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The effect of Maillard reaction products and yeast strain on the synthesis of key higher alcohols and esters in beer fermentations

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Abbreviations

MRP's, Maillard reaction products; MR, Maillard reaction; HMF, 5-hydroxymethyl furfural; TOF, Time-of-flight; FAN, Free amino nitrogen.

Keywords

Maillard reaction products; Brewing; Yeast; GC-MS; Higher alcohols and Esters

Chemical compounds studied in this article

2-Phenylethanol (PubChem CID: 6054); 3-Methylbutanol (PubChem CID: 31260); 2-Methylbutanol (PubChem CID: 8723); Ethyl hexanoate (PubChem CID: 31265); Ethyl octanoate (PubChem CID: 7799); Isobutyl acetate (PubChem CID: 8038); 3-Methylbutyl acetate (PubChem CID: 31276); Hexyl acetate (PubChem CID: 8908).

Abstract

The effect of Maillard reaction products (MRPs), formed during the production of dark malts, on the synthesis of higher alcohols and esters in beer fermentations was investigated by headspace solid-phase microextraction GC-MS. Higher alcohol levels were significantly ($p < 0.05$) higher in dark malt fermentations, while the synthesis of esters was inhibited, due to possible suppression of enzyme activity and/or gene expression linked to ester synthesis. Yeast strain also affected flavour synthesis with *Saccharomyces cerevisiae* strain A01 producing considerably lower levels of higher alcohols and esters than S288c and L04. S288c produced approximately double the higher alcohol levels and around twenty times more esters compared to L04. Further investigations into malt type–yeast strain interactions in relation to flavour development are required to gain better understanding of flavour synthesis that could assist in the development of new products and reduce R&D costs for the industry.

1 Introduction

Flavour is one of the key factors in assessment of beer quality and a crucial element of consumer acceptance (Fenaroli, 1994). The distinctive flavour profile of beers can be affected by many variables, such as yeast strain, malt type and fermentation parameters, while the perceived flavour of beer is attributed to the specific ratios of a range of different compounds, such as higher alcohols and esters, as opposed to the impact of a single compound (Cole & Noble, 1995). Both volatile (i.e. aldehydes, ketones) and non-volatile compounds (organic salts, sugars, amino acids, and organic/inorganic acids) contribute to the organoleptic profile of beer (Briggs, Stevens, Young, & Hough, 1981), but higher alcohols and esters are regarded as the most important flavour active compounds (He *et al.*, 2014; Verstrepen *et al.*, 2003). The principal higher alcohols found in beer are 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-1-propanol, 1-propanol and 2-phenylethanol (Meilgaard, 1975b; Quain & Duffield, 1985). Higher alcohols not only impact on flavour, but also provide the alcohol moiety required for the synthesis of desirable esters, which represent the largest and possibly most important group of flavour-active compounds in beer. Esters are present at low concentrations but can influence the organoleptic characteristics well below their flavour threshold, due to synergistic effects with other compounds (Meilgaard, 1975a). Esters present in beer can be divided into acetate esters and medium-chain fatty acid ethyl esters (FAEE) while the most desirable esters in beer are ethyl acetate, isoamyl acetate, isobutyl acetate, phenylethyl acetate, ethyl hexanoate and ethyl octanoate, all of which impart a fruity flavour (Verstrepen *et al.*, 2003). Processing parameters used in the production of different malt types can also influence the flavour and colour of beer, with speciality dark malts being important for the production of certain beer styles (Kieninger & West, 1982). These characteristics are mainly a result of the Maillard reaction (MR), which is initiated due to the high kilning temperatures used in the production of such malts. The flavour characteristics of dark and pale malt types have been studied by sensory and GC-MS analysis;

Coghe *et al.* (2003) showed that the processing parameters used in malt production significantly affected the Maillard reaction-derived flavour compounds present in wort.

