced viability in its dry form, this may have reduced cell growth after pitching. Cheung et al., have previously shown that yeast format has an impact on the yeasts ability to handle stressful conditions (Cheung, Brosnan, Phister, & Smart, 2012). This will increase the likelihood of stuck fermentations that do not fully attenuate. Bacteria (specifically *L. plantarum*) has been shown to increase the amount of flocculation occurring during the early stages of fermentations when both bacteria and yeast are pitched together under laboratory conditions (Makanjuola et al., 1992). Increased flocculation early in fermentation would increase the likelihood of an aberrant fermentation.

Fermentations with a low pH may be further limited due to a decline in enzymatic activity. Enzymes have been shown to continue to break down complex wort sugars throughout fermentation in whisky fermentations (Makanjuola et al., 1992; Vriesekoop, Rathband, MacKinlay, & Bryce, 2010). In the sterile (SS) sample, mashing enzymes will have been completely denatured during the boiling stage. Whereas, of the unsterile sour (US) fermentation, shifting the pH during fermentation (Figure 2b) may

have resulted in dextrin-degrading enzymes becoming inactive ceasing the breakdown wort sugars, resulting in the observed final gravity (Bryce, McCaffery, Cooper, & Brosnan, 2002; Vriesekoop et al., 2010).

3.2 Analytical Data - Fermentation

The organic acid profile during both treatment and fermentation is consistent among similar batches of wort (Supplementary 3). Large differences, particularly during treatment, show that the levels of lactic, acetic and succinic acids to increase in concentration up to five times (Figure 3, Table 1). Acetic acid had the greatest percentage increase in both sterile and unsterile worts but lactic acid had the highest final concentration of the acids measured (Figure 3). These concentrations of organic acids are then carried forward into the subsequent fermentation with *S. cerevisiae*, however, levels of acetic were found to be reduced at the start of the fermentation with yeast, particularly those in the US sample – where a drop of around 1000 mg L^{-1} during the first 24 hours of fermentation was observed (Supplementary 3). *S. cerevisiae* has the ability to metabolise acetic acid and has been shown to do so in lychee wine, particularly at 20 °C (Shang, Zeng, Zhu, & Zhong, 2016). Acetic acid may be taken up by the cell, reacting with acetyl-CoA where it could form secondary metabolites, contributing to spirit character such as higher alcohols, esters, fatty acids and other lipids. The trend of organic acids throughout treatment and fermentation showed that acetic acid declines rapidly during the first 40 hours of fermentation when yeast is producing ethanol and other higher alcohols which could react with the acetic acid, further supporting this postulation. The levels of acetic acid were observed to increase towards the end of the SS fermentation (after 30 hours) however, it is suggested that this is due to yeast autolysis and secretion of cellular compounds and organic acids into the wort.



335 Figure 3 – Measured concentrations of organic acids before treatment (pre-sour), in wort (start
336 fermentation) and wash. Organic acids analysed were A) malic, B) lactic, C) acetic, D) succinic and E)
337 fumaric. Error bars indicate the standard deviation on n = 3 samples.

Table 1 – Levels of lactic, acetic and succinic acid in wort and post treatment with *Lactobacillus* and differences in SS and US samples respectively.

Treatment	Acid	Average Concentration / mg L^{-1}		% Difference in Concentration
		Wort	End of Treatment	
SS	Lactic	4039	10031	248
	Acetic	425	2150	505
	Succinic	1050	4621	440
US	Lactic	3507	10163	290
	Acetic	434	2325	536
	Succinic	1127	5033	446

The level of both lactic and succinic acid is consistently higher in the SS fermentation due to a reduced lag phase in the bacterial fermentation, a result of boiling the wort. The boiled wort will also have a large reduction in competing bacteria (certainly most if not all vegetative bacteria will be removed) and therefore pitched *L. plantarum* can be expected to dominate this phase of fermentation.

3.3 Distillation Data

The fermented wash was then distilled twice, the first distillation in a hybrid (copper and glass) still to produce low wines and then distilled in a copper pot still to produce new make spirit.

