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Influence of valine and other amino acids on total diacetyl and 2,3-pentanedione levels during fermentation of brewer's wort

Kristoffer Krogerus · Brian R. Gibson

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Abstract Undesirable butter-tasting vicinal diketones are produced as by-products of valine and isoleucine biosynthesis during wort fermentation. One promising method of decreasing diacetyl production is through control of wort valine content since valine is involved in feedback inhibition of enzymes controlling the formation of diacetyl precursors. Here, the influence of valine supplementation, wort amino acid profile and free amino nitrogen content on diacetyl formation during wort fermentation with the lager yeast *Saccharomyces pastorianus* was investigated. Valine supplementation (100 to 300 mg L^{-1}) resulted in decreased maximum diacetyl concentrations (up to 37 % lower) and diacetyl concentrations at the end of fermentation (up to 33 % lower) in all trials. Composition of the amino acid spectrum of the wort also had an impact on diacetyl and 2,3-pentanedione production during fermentation. No direct correlation between the wort amino acid concentrations and diacetyl production was found, but rather a negative correlation between the uptake rate of valine (and also other branched-chain amino acids) and diacetyl production. Fermentation performance and yeast growth were unaffected by supplementations. Amino acid addition had a minor effect on higher alcohol and ester composition, suggesting that high levels of supplementation could affect the flavour profile of the beer. Modifying amino acid profile of wort, especially with respect to valine and the other branched-chain amino acids, may be an effective way of decreasing the amount of diacetyl formed during fermentation.

Keywords Diacetyl · Valine · Amino acid · Beer · Lager · Fermentation

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Introduction

During fermentation of alcoholic beverages, the vicinal diketones diacetyl and 2,3-pentanedione are produced by yeast from intermediates of valine, leucine and isoleucine biosynthesis. Vicinal diketones (VDK) impart a butter or toffee-like flavour which may be perceived positively or negatively depending on the beverage. VDKs are generally considered undesirable in lager-style beers which require a relatively 'clean' flavour profile. The flavour threshold of diacetyl is traditionally reported as 0.1–0.2 mg L^{-1} in lager and 0.1–0.4 mg L^{-1} in ales (Meilgaard 1975; Wainwright 1973), although flavour thresholds as low as 17 μg L^{-1} (Saison et al. 2009) and 14–61 μg L^{-1} (Kluba et al. 1993) have been reported. Diacetyl and 2,3-pentandione are formed extracellularly through the spontaneous non-enzymatic oxidative decarboxylation of α-acetohydroxy acids, which are intermediates in the valine and isoleucine biosynthesis pathways. Intracellular valine biosynthesis begins with the conversion of pyruvate into α-acetolactate, which is converted into valine through a series of three further reactions (Chuang and Collins 1968; Radhakrishnan and Snell 1960; Strassman et al. 1958; Suomalainen and Ronkainen 1968). The conversion of α-acetolactate to 2,3-dihydroxy-isovalerate is rate-limiting, and thus during fermentation and yeast growth, some α-acetolactate transfers out through the cell membrane into the wort, where it is non-enzymatically decarboxylated to form diacetyl (Dillemans et al. 1987). In addition to producing VDK precursors, yeast

cells are able to reduce diacetyl and 2,3-pentanedione to acetoin, 2,3-butanediol and 2,3-pentanediol, which in turn have higher flavour thresholds, and rarely influence the sensory properties of the beer. VDK reduction by yeast begins during primary lager fermentation but concentrations are typically still above threshold levels at the end of fermentation, necessitating a further secondary maturation or lagering stage to remove excess VDK. Diacetyl removal is one of the main purposes of beer maturation. This step in the lager beer production process is time-consuming and energy-demanding, and it is of interest for the breweries to decrease this maturation time, without affecting the quality of the final beer. Research has been conducted on understanding diacetyl formation and reducing diacetyl production, but the area still remains a challenge, especially in regard to new brewing technologies, such as continuous fermentation, high gravity brewing and, in particular, any process change which results in shorter fermentation time (Boulton and Quain 2001; Nienow et al. 2011; Verbelen et al. 2008, 2009).

One promising method of decreasing diacetyl production during fermentation, without the use of GM strains (Blomqvist et al. 1991; Dillemans et al. 1987; Duong et al. 2011; Kronlöf and Linko 1992; Kusunoki and Ogata 2012; Lu et al. 2012; Mithieux and Weiss 1995; Wang et al. 2008), is through the control of the valine content of the wort. Valine is involved in feedback inhibition of the enzyme acetohydroxy acid synthase (AHAS), which catalyses both the irreversible conversion of pyruvate to α-acetolactate (the precursor of diacetyl) and α-ketobutyrate to α-acetohydroxybutyrate, and is of importance regarding VDK production (Barton and Slaughter 1992; Magee and de Robichon-Szulmajster 1968). Disruption of the AHAS-encoding *ILV2* gene and/or the *ILV6* gene, encoding its regulatory subunit, have produced yeast strains with lower diacetyl productions rates (Duong et al. 2011; Kusunoki and Ogata 2012; Wang et al. 2008). Valine can thus be linked to the control of the formation of diacetyl precursors. The concentrations of other amino acids, especially branched-chain, in the wort may also indirectly affect diacetyl production, since they affect the uptake rate of valine into the cell and may also be involved in enzyme inhibition (Barton and Slaughter 1992; Didion et al. 1998; Kodama et al. 2001; Magee and de Robichon-Szulmajster 1968). Higher wort valine concentrations and greater valine uptake result in decreased diacetyl production during fermentation (Cyr et al. 2007; Nakatani et al. 1984; Petersen et al. 2004). However, no previous trials have been performed to determine the effects of alteration of background wort amino acid profile on the production of diacetyl during fermentation. Hence, the objective of this study was to investigate the influence of valine supplementation, wort amino acid profile and free amino nitrogen content on diacetyl formation and valine uptake rates during wort fermentation with the lager yeast *Saccharomyces pastorianus*.

Materials and methods

Yeast Strain and Medium

The experiments were carried out with a production lager yeast strain of *S. pastorianus* (A-63015) from the VTT Culture Collection, Finland. The yeast was propagated from a freezer stock maintained at −150 °C.