Some Maillard reaction products (MRPs) could have a negative effect on the growth of microorganisms. For example, melanoidins, being amongst the most widely studied MRPs, have been shown to inhibit the growth of bacteria due to chelation of important metal ions such as magnesium (Rufián-Henares & De la Cueva, 2009). Magnesium is an important ion in the brewing process, as it acts as an essential co-factor for a number of catalytic reactions, plays a major role in protecting yeast cells against environmental stresses such as ethanol, and is involved in gene expression (Pohl, 2008; Udeh, 2013). Amongst its many roles magnesium activates cytosolic aldehyde dehydrogenase ALD6, which is involved in the reduction of fusel alcohols to fusel acids, and acts as a cofactor for catalytic phosphoglycerate kinase involved in the formation of pyruvate and ATP (Berg, Tymoczko, & Stryer, 2002). In addition to the more widely studied melanoidins, other MRPs, such as furfural and 5-(hydroxymethyl)furfural (HMF), formed from pentose and hexose degradation respectively, have been shown to have a negative effect on yeast growth, inhibit glycolytic enzymes and induce DNA damage (De la Cueva *et al.*, 2017; Liu *et al.*, 2004; Modig, Lidén, & Taherzadeh, 2002; Palmqvist & Hahn-Hägerdal, 2000) while they have also been shown to inhibit aldehyde and pyruvate dehydrogenases involved in the Ehrlich pathway (Modig *et al.*, 2002).

Despite MRPs being shown to inhibit enzymes involved in the synthesis of key flavour compounds, such as higher alcohols, limited information exists in the literature on the effect of dark malts on the formation of important flavour compounds. Work by Coghe *et al.* (2005) showed a correlation between increasing wort colour and decreasing rate of ethyl acetate formation, while isoamyl acetate production was completely inhibited when fermenting dark malt wort. On the other hand, they showed that the synthesis of higher alcohols was not seen to be affected by dark-coloured wort.

The aim of this study was to compare synthesis levels of important higher alcohols and esters produced by three different yeast strains during fermentation of wort produced from

different coloured malt types, with a view to establish whether MRPs present in dark malts may affect the course of the fermentation in relation to flavour compound synthesis.

2 Materials and Methods

2.1 Yeast strains and fermentation conditions

Saccharomyces cerevisiae A01, a commercial ale strain and *Saccharomyces pastorianus* L04, a commercial lager strain, were used from Northumbria University's yeast strain bank (Newcastle-upon-Tyne, UK) while a laboratory strain, *Saccharomyces cerevisiae* S288c was obtained from ATCC (Manassas, VA). Commercially available pale, chocolate, and black malts were used for wort preparation. Wort samples were prepared using a single infusion mash in line with brewing practice, at a standard grain to liquor ratio of 2.5:1 liquor to malt. The sugar and amino acid content of different wort samples was analysed by HPAEC-PAD and GC-MS respectively, and all wort samples were supplemented with sugars and amino acids to match those of the pale malts. Wort samples were buffered to pH 4 prior to fermentation using a citrate buffer, to match the pH of the dark malt wort.

Fermentations were carried out using three yeast strains (A01, S288c and L04), at a starting density of 10×10^6 cells per mL in line with brewing practice. Each yeast strain was fermented independently in triplicate, using black, chocolate or pale malt wort as the carbohydrate source. Fermentations were carried out at 25 °C and 15 °C for strains of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*, respectively. Sampling was performed at three time points selected over the course of the fermentation (mid-exponential, end of exponential, and mid-stationary phase). Total viable cell counts for each fermentation were determined using a Miles & Misra plating method in triplicate at each time point, to allow for normalisation of flavour synthesis to cell number. For GC-MS analysis 500 µL of culture were taken, samples were centrifuged for 5 min at 4,000 g at 4 °C to pellet the yeast cells, the supernatant was snap frozen in liquid nitrogen and stored at -80 °C until further analysis. Higher alcohol content was determined at all three

time points, while ester content was only determined at time points two and three, due to the later stage of fermentation at which they are produced.

2.2 Analysis of saccharides by high-performance anion exchange chromatography with pulsed amperometric detection

An in-house method used for the quantification of saccharides was optimised for the quantification of fermentable sugars in wort. Wort samples were diluted 1:200, in 18.2 MΩ/cm H₂O. A HPAEC-PAD Dionex 500 system (Dionex Corp., Sunnyvale, CA) comprised of an LC25 chromatographic oven, a GP50 gradient pump and a pulsed amperometric detector (ED40) was used for the analysis. Samples were injected onto a Dionex CarboPac PA100 (250 mm (L) × 4.6 mm (ID) BioLC column using an AS40 autosampler and a 150-μL loop. The eluents used for the equilibration of the column and separation of the compounds comprised: eluent **A**, 500 mM sodium hydroxide; eluent **B**, 500 mM sodium hydroxide in 1 M sodium acetate; and eluent **C** 18.2 MΩ/cm grade water. An isocratic gradient was used as follows: time (min) (**A**, **B**, **C**): 0–20 (50:0:50), 20–30 (15:35:50), 30–50 (0:100:0), 50–70 (50:0:50). Saccharides were quantified by the use of external calibration curves.