Low wines were found to demonstrate a significant decrease ($p < 0.05$) in litres of pure alcohol (LPA) between the control and treated samples, ranging from 1.79 LPA (control) to 1.46 LPA in the US wort. This demonstrates that during fermentation, less alcohol was produced from all wort samples pitched with bacteria, mainly due to the lack of fermentable sugars.

LPA of both foreshots and spirit cuts showed no significant difference ($p > 0.05$) between fermentations. This suggests that appropriate rectification was achieved on all distillation runs,

however, a reduction in spirit yield (LPA per kg malt) was observed from the fermentations using treated (SS and US). This result was not unexpected as consumption of sugar by bacteria will lead to a reduction of alcohol per molecule of sugar consumed (Wilson, 2014).

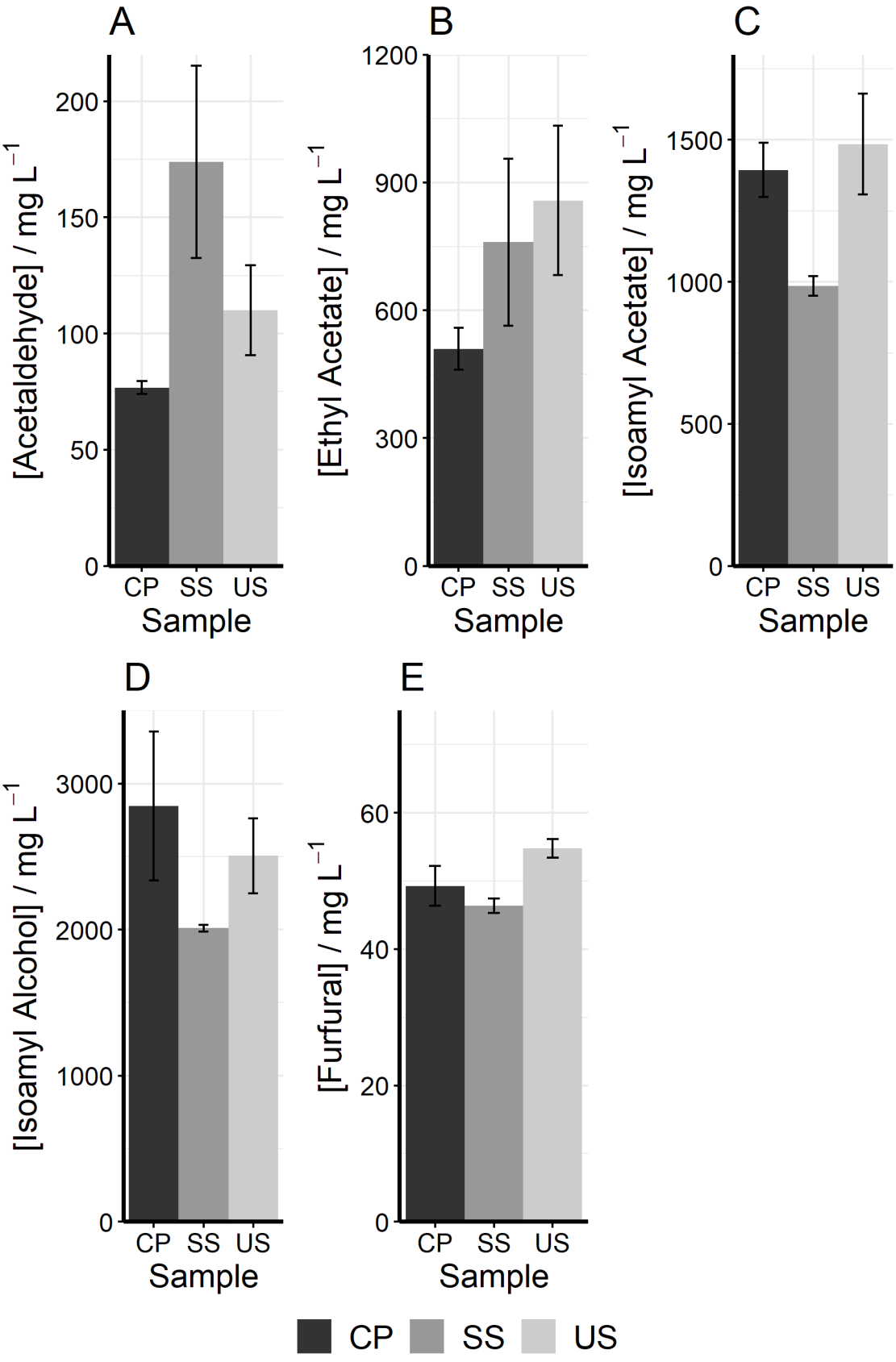
Chemical analysis of the distilled wash (low wines) showed an increase in concentration of ethyl acetate, significantly ($p < 0.05$) in the US sample compared with the control (103 ± 23 mg L^{-1} and 63 ± 13 mg L^{-1} respectively), with an increase in SS sample also observed (79 ± 22 mg L^{-1}). Furthermore, the higher alcohols propanol, isobutanol and isoamyl alcohol were detected at lower concentrations in soured low wines samples than in the control.