All-malt wort with an extract content of 15.0 °Plato (68 g maltose, 19 g maltotriose, 19 g glucose, 4.7 g fructose and 1.5 g sucrose per litre) and FAN content of 408 mg L^{-1} (alanine 248.4, arginine 303.4, asparagine 210.5, aspartic acid 128.8, glutamine 54.8, glutamic acid 103.4, glycine 82.5, histidine 98.8, isoleucine 161.2, leucine 380.3, lysine 253.5, methionine 68.1, phenylalanine 283.2, proline 752.5, serine 146.5, threonine 133.2, tryptophan 77.3, tyrosine 273.4 and valine 263.7 mg L^{-1}) was prepared at the VTT Pilot Brewery. Part of the all-malt wort was diluted to a semi-synthetic wort with an extract content of 13.3 °Plato (77.5 g maltose, 8.5 g maltotriose, 19 g glucose, 4.7 g fructose and 1.5 g sucrose L^{-1}) and a FAN content of 204 mg L^{-1} using a sterile sugar solution made up of maltose (Sigma-Aldrich, Finland), glucose (VWR, USA), fructose (Merck KGaA, Germany) and sucrose (VWR, USA) in deionized water. Amino acids (Sigma-Aldrich, Finland) were supplemented to the worts from sterile-filtered stock solutions prepared in deionized water. The FAN content was determined using method 9.24.2 described in EBC-Analytica (European Brewery Convention 2008).

Fermentation conditions

Three different fermentation trials were performed, the first investigating the effect of supplementing various amounts of valine (100, 200 and 300 mg L^{-1}) to the wort, the second investigating the effect of supplementing valine (300 mg L^{-1}) to worts with standard (408 mg L^{-1}) and reduced FAN content (204 mg L^{-1}), and the third investigating the effects of supplementing various groups of amino acids to the wort (amino acids were supplemented to double their concentration in the wort, with the exception of tyrosine, which concentration was increased by only 10 % because of poor aqueous solubility), on the production of diacetyl and diacetyl precursors and the change of wort valine concentration during fermentation. The amino acids were grouped into the three groups based on their absorption rates or structures (Table 1). The first group, preferred amino acids (PAA), contains the amino acids which had a higher uptake rate than valine during the first 25 h of fermentation. The second group, non-preferred amino acids (NPAA), contains the amino acids which had a lower uptake rate than valine during the first 25 h of fermentation. The third group, branched-chain amino acids (BCAA), contains leucine and isoleucine, which have a

Table 1 The grouping and average linear change in concentrations during the first 25 h of fermentation of amino acids for fermentations. Supplemented amino acids concentrations are equal to the concentrations found in all-malt wort (with the exception of tyrosine), i.e. the concentrations were doubled after supplementation

Amino acid	Decrease in concentration		Groups
	μmol L^{-1} min^{-1}	mg L^{-1} min^{-1}	
Asparagine	0.575	0.076	Preferred amino acid
Serine	0.465	0.049	Preferred amino acid
Threonine	0.382	0.046	Preferred amino acid
Leucine	0.368	0.048	Preferred amino acid, branched-chain amino acid
Lysine	0.276	0.040	Preferred amino acid
Arginine	0.161	0.028	Preferred amino acid
Phenylalanine	0.124	0.021	Preferred amino acid
Glutamine	0.107	0.016	Preferred amino acid
Aspartic acid	0.106	0.014	Preferred amino acid
Valine	0.104	0.014	–
Isoleucine	0.104	0.012	Non-preferred amino acid, branched-chain amino acid
Histidine	0.059	0.009	Non-preferred amino acid
Glycine	0.053	0.004	Non-preferred amino acid
Glutamic acid	0.039	0.006	Non-preferred amino acid
Tryptophan	0.007	0.002	Non-preferred amino acid
Alanine	−0.012	−0.001	Non-preferred amino acid
Methionine	−0.030	−0.005	Non-preferred amino acid
Tyrosine	−0.046	−0.008	Non-preferred amino acid

similar structure to valine. The pH of the PAA-supplemented wort was adjusted to that of the control wort with 90 % lactic acid (Merck KGaA, Germany).

Yeast propagation was carried out essentially as previously described (Ekberg et al. 2013). Briefly, frozen yeast suspensions in 30 % glycerol were thawed and used to inoculate 500 mL autoclaved YP medium containing 40 g maltose L^{-1} in 1L Erlenmeyer flasks. Cultures were incubated overnight at 25 °C with shaking (120 rpm) and then transferred to 1.5 L of 15 °P wort to achieve an OD_{600} of 0.15. These cultures were incubated at 16 °C with shaking for 48 h and then moved to 0 °C. After 16 h, the sedimented yeast was diluted with decanted supernatant to 20 g centrifuged yeast mass/100 g of slurry. Cylindroconical fermentation vessels containing approx. 10 L of oxygenated (10 mg dissolved oxygen L^{-1}) 15 °P wort were pitched with this 'generation 0' slurry to a concentration of 5 g fresh centrifuged yeast L^{-1}. This fermentation was allowed to proceed in a room at 15 °C until 80 % apparent attenuation was reached. The partially settled yeast was then cropped from the bottom of the vessels as a slurry mixed with beer (approximately 2 L), transferred to 0 °C and allowed to sediment for 16 h. A slurry containing 200 g centrifuged yeast mass L^{-1} was prepared as described above and used within 2 h to pitch the 'generation 1' experimental yeast fermentations. These 'repitched' yeast fermentations were carried out so that the yeast condition would approximate that of yeast used to start industrial fermentations.

The experimental fermentations were carried out in duplicate, in 2-L cylindroconical stainless steel fermenting vessels, containing 1.5 L of wort medium. Yeast was inoculated at a rate of 5 g fresh yeast per litre of wort (corresponding to 20×10^6 viable cells mL^{-1}). The wort was oxygenated to 9 mg L^{-1} prior to pitching. The fermentations were carried out at 15 °C for 8 days. Wort samples were regularly drawn from the fermentation vessels with a syringe, and placed directly on ice, after which the yeast was separated from the fermenting wort by centrifugation (9,000×*g*, 10 min, 1 °C). Fermentations were stopped once apparent attenuation of the all-malt wort had reached 80 % or the apparent attenuation of the semi-synthetic wort had reached 95 % (approximate alcohol content of 6.7 %), and the beer was collected in sterile flasks.

Fermentation analysis

The density, specific gravity, ethanol concentration and pH of samples was determined from the centrifuged and degassed fermentation samples using an Anton Paar Density Meter DMA 5000 M (Anton Paar GmbH, Austria) with Alcolyzer Beer ME and pH ME modules (Anton Paar GmbH, Austria). The apparent extract (AE; in degree Plato) of the samples was estimated from the previously measured specific gravities (SG) using the approximations from Kobayashi et al. (2005b). The apparent attenuation (AA; %) of the samples was estimated from the apparent and original extract (i.e. the apparent extract of the wort at the

time of pitching) as described in Vidgren et al. (2009). The real extract (in degree Plato) of the samples was estimated from the AE (in degree Plato) and the ethanol content (A_{ABW}; % (*w*/*w*)) using an approximation proposed by Hackbarth (2009).