2.3 Amino acid quantification by EZ:FAAST

Individual amino acids were identified and quantified using the EZ:FAAST amino acid kit for GC-MS (Phenomenex, CA) according to the manufacturer's instructions. Sample preparation briefly comprised a solid-phase extraction step followed by derivatisation and a liquid-liquid extraction. An Agilent Technologies 6890N Network gas chromatograph coupled to a 5973N mass selective detector and GC Sampler 80 autosampler was used for the analysis. Two microlitres of sample were injected at 250 °C in split mode (10:1) onto a 10 cm × 0.25 mm Zebron ZB-AAA column. The oven temperature was held at 110 °C for 1 minute, then increased at 30 °C/min to 320 °C, and held for 2 minutes. The temperatures of the ion source and transfer line were 230 °C and 300 °C, respectively. The carrier gas (helium) was kept at a constant flow of 1.1 mL/min throughout the run. Electron impact mass spectra were obtained at

70 eV with 500 amu/s scan rate. Identification of the amino acids was based on the retention times and mass spectra of the standard compounds prepared in unfermented wort. Quantification was performed using selected ions and norvaline as an internal standard.

2.2.14 Headspace solid phase microextraction GC-MS analysis of higher alcohols and esters

Higher alcohols and esters analysed were selected for their known contribution to the organoleptic profile of beer. Full method development and validation was undertaken to develop a methodology suitable for the identification and quantification of the selected esters and higher alcohols (data not shown). Analysis of volatile compounds was performed on an Agilent Technologies 7890A GC (Agilent Technologies, Santa Clara, CA) coupled to a BenchTOF-dx (Almsco International, Llantrisant, UK) time of flight (TOF) mass spectrometer and a CTC-CombiPal autosampler (CTC, Zwingen, Switzerland), equipped with a heated sample agitator. For the analysis of higher alcohols and esters 50 μL and 200 μL , respectively, were added to 1 mL of 30% sodium chloride solution to aid partitioning of more polar volatiles into the headspace (Câmara, Alves, & Marques, 2006). Samples were incubated for 40 min at 40 °C and agitated at 500 rpm for 5 s followed by a 2 s stationary period for the duration of the incubation period. A PDMS/DVB SPME fibre (Supelco Analytical, Bellefonte, PA) was used to extract the volatiles for 1 min under agitation followed by desorption (5 min) in a split-splitless injector equipped with a 1.2 mm ID glass liner (SGE Analytical Science, Milton Keynes, UK) and operated in splitless mode at 250 °C. Analytes were separated on an Agilent J&W VF-WAXms GC Column (60 m \times 0.25 mm \times 0.25 μm) using helium as a carrier gas at a constant flow rate of 1 mL/min. The GC oven temperature was held at 40 °C for 5 min, then increased at 4 °C/min to 200 °C, held for 1 min, then increased at 10 °C/min to 260 °C and held for 5 min. An ionisation energy of 70 eV was used. Quantification of higher alcohols was performed using a standard addition method in unfermented wort to account for matrix effects; levels of higher alcohols were normalised by cell number to account for differences in overall yeast cell number in the different malt types. Low levels of esters in beer meant that in most

cases levels were above the limits of detection but below the limit of quantification as determined during method development experiments; thus analysis of esters was semi-quantitative in nature.

2.4 Statistical analysis

Statistical analyses were carried out using SPSS (IBM SPSS Statistics for Windows, Version 19.0, New York, NY). Normal distribution was tested and one-way ANOVA with post-hoc multiple comparisons (Tukey's test/Dunnett's T3) were used where appropriate; where normal distribution was not observed a Kruskal-Wallis test was used.

3. Results and discussion

The aim of this work was to comprehensively determine the interaction of different malt types and yeast strains on the formation of higher alcohols and esters at different stages of various wort fermentations. To achieve this, nitrogen availability and carbohydrate sources were matched prior to fermentation. The synthesis of both higher alcohols and esters are known to be directly affected by these two parameters. Despite research suggesting that MRPs can affect the activity of enzymes involved in the synthesis of higher alcohols (Modig *et al.*, 2002) and antimicrobial properties of MRPs against a number of microorganisms, to date only one study has reported the effect of dark speciality malts on yeast fermentation and volatile metabolite synthesis (Coghe *et al.*, 2005).