Chemical analysis from the second distillation (Figure 4) found that low levels of butanol were only detected in the control sample at mean concentrations of 35.8 mg L^{-1} and 28.4 mg L^{-1} in the foreshots and spirit cut respectively. Propanol was detected a concentration of 124.1 mg L^{-1} in the US spirit sample (Figure 4).

In both soured fermentation regimes, levels of acetaldehyde and ethyl acetate were enhanced substantially in comparison to those of the control sample. Acetaldehyde, associated an ethereal aroma (Burdock, 2016) is not generally perceived as a positive attribute to new-make spirit. Heightened levels of ethyl acetate in both sour samples are shown could be a result of initially produced acetic acid (by *Lactobacillus*) becoming subsequently metabolised by yeast or may be formed by esterification during one of the distillation stages. Typically, acetate esters confer fruity notes in new-make spirit. These results concur with work by Ensor, Bryce, & Hill, (2015) who demonstrated that higher concentrations of acetaldehyde and ethyl acetate are achieved when a distillery fermentation is co-inoculated with *Lactobacillus* (Ensor, et al., 2015).

The reduction seen in higher alcohols may suggest that the organic acids produced during souring treatment remove these higher alcohols in the form of esters, removing such analytes from the samples produced. By changing the conditions during fermentation it is suggested that the metabolic

pathway of yeast changed and this led to a reduction in higher alcohols formed during treated fermentations. Furthermore, reduced levels of amino acids (primarily due to metabolism by bacteria) present in the wort may have influenced the amount of keto-acids produced within the yeast cell. This would inhibit aldehyde formation, thus making producing less of the corresponding reduced product. This would have had a positive influence on the aroma of the spirit.



383 Figure 4 - Concentration of key volatiles in spirit. Congeners analysed were A) acetaldehyde, B) ethyl
384 acetate, C) isoamyl acetate, D) isoamyl alcohol and E) furfural. Error bars indicate the standard
385 deviation on n = 3 samples.

386 Trace ester analysis of the main-cut fraction was also conducted using SPME-GC/MS. From the GC/MS
387 results, levels of all ethyl esters – excluding ethyl lactate and ethyl dodecanoate – were higher in the
388 control when compared with both soured samples (Supplementary 10).

389 This discrepancy somewhat disagrees with the literature on the subject (Geddes et al., 1989; Priest et
390 al., 2002), however, there are several aspects of the experimental set-up which may explain the
391 difference. Through the action of pre-souring the wort prior to fermentation by yeast, the yeast will
392 both produce less biomass due to a lack of nutrients available and, also due to the stressful
393 environment present from high levels of organic acids. Thus, levels of fatty acid esters such as ethyl
394 octanoate and ethyl decanoate – derived from the by-products of lipid synthesis and alcohols will be
395 reduced in the final distillate. Levels of ethanol are also higher in the control sample though this is
396 unlikely to have as great an impact as ethanol is the most abundant solvent in all samples.