Fermentable sugars were analysed by high-performance anion exchange chromatography (HPAEC) (Dionex ICS-3000) with pulse amperometric detection using CarboPac PA-1 (4 mm×250 mm) analytical column and CarboPac PA-1 (4 mm×50 mm) guard column at 30 °C (Dionex Corp, USA). The system was equilibrated with 100 mM NaOH. After injection of a 100 μL filtered (0.45 μm), diluted sample, 100 mM NaOH was run through the column (5 min). Separation was with a gradient (1 mL min^{-1}) of 100 mM to 300 mM NaOH in 3 min and then 300 mM NaOH to 250 mM NaOH + 75 mM Na-acetate in 15 min and washing was with 100 mM NaOH + 300 mM Na-acetate and 300 mM NaOH. The flow rate was 1 mL min^{-1}. The results were confirmed by MSQ detection (HPAEC-MS) using a CarboPac PA200 (3 mm×250 mm) with a CarboPac PA200 guard (3 mm×50 mm) column (Dionex) with a configuration as described by Bruggink et al. (2005) and a gradient as described by Mikkelson et al. (2013).

The yeast dry mass content of the samples was determined by suspending the yeast pellet gained from centrifugation in a total of 6 mL H_2O (water was deionized and filtered through active carbon (MilliQ Water System; Millipore Corporation, MA, USA). The suspension was then transferred to a pre-weighed porcelain crucible, and was dried overnight at 105 °C and allowed to cool in a desiccator, before the change of mass was measured.

Vicinal diketone analysis

Total VDKs (free and acetohydroxy acid form) were measured for the centrifuged fermentation samples according to Analytica-EBC method 9.10 (European Brewery Convention 2008). Samples were heated to 60 °C, where they were kept for 90 min, in a headspace auto sampling unit (Headspace Autosampler 7000 HT, Tekmar-Dohrmann, USA). Heating to 60 °C results in the conversion of acetohydroxy acids to VDK. The samples were then analysed by headspace gas chromatography (HP 6890 Series GC System, Hewlett-Packard, USA; HP-5 50 m×320 μm×1.05 μm column, Agilent, USA) with 2,3-hexanedione as an internal standard.

Amino acid analysis

Centrifuged fermentation samples were diluted to 1:40. A 10-μL volume of the diluted sample was taken and mixed with 10 μL of norvaline (250 μM, internal standard) and 70 μL of boric acid buffer. The mixture was then vortexed for 30 s. Derivatization was done with AccQ·Fluor reagent kit (Waters Corporation, USA). The AccQ·Fluor reagent was reconstituted with acetonitrile (1 mL), and vortexed for 30 s. The mixture was heated to 55 °C for 8 min, kept in an ultrasound bath for 5 min and finally vortexed for 60 s. The AccQ·Fluor reagent (10 μL) was added to the sample mixture, which was instantly vortexed for 60 s. Samples were kept at 5 °C before and during analysis. Analysis was performed on an Acquity UPLC system (Waters Corporation, USA) with UV detector. Chromatography was performed using an Acquity Mass Trak™ (2.1×150 mm, 1.7 μm) column (Waters Corporation, USA), kept at 43 °C. Injection volume was 2.0 μL. Separation was performed using gradient elution with 10 % (*v*/*v*) Amino Acid Analysis Concentrate A in water and Amino Acid Analysis Eluent B at a flow rate of 0.4 mL/min. The signal was detected at 260 nm (2.4 nm resolution, 20 points/s).

Aroma compounds analysis

The concentrations of various yeast-derived aroma compounds (acetaldehyde, alcohols, and esters) in the wort samples were determined by headspace-GC/MS. A 10-mL volume of the supernatant was filtered (0.45 μm cellulose acetate filter) before analysis. For analysis, the samples were first thawed and then incubated at 60 °C for 30 min. A 1-mL volume of sample was then injected in the splitless injector (260 °C; flow 14.9 mL min^{-1}) of the gas chromatograph (Agilent 6890 Series; Palo Alto, CA, USA) combined with an MS detector (Agilent 5973 Network MSD, USA) and SPME autosampler (Combipal, Varian Inc., USA). Analytes were separated on a BPX5 capillary column of 60 m×0.25 mm with phase thickness 1.0 μm (SGE Analytical Science Pty Ltd., Australia). Helium was used as carrier gas on constant flow mode 1.7 mL min^{-1}. The temperature program was started at 50 °C for 3 min, then 10 °C min^{-1} to 100 °C, followed by 5 °C min^{-1} to 140 °C and finally 15 °C min^{-1} to 260 °C, where the temperature was kept for 1 min. MSD was operated in electron-impact mode at 70 eV, in the full scan *m*/*z* 40–550. The ion source temperature was 230 °C and the interface was 280 °C. Compounds were identified with retention times of corresponding standards and by comparing the mass spectra on Palisade Complete 600 K Mass Spectral Library (Palisade Mass Spectrometry, USA) and were quantitated with a standard curve. 1-Butanol was used as internal standard.

Results

Supplementing various amounts of valine to brewer's wort

Valine supplementation had no effect on either fermentation rate or final attenuation level (Fig. 1a). At the end of

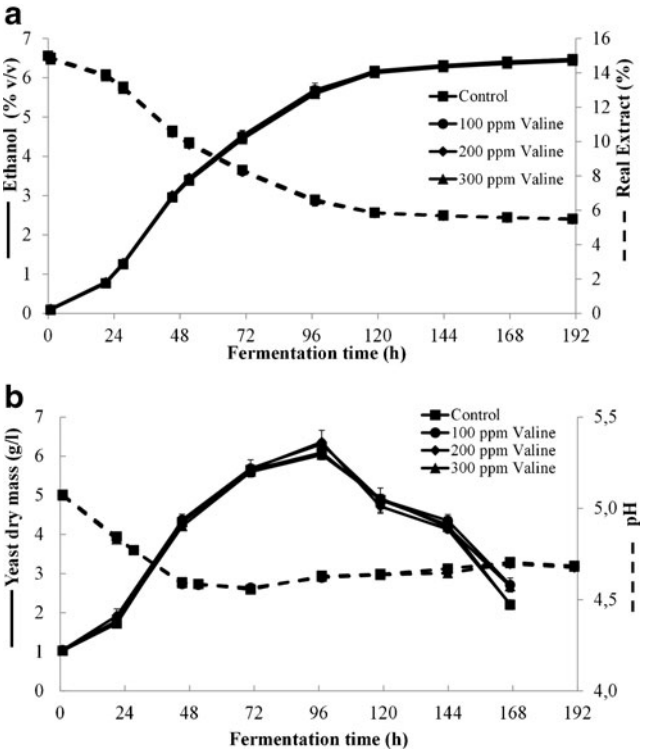

Fig. 1 **a** The ethanol content (% *v*/*v*; *solid line*) and real extract (weight %; *dashed line*), and **b** yeast dry mass (in gram per litre; *solid line*) and pH (*dashed line*) of the worts supplemented with various amounts of valine as a function of fermentation time (hour). Values are means from two independent fermentations. *Error bars* where visible represent the range