3.1 Saccharide and amino acid profiling of wort samples by HPAEC-PAD and GC-MS

Saccharide and amino acid levels were determined in all worts, in order to allow for modification of the fermentations that will negate any potential effect of loss of nutrients that might affect yeast growth and secondary metabolism. The level of saccharides in wort is dependent on a number of factors, the most important being the level of starch in the malt, the extent of gelatinisation of the starch, the enzymatic breakdown of starch into fermentable saccharides during the mashing step, and the involvement of the saccharides in non-enzymatic

browning reactions (Osman, 2002). Free fructose and sucrose are abundant in malted barley and subsequently the wort, while glucose, maltose and maltotriose are released from the amylolysis of starch during the mashing step (Zarnkow, Keßler, Back, Arendt, & Gastl, 2010). Intense heating used in the production of dark malts reduces the diastatic activity of the malt, reducing the breakdown of starch to fermentable sugars. In addition to reduced enzyme activity, the rate of the MR is also strongly accelerated at high temperatures, leading to further losses in fermentable saccharides in wort produced from dark malts. The carbohydrate profile of each wort sample was determined by HPAEC-PAD and the saccharide profiles are illustrated in Table 1. Maltose and maltotriose were found to be the two most abundant saccharides in all wort samples, followed by glucose. As expected, dark malt wort contained significantly ($p < 0.01$) lower levels of all fermentable sugars than the pale malt wort, with the black malt wort containing the lowest levels.

<Insert Table 1>

Amino acid levels in wort are dependent on a number of factors, such as barley variety, growing conditions, season of crop, malting conditions (steeping, germination, kilning), modification of the endosperm and the enzymatic release of amino acids during the mashing stage. Due to the intense heating used in the production of dark malts, enzymes are often denatured and due to the elevated temperatures, the rate of the Maillard reaction increases exponentially, which utilises amino acids in the first stage of the reaction. An inverse relationship between the levels of amino acids and the amount of heat applied during production of the malt (Coghe *et al.*, 2003; Hough, Briggs, Stevens, & Young, 1982; Samaras, Camburn, Chandra, Gordon, & Ames, 2005) has been previously reported.

Individual amino acids were quantified using an EZ:FAAST kit for GC-MS and the results are summarised in Table 2. As expected and in agreement with previous work, the dark malt wort contained significantly ($p < 0.01$) lower levels of all amino acids than the pale malt, while proline was found to be the most abundant amino acid in the malt samples as previously reported (Samaras *et al.*, 2005). The total free amino acids content of the pale malt wort was 147 mg/L. In order to ensure that any observed effect on the synthesis of higher alcohols and esters was not due to a difference in free amino nitrogen (FAN) availability, dark malt worts were subsequently supplemented to match the amino acid profile of the pale malt.

<Insert Table 2<

3.2 Higher alcohol and ester content of fermented wort samples using three different yeast strains

Higher alcohol and ester formation was monitored by GC-MS in three wort samples (black, chocolate and pale malt wort) with matched levels of sugars and amino acids, fermented by three strains of yeast in biological triplicate. Method optimisation (data not shown) showed that 2-methyl-1-butanol and 3-methyl-1-butanol co-eluted, and will be referred to as amyl alcohols. Moreover, preliminary data showed that growth of yeast, determined by Miles & Misra plates, was significantly lower ($p < 0.05$) in the dark malt wort than in the pale malt wort (data not shown); therefore higher alcohol and ester levels were normalised by cell number to ensure any effect observed was not attributed to the lower CFU/mL in these samples. The higher alcohol profile of different wort samples and strains is illustrated in Table 3; as previously mentioned the ester levels were analysed in a semi-quantitative manner and are discussed below. Strain and malt type were found to significantly affect the levels of both higher alcohols and esters; in most cases the higher alcohols were more abundant in the fermented dark malt wort than the fermented pale malt wort, while esters were generally more abundant in the pale malt beer.