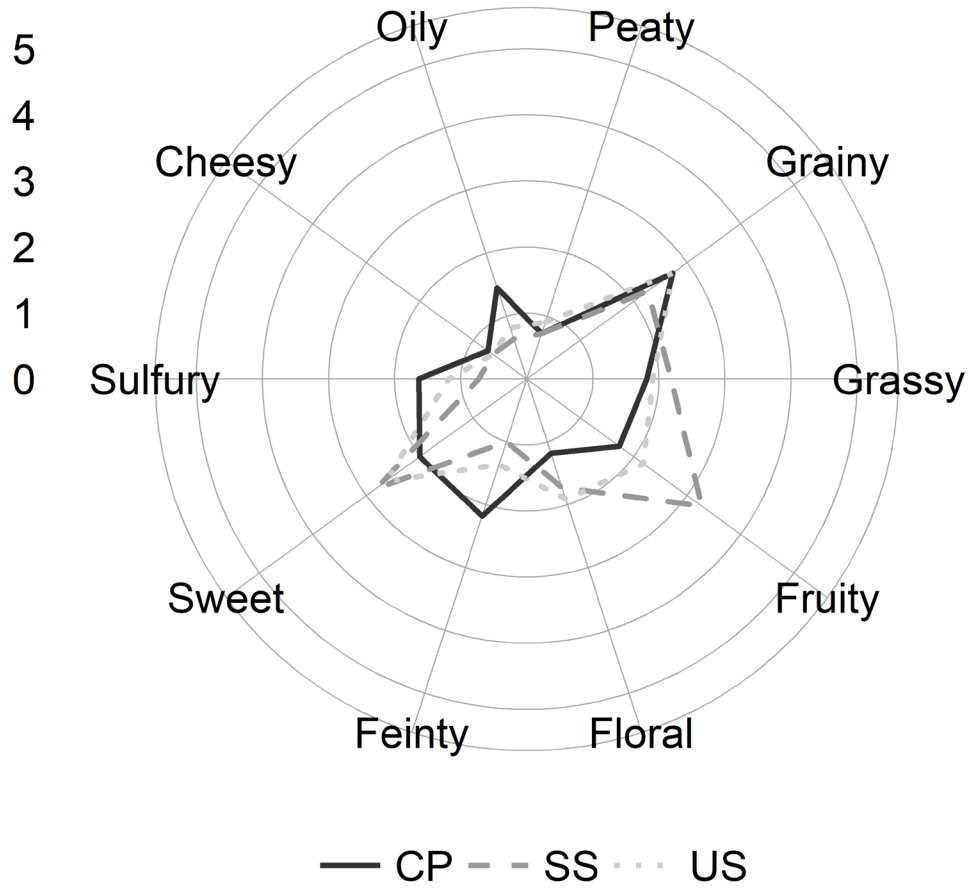
397 Levels of ethyl lactate were significantly ($p < 0.05$) higher in treated samples due to esterification of
398 the produced lactic acid with ethanol with levels in the control, SS and US samples being 3, 20 and 21
399 $\mu g\ L^{-1}$ respectively were observed. Similarly, phenylethyl acetate was shown to present at significantly
400 ($p < 0.05$) higher concentrations through similar reactions with phenylethyl alcohol this ester
401 contributes sweetness, floral and fruity characteristics to spirit (Burdock, 2016). Ethyl lactate confers
402 a waxy aroma, however, has not been seen to have a significant impact on the quality of new-make
403 spirit (Wilson, 2008).

404 Boiled wort will have a reduced level of proteins and other wort nutrients due to their removal during
405 the formation of hot break (precipitated proteins and other materials). Studies have shown that
406 reduced levels of such wort constituents increase levels of esters and promote the quality of the
407 subsequent spirit (Martin, 2002). Furthermore, production of esters and other metabolites derived
408 from yeast will be reduced in treated samples due to the reduction of fermentable sugar in comparison
409 with the control sample. Thus, the decrease in many levels of esters is to be expected. By boiling the
410 wort, wort sugars become more concentrated and the gravity (i.e., density) increases. Gravity

411 measurements in the SS sample at the end of treatment - t = 0 (Figure 2a) - is more similar to the
412 original gravity of the control fermentation. As a result, the ester profile of the control and sample SS
413 are similarly distributed.