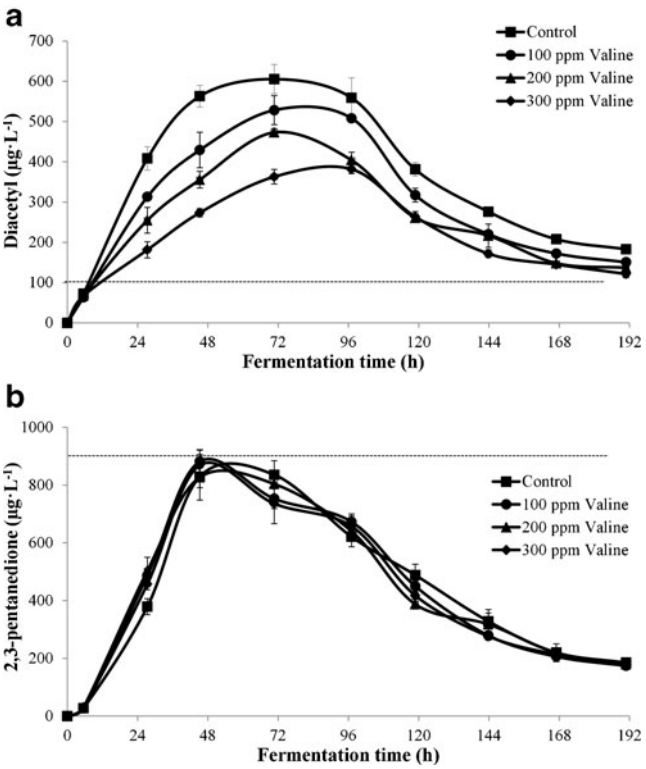

Fig. 2 The **a** diacetyl and **b** 2,3-pentanedione concentrations (in microgram per litre) of the worts supplemented with various amounts of valine as a function of fermentation time (in hour). The *dotted lines* at 100 and 900 µg L^{-1} depicts the flavour threshold (Meilgaard 1975). Values are means from two independent fermentations. *Error bars* where visible represent the range

fermentation, all beers contained an alcohol content of around 6.5 % (*v*/*v*). Likewise, valine supplementation had no effect on either the amount of yeast biomass produced during fermentation, nor on the pH of the worts (Fig. 1b).

All amounts of valine supplementation lowered the maximum diacetyl concentration produced during fermentation, but it did not have as large impact on the production of 2,3-pentanedione (Fig. 2). Increasing the amount of supplemented valine reduced the maximum concentration of diacetyl produced during fermentation. The diacetyl concentrations at the end of the fermentation (192 h) were also lower for the valine-supplemented worts compared to the control wort. The diacetyl removal rate at the end of fermentation was similar for all worts. The diacetyl concentrations in the beer at the end of fermentation were not reduced to levels under the flavour threshold (below 100 µg L^{-1} in lager beers), however the diacetyl concentration (121.6 µg L^{-1}) of the beer fermented from the wort supplemented with 300 mg L^{-1} valine was closest to the threshold. The 2,3-pentanedione concentrations remained under the flavour threshold of 900–1,000 µg L^{-1} for all the worts during the entire fermentation.

Supplementing valine to worts with standard and reduced FAN content

Valine supplementation again had no effect on either fermentation rate or final attenuation level of standard and reduced FAN worts, but the reduced FAN content resulted in a slightly decreased fermentation rate up to 140 h and a higher ethanol content (Fig. 3a). At the end of fermentation, the beers produced from all-malt wort contained an alcohol content of around 6.3 % (*v*/*v*), while the beers produced from semi-synthetic wort contained an alcohol content of around 6.9 % (*v*/*v*). Valine supplementation had no effect on either the amount of yeast biomass produced during fermentation nor on the pH of the worts. The all-malt wort fermentation produced around 20 % more biomass than the semi-synthetic wort, while the pH of the semi-synthetic worts during fermentation was lower than that of the all-malt worts, most likely due to loss of buffer capacity through dilution.

Valine supplementation of both the all-malt wort (Standard FAN) and the semi-synthetic wort (Reduced FAN) lowered the maximum diacetyl concentration produced during fermentation, while it did not have as large of an impact

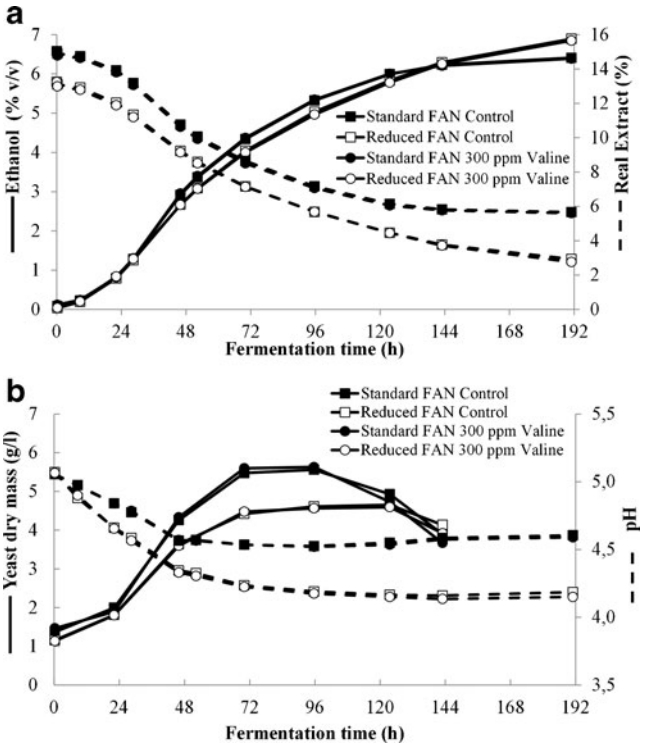

Fig. 3 **a** The ethanol content (% *v/v*; *solid line*) and real extract (weight %; *dashed line*), and **b** yeast dry mass (in microgram per litre; *solid line*) and pH (*dashed line*) of the all-malt (Standard FAN) and semi-synthetic (Reduced FAN) worts supplemented with valine as a function of fermentation time (in hour). Values are means from two independent fermentations. *Error bars* where visible represent the range