Different yeast strains are known to produce different levels of flavour-active compounds (Äyräpää, 1968), as shown in this experiment. The most prominent difference observed was between the different yeast strains with *S. cerevisiae* strain A01 producing much lower levels of both higher alcohols and esters than S288c and L04. Similar levels of the higher alcohols were found in wort fermented by S288c and L04, but much higher levels of esters were synthesised by S288c.

The effect of different levels of higher alcohols and esters on the overall profile of the beer is complex due to synergistic and antagonistic effects (Meilgaard, 1975b). This is

reflected in previously reported disparities of flavour thresholds for the higher alcohols studied. The reported flavour threshold for 2-phenylethanol is 100–125 ppm (Hough *et al.*, 1982; Meilgaard, 1982), amyl alcohols have thresholds in the region of 50–70 ppm (Meilgaard, 1982; Siebert, 1988) while the range of threshold values reported for 2-methyl-1-propanol (Fazzalari, 1978; Salo, 1970) is spread across two orders of magnitude (7–200 ppm). Although higher alcohols in beer are desirable, the presence of these compounds above their flavour thresholds are linked to off flavours (Hazelwood, Daran, Van Maris, Pronk, & Dickinson, 2008). The levels of the higher alcohols analysed in this study fell well below those of their reported flavour thresholds.

<Insert Table 3<

Results presented in this study showed greater abundance of higher alcohols in the dark malt is contrary to work by Coghe *et al.* (2005), who showed that dark malts had little effect on formation of isobutanol and isoamyl alcohol. Due to a number of differences in the experimental parameters between the two studies, direct comparison of the results is difficult. Different malt and yeast strains were utilised in the two studies, and these two parameters have been shown to affect flavour compound formation (Äyräpää, 1968; Coghe *et al.*, 2005; Coghe, Martens, D'Hollander, Dirinck, & Delvaux, 2004). The work by Coghe used additional malt in the mash to obtain the same original gravity whereas in this study individual sugars and amino acids were matched across the different wort samples. This can directly affect the synthesis of compounds such as higher alcohols. A higher boiling temperature was used for wort preparation by Coghe *et al.* (2004), which may lead to evaporation of volatile components from the wort, and finally the fermentation time and temperature was also longer/higher in their study. It is hypothesised that results observed in this present study are a result of MRPs in dark malts affecting gene expression and/or enzyme activity leading to an effect on the synthesis of higher alcohols. Firstly, it is proposed that MRPs may have a negative effect on the activity of enzymes involved in the Ehrlich pathway. Work by Modig *et al.* (2002)

showed that the activity of aldehyde dehydrogenase (AIDH) was decreased by around 80% in the presence of 1.3 mM furfural, while alcohol dehydrogenases (ADH) on the other hand maintained much of their activity. Down-regulation of AIDH may lead to more fusel aldehydes being reduced to the corresponding alcohol as opposed to being oxidised to fusel acids. Secondly, it is proposed that MRPs may also effect the expression levels of genes involved in the synthesis of higher alcohols. During fermentation a number of environmental factors such as temperature, nutrient availability and oxygen levels, can influence the expression levels of genes involved in aroma metabolism and have a knock-on effect on the amount of metabolites synthesised (Procopio, Qian, & Becker, 2011). The formation of higher alcohols is dependent not only on the expression levels of genes directly involved in the reduction of aldehydes to alcohols, but also the genes involved in the earlier steps of the Ehrlich pathway. Expression studies have shown that overexpression of the genes encoding branched chain amino acid amino transferases (Bat1p & Bat2p) and alcohol dehydrogenases (Adh6p & Adh7p) encoded by BAT1, BAT2 ADH6 and ADH7 respectively, lead to an increase in higher alcohol formation (Kondo *et al.*, 2012; Lilly, Bauer, Styger, Lambrechts, & Pretorius, 2006). A multi-omics approach including proteomic and metatranscriptomics could help gain a greater insight into the effects of MRPs on gene expression and enzyme activity in specified pathways.

Although esters represent the largest and possibly most important group of flavour-active compounds in fermented beverages, to the best of our knowledge work by Coghe *et al.* (2005) is the only paper to report the effect of dark malts on acetate ester formation during wort fermentation. The formation of acetate esters is highly dependent upon enzyme activity, while FAEEs are more reliant on substrate availability (Malcorps, Cheval, Jamil, & Dufour, 1991). The main enzymes identified for their role in the formation of acetate esters in *S. cerevisiae* are alcohol acetyltransferases I and II (encoded by ATF1 and ATF2 respectively) and Ig-Atf1 found in strains of *S. pastorianus*. The formation of the ethyl esters is catalysed by the bi-functional enzymes acyl-CoA ethanol

O-acyltransferases (AEATases) EHT1 and EHT2. These enzymes are responsible for both the synthesis and the hydrolysis of FAEEs (Saerens *et al.*, 2006).