414 *3.4 Sensory Data*

415 Figure 5 shows the average results of the quantitative descriptive analysis (QDA) conducted by the
416 same panel.



417 Figure 5 – Spider diagram showing the mean result from the QDA of the new-make spirit samples
418 produced, candidates ranked each component 0 - 5 on an arbitrary scale, n = 12.

419 Candidates selected spirit sample SS as their preference at 67 %, with a further 25 % selecting US as
420 their preferred spirit. A final 8 % of candidates selected the control (CP) as their preference.

Sensory results depicted that all three spirits showed slight evidence of apparent differences between samples when assessed by BS EN ISO 4120 (CI ≥ 90 %, Supplementary 11) (British Standard, 2004). Furthermore, evidence of a moderate apparent difference between the control sample and SS was observed. It is postulated that a trained spirit sensory team is likely to have produced results with a higher confidence interval, as random, untrained panels have been shown to lack consistency (Worch, Lê, & Punter, 2010).

Further evidence for differences in spirit is shown by the quantitative descriptive analysis (Figure 5). The panel detected greater fruity, floral and sweet aromas from both samples – attributes generally associated with positive spirit character – with both soured samples. Typically, panellists ranked sample SS higher in these categories compared with the US distillate. Studies involving *L. plantarum* have shown previously that both the fruity and floral notes have been enhanced in new-make spirit with inclusion of this species during fermentation (Takatani & Ikemoto, 2002). Heavier characteristics, such as feinty, oily and sulfury were more generally associated with the control sample.

Following sensory testing 67 % of panellists preferred the sterile sample, with a further 25 % choosing the unsterile sour sample and one panellist (8 %) choosing the control sample. Although the preference test is subjective, as no instruction was given on what to base their opinion on, it does demonstrate that preference correlated with positive attributes mentioned.

4. Conclusion

By use of a pre-fermentation treatment step to lower pH (souring), using a commercial strain of *L. plantarum*, the organic acid profile of a typical Scotch whisky distillery wash was influenced. The bacterial species produced lactic, acetic and succinic acids whose concentrations remained high through subsequent fermentation using a known strain of *S. cerevisiae*. Souring significantly reduced the pH of fermenting media and so, fermentability of the wort was reduced. Although the yields of alcohol are significantly lower in the soured samples, a shorter souring period or earlier yeast pitching time could be employed, which has the potential to reduce the final pH and allow the yeast to remain viable and active until fermentation is completed.

The distillate of soured samples showed higher levels of acetate esters, particularly ethyl acetate and phenylethyl acetate. Ethyl lactate – produced via the reaction of ethanol and lactic acid – was also detected at elevated concentrations in treated distillates, depicting that organic acids produced by the bacterial species can increase ester concentration in spirit. Levels of higher alcohols were also reduced compared with a non-soured control.

A sensory panel determined that the three regimes employed gave spirit that was statistically different by use of a triangle test. Soured samples produced spirit of a sweet, floral and fruity characteristic.

Organic acids produced during fermentation may limit production of such esters during distillation. By incorporating yeast or bacterial strains or fermentation conditions which encourage the production of such acids (such as acetic) may produce distillate of a quality that is perceived as having increased fruity and sweet notes. It is likely that the sensory contribution that bacteria has on new-make spirit is one of both primary effects via metabolism and also secondary effects on the fermentation of yeast (Takatani et al., 2002).

Although the Scotch Whisky Technical File (DEFRA, 2013) does not permit the specific addition of bacterial species to wort directly, this process may have use in other markets where such restrictions

462 do not apply (CAN, IND, JPN or USA) thus giving enhanced control over the bacteria which may
463 inoculate their malt whisky production. Furthermore, organic acid producing yeasts are becoming
464 increasingly popular amongst craft brewers as an alternative to standard kettle souring bacteria. As
465 the Scotch whisky regulations do not specify which strain of yeast must be used to produce whisky
466 this work shows that yeast strains with the ability to produce organic acids may be of benefit to the
467 whisky new-make spirit quality.

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Conflict-of-interest statement

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