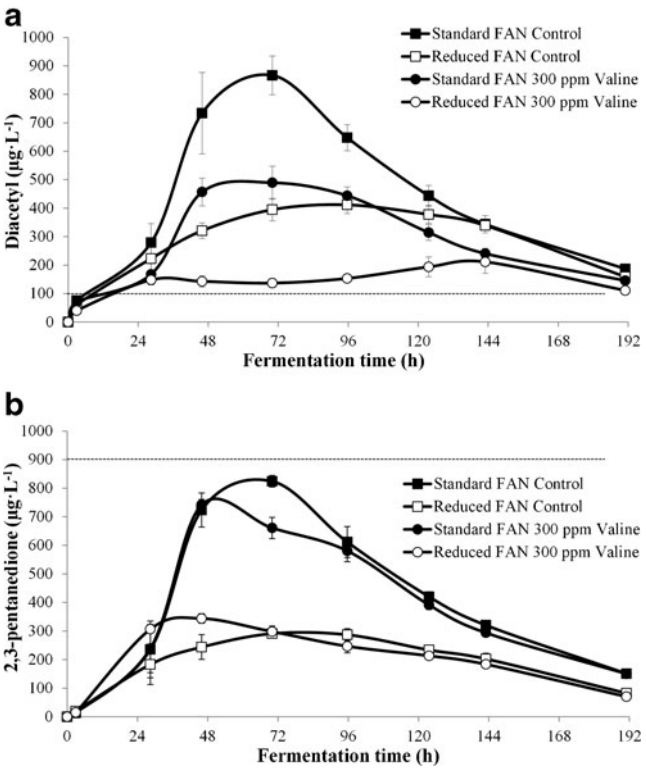

Fig. 4 The **a** diacetyl and **b** 2,3-pentanedione concentrations (in microgram per litre) of the all-malt (Standard FAN) and semi-synthetic (Reduced FAN) worts supplemented with valine as a function of fermentation time (in hour). The *dotted lines* at 100 and 900 µg L^{-1} depicts the flavour threshold (Meilgaard 1975). Values are means from two independent fermentations. *Error bars* where visible represent the range

on the production of 2,3-pentanedione (Fig. 4). The diacetyl concentrations at the end of active fermentation (143 h for the all-malt worts and 191 h for semi-synthetic worts) were also lower for both the valine-supplemented worts compared to their respective control worts. The diacetyl removal rate at the end of fermentation was greater in the semi-synthetic wort fermentations. The broader and later diacetyl peak of the semi-synthetic worts compared to the all-malt worts is reflected by the broader biomass peak in these fermentations. The valine-supplemented semi-synthetic wort (Reduced FAN 300 mg L^{-1} valine) had the lowest diacetyl concentrations during peak fermentation (around 30 to 96 h). The diacetyl concentrations of the worts were not reduced to levels under the flavour threshold (below 100 µg L^{-1} in lager beers) during the observed fermentation time period. The 2,3-pentanedione concentrations remained under the flavour threshold of 900–1,000 µg L^{-1} for all the worts during the entire fermentation.

The valine uptake rate of the yeast during the first 3 days of fermentation was higher (i.e. the change in wort valine concentration is more negative) in the valine-supplemented wort compared to the control wort (Fig. 5), while the uptake rate of isoleucine and leucine was not as affected by valine supplementation (results not shown). Concurrently, the diacetyl production rate of the yeast was lower in the valine-supplemented wort compared to the control wort, suggesting that valine uptake rate negatively correlates with the amount of diacetyl produced during the growth phase of fermentation. The difference between the initial valine concentration and the valine concentration at the end of fermentation (191 h) was

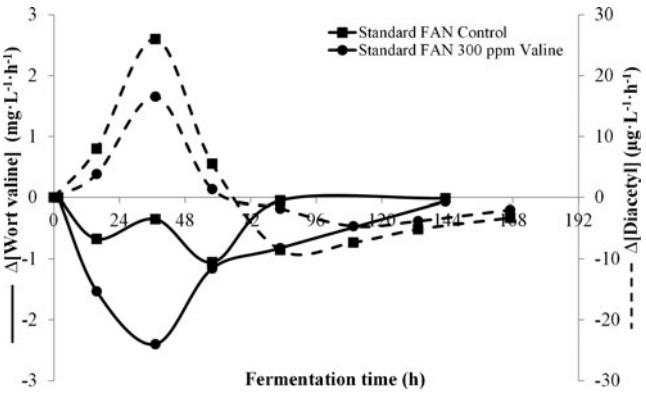

Fig. 5 The change in valine concentration (in milligram per litre per hour) and diacetyl concentration (in microgram per litre per hour) of the all-malt (Standard FAN) worts supplemented with valine as a function of fermentation time (in hour). Values are means from two independent fermentations

also greater in the valine-supplemented wort compared to the control wort (96.7 and 31.1 mg L^{-1}, respectively).

Supplementing various groups of amino acids to the brewer's wort

Amino acid supplementation had no effect on either fermentation rate or final attenuation level. At the end of fermentation, all beers contained an alcohol content of around 6.2 % (*v*/*v*). Amino acid supplementation also had no effect on the amount of yeast biomass produced during fermentation, even though the amount of assimilable nitrogen available for yeast growth increased with supplemented amino acids, but there was a slight variation of the pH of the worts (under 0.1 units throughout the fermentation), most likely caused by the acidity and alkalinity of certain supplemented amino acids (data not shown).

Supplementation of PAA and NPAA resulted in increased diacetyl concentrations compared to the control wort during fermentation, while supplementation of BCAA resulted in an initial increase (up until 50 h), but a later decrease in diacetyl concentrations compared to the control wort (Fig. 6). Supplementation with NPAA and BCAA reduced the relative amount of 2,3-pentanedione produced, while supplementation of PAA increased the amount of 2,3-pentanedione produced during fermentation. The diacetyl concentrations at the end of the fermentation (192 h) were lowest for the BCAA-supplemented worts, but higher for the NPAA- and PAA-supplemented worts, compared to the control wort.