In this work it has been shown that fermented dark malt wort contained lower levels of the acetate esters and ethyl octanoate than the fermented pale wort, while ethyl hexanoate levels were similar in all three malt types. These results are in line with the work by Coghe *et al.* (2005), who showed that levels of acetate esters, isoamyl acetate and ethyl acetate were lower in dark malt beer, which was linked to down-regulation of ATF1 or reduced enzyme activity.

Due to the low flavour thresholds of esters in beer they can have a large impact on the flavour profile at a relatively low abundance. However; although esters were detected in all samples they often fell below the limit of quantification (LOQ) as determined during method development. Isobutyl acetate was found to be above the LOQ in 16.7% of the samples, all of which were pale malt fermentations, while peak areas of the pale malt fermentations were around 7.5 times and 4.9 times higher at the end of exponential and mid-stationary phase, respectively. Those containing the highest levels of this ester were the pale malt fermentations using S288c and L04, with L04 producing the most. In line with the previous work on higher alcohols, differences in ester formation across the three strains demonstrated that yeast strain is a key factor in understanding and manipulating flavour profiles. This trend was also seen with 3-methylbutyl acetate, in which 69.4% and 75% of samples were found to have levels of this ester exceeding the LOQ at the end of exponential phase and mid-stationary phase, respectively. Pale malt and chocolate malt fermentations using all 3 strains and black malt fermented by L04 were found to exceed the LOQ. Pale malt fermentations contained the highest level of 3-methylbutyl acetate, with S288c producing the most of this ester followed closely by L04, A01 was again found to produce significantly lower levels of the ester in all 3 malt types. Hexyl acetate levels were found to be below the LOQ in all samples, although levels detected showed a similar pattern to those observed with the other acetate esters, with

peak areas highest in pale malt fermentations. Work by Verstrepn *et al.* (2003) showed that a difference of two amino acids in the ATF sequence caused a 50–100% difference in acetate ester production, which may explain strain differences observed in this study. Based on the higher alcohol content the differences seen between the strains could also be attributed to substrate availability, as the synthesis of acetate esters requires higher alcohol, acetyl CoA and a number of enzymes (Malcorps *et al.*, 1991). Higher alcohol levels in the A01 fermentations were much lower than the other two strains, which could have been a limiting factor for the production of acetate esters. However, previous work has implied that while substrate availability certainly influences acetate ester production, the expression levels/activity of alcohol acetyltransferases (AAtases) ATF1, ATF2 and Ig- ATF1 is of greater importance (Malcorps *et al.*, 1991; Verstrepn *et al.*, 2003). ATF1 and ATF2 are involved in the production of ethyl acetate, isoamyl acetate, isobutyl acetate, propyl acetate and phenylethyl acetate (Verstrepn *et al.*, 2003) and it is proposed that the lower level of esters produced in the dark malts could be due to differences in the total enzyme activity caused by MRPs or down-regulation of the genes encoding the enzymes required. A number of factors, such as temperature, carbohydrate availability, malt type and nitrogen sources, can affect expression of ATF1 and ATF2 and the activity of AAtases, while deletion of ATF1 reduced isoamyl acetate and ethyl acetate production by 80% and 30% respectively (Fujii, Yoshimoto, & Tamai, 1996). As temperature, nitrogen availability and carbohydrate sources were matched prior to wort fermentation, it would appear that the presence of dark malt had a negative effect on flavour synthesis. Should it be demonstrated that enzyme activity, rather than gene expression is the cause of reduced acetate ester production, then it is possible that the effect may be caused by the MRPs present in the dark malts and would support previous work that suggested that MRPs can inhibit enzyme activity (Rufián-Henares & Morales, 2007).