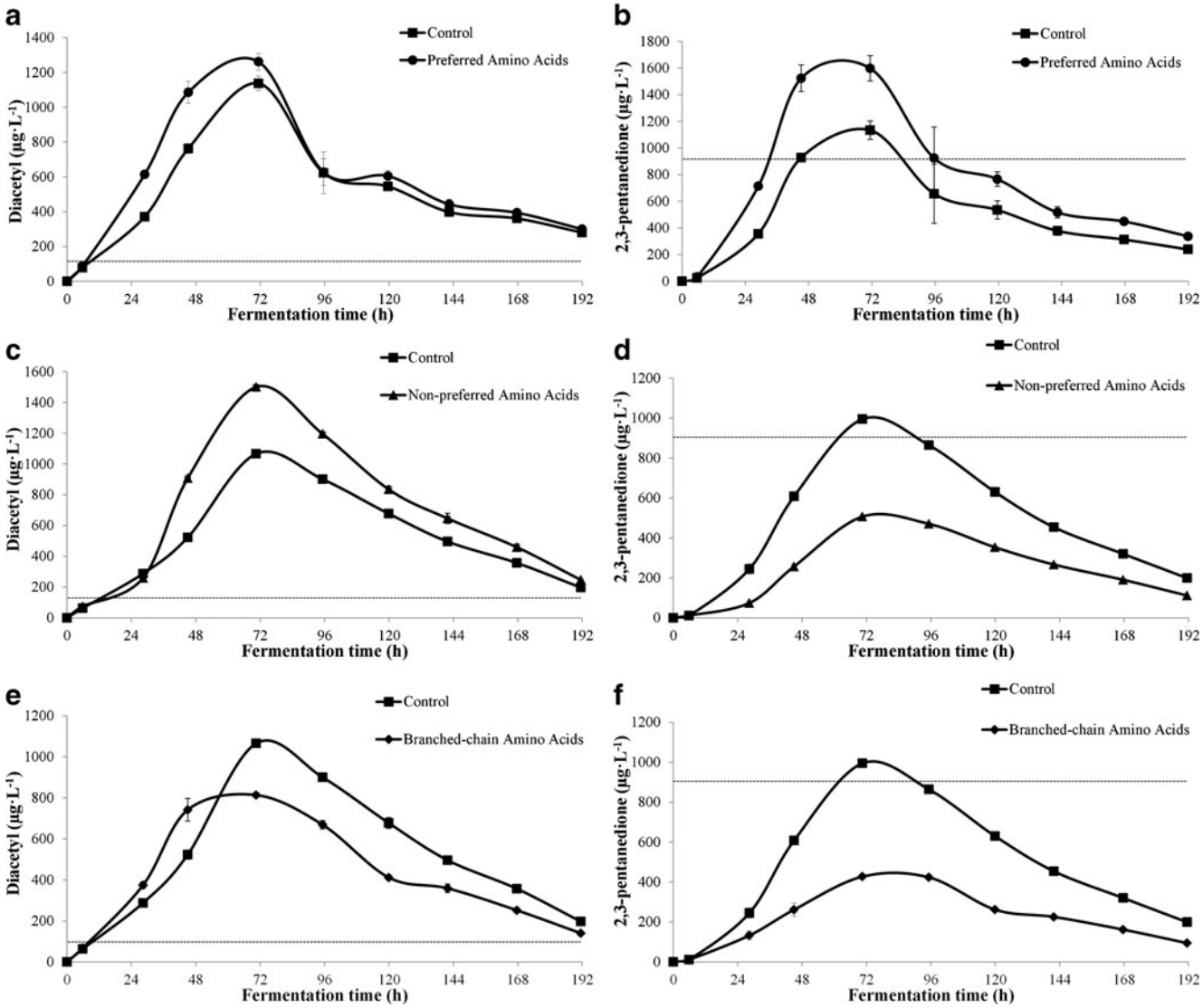

Fig. 6 The (**a**, **c**, **e**) diacetyl and (**b**, **d**, **f**) 2,3-pentanedione concentrations (in microgram per litre) of the worts supplemented with various groups of amino acids as a function of fermentation time (in hour). The *dotted lines* at 100 and 900 µg L^{-1} depict the flavour threshold (Meilgaard 1975). Values are means from two independent fermentations. *Error bars* where visible represent the range

The valine uptake rate of the yeast was higher (i.e. the change in valine concentration is more negative) and diacetyl production rate lower in the NPAA-supplemented wort compared to the control wort during the first 20 h of fermentation, while lower valine uptake and higher diacetyl production was observed in both the BCAA- and PAA-supplemented worts (Fig. 7). Between 24 and 48 h, all supplemented worts showed similar trends with a higher diacetyl production rate and lower valine uptake rate than that of the control wort. Earlier diacetyl production peaks were observed in the BCAA and PAA-supplemented worts (36 h), despite lower valine uptake rates compared to the control wort. The results suggest that valine uptake rate negatively correlates with the amount of diacetyl formed during the growth phase of fermentation, but other amino acids may be responsible for the decrease of diacetyl production as well. The leucine uptake rate increased with the supplementation of PAA and BCAA (both containing leucine) and remained unaffected by the supplementation of NPAA, while the isoleucine uptake rate increased with the supplementation of NPAA and BCAA (both containing isoleucine), but decreased with the supplementation of PAA (results not shown).

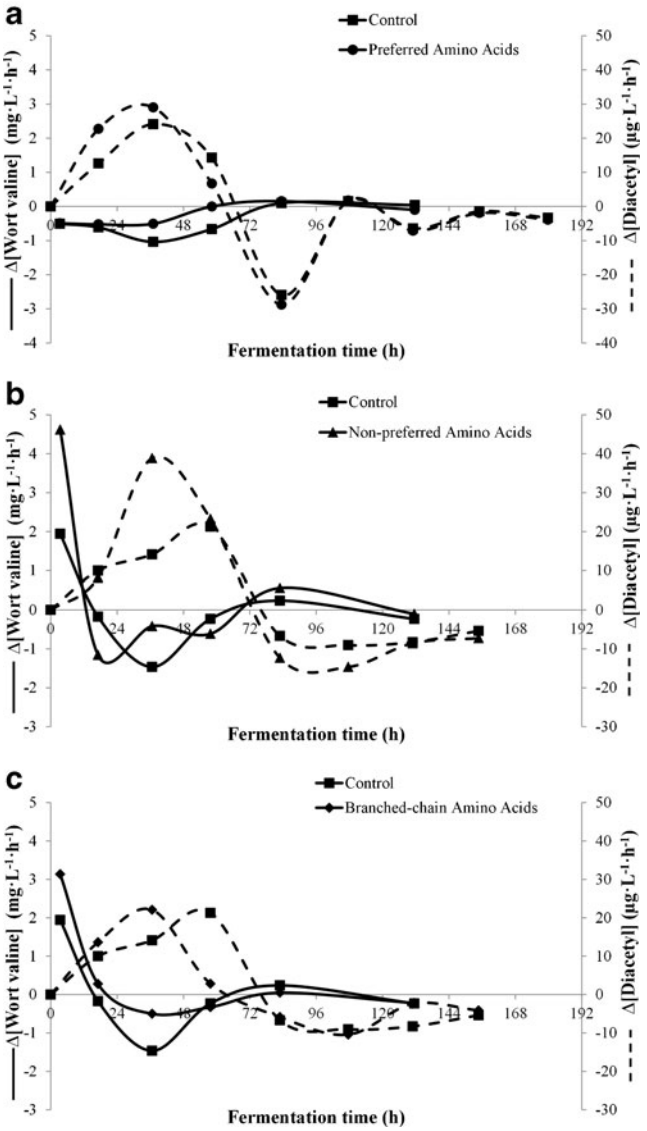

Fig. 7 The change in valine concentration (in milligram per litre per hour) and diacetyl concentration (in microgram per litre per hour) of the worts supplemented with various groups of amino acids as a function of fermentation time (in hour). Values are means from two independent fermentations

The concentrations of aroma compounds in the worts supplemented with various groups of amino acids at the end of fermentation are presented in Table 2. The concentration of 2-methylpropanol, formed from valine, was similar in all the worts, while the concentration of 3-methylbutanol, formed from leucine, was higher in both the BCAA- and PAA-supplemented worts, suggesting that leucine uptake rate of the yeast is positively correlated with the amount of 3-methylbutanol produced during fermentation. Similar results were obtained with the concentrations of 2-methylbutanol, formed from isoleucine, where higher concentrations of 2-methylbutanol were observed in the beer fermented from the NPAA-supplemented worts compared to control wort. An increased concentration of 2-methylbutanol was not however observed in the BCAA-supplemented wort, despite an increased overall isoleucine uptake, which could possibly be explained by similar isoleucine uptake rates during the first 24 h of fermentation between the BCAA-supplemented and control all-malt worts.

Discussion

The purpose of this study was to investigate whether the supplementation of valine to brewer's wort or the modification of wort amino acid profile could influence the amount of vicinal diketones produced during fermentation. By supplementing valine to brewer's wort, it was possible to decrease both the maximum diacetyl concentration observed during fermentation and the diacetyl concentration at the end of fermentation, suggesting beer maturation times could be shortened, which in turn could benefit breweries economically. The composition of the amino acid spectrum of the wort also had a large impact on diacetyl and 2,3-pentanedione production, suggesting that diacetyl production could be reduced by modifying the wort amino acid spectrum through raw material choices, adjuncts, malting conditions, or mashing conditions. The results from all three trials showed that fermentation performance and yeast growth are not affected by the amino acid supplementations, implying that supplementation of valine or other amino acids will not affect the primary fermentation time nor the attenuation level achieved.

Supplementation with valine resulted in an increased uptake rate of valine into the yeast cells, suggesting that

Table 2 Concentrations of aroma compounds in the green beer fermented from worts supplemented with various groups of amino acids (all values in milligram per litre). Values are means from two independent fermentations. Error represents standard deviation of mean

Compound	Control	NPAA	BCAA	Control	PAA
1-Propanol	10.2±0.3	8.54±0.13	8.32±0.12	11.0±0.7	11.0±0.7
2-Methylpropanol	19.8±0.3	18.5±0.3	16.2±0.3	18.8±0.2	15.6±0.4
3-Methylbutanol	28.8±1.2	25.6±0.7	39.8±0.7	30.8±0.8	39.8±1.3
2-Methylbutanol	49.0±1.7	59.2±1.7	48.7±1.3	49.6±1.3	42.7±0.9
2-Phenylethylalcohol	1.62±0.21	2.48±0.82	2.04±0.74	2.39±1.34	2.58±1.16
Ethyl acetate	24.2±0.9	24.6±1.8	23.4±2.1	24.2±1.2	22.8±3.1
Ethyl caproate	0.29±0.03	0.30±0.03	0.27±0.03	0.30±0.03	0.27±0.06
Ethyl caprylate	0.83±0.18	0.93±0.11	0.86±0.14	0.94±0.2	0.91±0.23
Ethyl decanoate	2.74±2.05	1.90±0.46	1.85±0.29	1.71±0.72	1.44±0.22
3-Methylbutylacetate	2.26±0.10	1.93±0.11	3.09±0.36	2.37±0.06	2.93±0.56
2-Phenylethylacetate	0.48±0.41	0.20±0.03	0.21±0.03	0.30±0.25	0.23±0.04

the decreased diacetyl concentrations during fermentation are a result of less pyruvate being converted into α-acetolactate because of the inhibition of AHAS by valine (Magee and de Robichon-Szulmajster 1968). These results agree with those presented by Nakatani et al. (1984), where increased amounts of valine supplementation resulted in increased valine uptake and decreased maximum valine concentrations observed during fermentation. According to the results observed by Didion et al. (1996), valine does not have any significant inducing effect on the expression of *BAP2* in *Saccharomyces cerevisiae*, while de Boer et al. (1998) observed that a number of amino acids, not only branched-chained amino acids, induced the expression of *BAP3* in *S. cerevisiae*, suggesting that increased expression of specific branched-chain amino acid permease-encoding genes may only be a minor cause of the increased valine uptake caused by valine supplementation. Since the uptake rate of leucine and isoleucine slightly decreased in the beginning of the fermentation, the increased valine uptake rate can most likely be explained by increased interactions between valine and the amino acid permeases caused by the increased ratio of valine to other branched-chain amino acids following valine supplementation. The transcriptional regulation of *BAP2* and other genes encoding branched-chain amino acid transporting permeases (*BAP3* and *TAT1*) is complex however, with several transcription factors, mainly the amino acid sensing Ssy1p protein, controlling the induced transcription of these genes (Nielsen et al. 2001).