To the best of our knowledge the synthesis of FAEEs in dark malt wort has not previously been reported. Levels of ethyl octanoate were affected by the presence of dark

malts in the wort similarly to the acetate esters, with lower levels being detected in the wort produced from dark malts, whereas ethyl hexanoate appeared to be less affected by malt type. Ethyl octanoate was quantified in 16.7% and 30.5% of samples at the end of exponential phase and mid-stationary phase, respectively. All samples in which these esters were quantified were pale malt fermentations with S288c and L04 producing similar levels and again significantly higher than A01. In contrast to all other esters ethyl hexanoate was affected only by yeast strain, with L04 producing the most, while levels of ethyl hexanoate were similar across all three malt types. It has previously been demonstrated that ethyl hexanoate responds differently to changes in fermentation parameters than ethyl octanoate and decanoate (Saerens *et al.*, 2008), while factors known to affect FAEE synthesis include the presence of free fatty acids and substrate availability, such as ethanol and the acyl-CoA moiety. Moreover, unlike the acetate esters, FAEE formation is more dependent on substrate availability than enzyme activity (Saerens *et al.*, 2008; Verstrepen *et al.*, 2003). In this study the levels of the substrates required for the synthesis of FAEEs were not monitored. The analysis of these substrates, in addition to monitoring the synthesis of more ethyl esters, may assist in explaining the differences between ethyl hexanoate and ethyl octanoate levels. Fatty acids are toxic to yeast and the conversion to FAEEs is a detoxification mechanism (Edwards, Beelman, Bartley, & McConnell, 1990), while in the present experiment the more abundant levels of ethyl hexanoate, as opposed to ethyl octanoate, may reflect the levels of the fatty acids required for their synthesis. As with the higher alcohols and acetate esters the levels of FAEEs were lower in the wort fermented by A01 than the other two strains.

4. Conclusions

It has been shown that both malt type and yeast strain significantly affect the synthesis of key higher alcohols and esters in beer fermentation. In this study *Saccharomyces cerevisiae* S288c and *Saccharomyces pastorianus* L04 consistently produced higher levels of the higher alcohols and esters studied than *Saccharomyces cerevisiae* strain A01,

irrespective of malt type. For all three yeast strains it was found that the higher alcohol levels were greater in the dark malt fermentations than the pale malt, thought to be due to MRPs affecting gene expression and/or enzyme activity. One such example could be the down-regulation of AIDH, leading to fusel aldehydes preferentially being reduced to the corresponding alcohols. In contrast, most of the esters studied were found to decrease with the use of dark malts, despite the higher alcohols providing precursors for acetate ester synthesis. As substrate availability was not a limiting factor for decreased ester synthesis, it is likely that the metal-chelating properties of MRPs, in particular melanoidins, may cause an overall reduction in enzyme activity, possibly through chelation of co-factors such as magnesium. Further investigations with a multi-omics approach could help to draw conclusions as to why MRPs negatively affect flavour synthesis. The work presented in this study highlights the need to gain a greater understanding of the parameters that affect flavour compound synthesis in beer and could help reduce R&D costs for microbrewers through a more thorough understanding of the importance of yeast and malt selection. These findings also contribute to an ongoing debate relating to the positive and negative impact MRPs might have on both food and beverage production and gut health, with conflicting evidence relating to their properties.

Conflict of interest

The authors declare that they have no conflict of interest.

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Highlights

- The synthesis of higher alcohols and esters was investigated by GC-MS.
- Alcohol levels were generally higher in beers produced using dark malts.
- Esters were generally more abundant in the beers produced using pale malts.
- Yeast strain is vital for understanding the flavour profile of the final product.

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Table 1. Concentration of main sugars in different wort samples ($\mu\text{mol/L}$) (mean \pm STDEV of 3 replicates as analysed by HPAEC-PAD)

Wort Sample	Glucose	Fructose	Sucrose	Maltose	Maltotriose
Pale	763 \pm 47.2	274 \pm 33.3	226 \pm 14.9	645 \pm 79.8	672 \pm 142
Black	5.00 \pm 6.11*	-	-	-	28.9 \pm 3.17*
Chocolate	9.44 \pm 0.56*	46.6 \pm 5.56*	-	-	82.7 \pm 14.9 [†]

One-way ANOVA was used to compare sugar levels in all conditions. Asterisks indicate sugars that were significantly lower in the black or chocolate wort compared to the pale malt wort (* $p > 0.01$). Letters indicate a significant difference between black and chocolate malt wort ([†] $p > 0.01$).

Table 2. Levels of individual free amino acids in different wort samples (Mean (μmole) \pm SD of three replicates) determined by EZ:FAAST for GC-MS

	Pale	Black	Chocolate
ALA	2750 \pm 91.1	14.0 \pm 3.03*	24.9 \pm 1.12* [†]
GLY	759 \pm 51.3	-	-
VAL	3073 \pm 119	-	-
LEU	2874 \pm 104	1.83 \pm 1.91*	1.68 \pm 0.23*
ILE	1555 \pm 76.2	2.29 \pm 1.21*	1.68 \pm 0.22*
THR	1587 \pm 12.0	-	-
SER	2674 \pm 117	-	-
PRO	18631 \pm 196	14.0 \pm 4.26*	-
ASP	1488 \pm 63.3	11.3 \pm 5.41*	-
MET	221 \pm 6.57	-	-
HYP	-	-	-
GLU	1427 \pm 91.1	-	-
PHE	1701 \pm 41.0	-	-
LYS	1033 \pm 9.85	-	-
HIS	1528 \pm 24.1	-	-
HLY	-	-	-
TYR	1275 \pm 41.6	-	-
TRP	1895 \pm 44.0	-	-
C-C	-	-	-
CYS	-	-	-
ASN	2074 \pm 70.0	-	-

One way ANOVA was used to compare sugar levels in all conditions. Asterisks indicate amino acids that were significantly lower in the black or chocolate wort compared to the pale malt wort (* $p > 0.01$). Letters indicate a significant difference in amino acid levels between black and chocolate malt wort ([†] $p > 0.01$).

Table 3. Comparison of higher alcohol levels (mmole/10 million cells at three time points) in three wort samples fermented by three different yeast strains (mean value \pm SD of 3 replicates as analysed by GC-MS, ME = mid-exponential phase, EE = end of exponential phase, MS = mid-stationary phase; P = pale, B = black, C = chocolate, A = A01, S = S288c, L = L04)

	2-phenylethanol			Amyl alcohols		
	ME	EE	MS	ME	EE	MS
PA	3.83 \pm 1.11	0.75 \pm 0.04	0.74 \pm 0.05	1.40 \pm 0.20	0.75 \pm 0.05	1.18 \pm 0.22
PS	54.9 \pm 12.7	52.8 \pm 2.82	40.7 \pm 4.68	49.5 \pm 2.68	45.7 \pm 0.86	42.5 \pm 3.80
PL	35.9 \pm 6.09	25.4 \pm 2.06	11.5 \pm 1.47	56.4 \pm 7.90	29.8 \pm 1.15	15.3 \pm 2.06
BA	1.64 \pm 0.04*	1.15 \pm 0.09**	1.17 \pm 0.07*	3.43 \pm 0.44**	2.38 \pm 0.31**	1.94 \pm 0.19**
BS	72.4 \pm 18.1*	48.5 \pm 15.0	36.6 \pm 7.33	254 \pm 55.6**	150 \pm 29.3**	160 \pm 28.0**
BL	67.0 \pm 6.88**	43.1 \pm 10.4*	36.4 \pm 8.76**	132 \pm 11.3**	45.4 \pm 10.4*	39.6 \pm 7.11**
CA	4.48 \pm 1.61 [†]	1.11 \pm 0.04**	1.81 \pm 0.57**	8.34 \pm 1.13** [‡]	4.42 \pm 0.30** [‡]	4.03 \pm 0.28** [‡]
CS	34.3 \pm 1.29 [†]	42.6 \pm 12.7	25.8 \pm 7.38*	218 \pm 14.3**	163 \pm 12.0**	177 \pm 15.7**
CL	47.6 \pm 8.51 [†]	38.6 \pm 3.27**	34.0 \pm 5.58**	177 \pm 28.8** [†]	57.3 \pm 7.06**	61.7 \pm 7.20** [‡]

One-way ANOVA (or Kruskal-Wallis if data was not normally distributed) was used to compare the synthesis of higher alcohols in different malt types at different stages of growth by 3 yeast strains. Asterisks indicate compounds that were significantly different in the black or chocolate malt wort (* $p > 0.05$ and ** $p > 0.01$) compared to the pale malt equivalent (strain and time point). Daggers indicate differences between black and chocolate malt fermentations ([†] $p > 0.05$ and [‡] $p > 0.01$) at the equivalent time point and yeast strain. Further statistical analysis showed significant difference between yeast strains, this information is not displayed as this is a widely accepted phenomenon.