Decreasing the free amino nitrogen content of wort lowered the amount of diacetyl produced during fermentation despite a reduced valine concentration. Pugh et al. (1997) also observed decreased diacetyl concentrations with wort FAN content decreasing from 216 to 144 mg L^{-1}, after which diacetyl concentrations increased again as FAN content was decreased to 122 mg L^{-1}. Nakatani et al. (1984) on the other hand report a negative correlation between the initial wort FAN content and the maximum VDK concentration observed during fermentation. Lei et al. (2013) observed that the amount of valine absorbed during fermentation decreased when FAN content was increased from 264 ppm to 384, 398 and 433 ppm by adding protease enzymes during mashing, despite the increase in total valine concentration. These apparent discrepancies are due to differences in valine uptake. At high FAN levels the yeast cell utilizes the preferred amino acids and less valine is taken up as a result (resulting in higher α-acetolactate production). At very low FAN levels, many amino acids will be entirely removed from the system. If valine is depleted in this fashion then the demand for anabolic valine synthesis is increased and the α-acetolactate level increases as a result. It would appear from the values available in the literature that a FAN level of approx. 150 ppm is required if high diacetyl levels are to be avoided. The valine concentrations of the semi-synthetic worts were not analysed (initial concentrations 132.7 and 422.7 mg L^{-1} in the control and valine-supplemented worts, respectively), but the decreased diacetyl production in the semi-synthetic worts is most likely caused by an increased valine uptake rate resulting from rapid depletion of preferred amino acids. Hence, it becomes evident that it is not the valine concentration per se that is of central importance regarding the production rate of diacetyl during fermentation, but rather the uptake rate. Since the pH of the semi-synthetic worts was lower during fermentation than that of the all-malt worts (maximum difference 0.4 units), most likely due to loss of buffer capacity through dilution, the lower diacetyl concentrations produced with wort containing a reduced FAN content may also be influenced in this case by an increased reaction rate for the spontaneous decarboxylation of α-acetolactate into diacetyl (Garcia et al. 1994; Kobayashi et al. 2005a; Rondags et al. 1996).

Supplementing the preferred amino acids to all-malt wort resulted in an increase in wort diacetyl relative to the control wort at the beginning of fermentation. Supplementing the non-preferred amino acids, i.e. those that had been absorbed in lesser amounts than valine during the first 25 h of fermentation, to all-malt wort however resulted in negligible difference during the first 24 h compared to the control wort. This was followed by an increase mid-fermentation (24 to

120 h). These results suggest that high concentrations of amino acids that are quickly absorbed have little effect on diacetyl concentration in beer, but high concentrations of amino acids that are slowly absorbed, and compete with valine for transporters, have a larger effect on the diacetyl concentration at the end of fermentation.

Increased uptake of leucine and isoleucine can also potentially decrease the production rate of diacetyl during fermentation. Since the maximum and final diacetyl concentrations were lower in the BCAA-supplemented all-malt worts, and the valine uptake was decreased in the BCAA-supplemented wort compared to the control wort, it is evident that valine is not the only amino acid responsible for reduced diacetyl production. Studies have shown varying data on the inhibiting effect of other branched-chain amino acids on AHAS, as Barton and Slaughter (1992) and Magee and de Robichon-Szulmajster (1968) observed that leucine also had an inhibiting effect on the AHAS enzyme's ability to produce α-acetolactate from pyruvate, though not as strong as the inhibiting effect of valine. No inhibiting effect of isoleucine on the AHAS enzyme's ability to produce α-acetolactate from pyruvate was found. Pang and Duggleby (2001) observed the opposite, i.e. that isoleucine had a slight inhibiting effect and leucine had no inhibiting effect on the AHAS enzyme's ability to produce α-acetolactate from pyruvate. The uptake rate of both isoleucine and leucine were increased in the BCAA-supplemented wort compared to the control wort, suggesting that the lower diacetyl production observed in the BCAA-supplemented wort could result from AHAS inhibition by leucine. This might also explain the similar diacetyl production rates towards the latter half of fermentation observed in the PAA-supplemented wort, containing increased concentrations of leucine, and its control wort. The initial diacetyl production rate of the BCAA-supplemented wort was however higher than the control wort, which presumably is a result of a combination of lower inhibiting effect on AHAS of leucine than valine and the decreased total uptake rate of branched-chain amino acids during the first approximately 12 h of fermentation, most likely caused by the increased competition for permease interactions. The total uptake rate of branched-chain amino acids increased towards the middle of fermentation in the BCAA-supplemented wort, perhaps from increased expression of genes encoding amino acid permeases (e.g. *BAP2* and *BAP3*) as a result of increased amino acid concentrations (Didion et al. 1996).

Supplementation of the amino acid groups containing isoleucine, i.e. NPAA and BCAA, resulted in lowered 2,3-pentanedione concentrations compared to the control worts. Because of the high flavour threshold of 2,3-pentanedione, the lowered concentrations will not impact on beer quality. Hence, the advantages gained from decreased 2,3-pentanedione concentrations, do not outweigh the disadvantages gained from potentially increased diacetyl concentrations resulting from decreased valine and leucine uptake rate. Results from the first experiment suggest that despite the presumably decreased activity of the AHAS enzyme, it can still actively catalyse the formation of α-acetohydroxybutyrate from α-ketobutyrate, since the concentrations of 2,3-pentanedione were not affected by the valine supplementations. It is unclear whether inhibition of the AHAS enzyme by valine still allows the α-ketobutyrate to α-acetohydroxybutyrate reaction to be active, and if isoleucine or the other branched-chain amino acids have any inhibiting effect on the α-ketobutyrate to α-acetohydroxybutyrate reaction. Results from the study by Epelbaum et al. (1996) on the effect of sulphometuron methyl on the activity of enzymes in the valine and isoleucine synthesis pathways in *Salmonella typhimurium*, suggest that sulphometuron methyl only inhibits the pyruvate to α-acetolactate reaction of the AHAS enzyme, while the α-ketobutyrate to α-acetohydroxybutyrate reaction remains active. Since studies on the AHAS activity (e.g. Byrne and Meacock 2001; Duong et al. 2011; Magee and de Robichon-Szulmajster 1968; Pang and Duggleby 2001) revolve around an assay based on the ability of AHAS to convert pyruvate into α-acetolactate, it would be of interest to measure the activity of the α-ketobutyrate to α-acetohydroxybutyrate reaction as well, and the effect of various amino acids on its activity.

The present study has focussed on total VDK (acetohydroxy acid and free VDK) concentration. It is therefore not possible to determine from the results to what extent VDK removal rate is due to spontaneous decarboxylation of the acetohydroxy acid or reduction of the free VDK compounds by yeast. The processes responsible for the lowering of VDK levels in mid- to late-fermentation are poorly understood compared to those processes involved in VDK generation (Bamforth and Kanauchi 2004) and further research is necessary to elucidate these important steps, particularly since, as seen in the current study, much of the advantage of valine supplementation is reduced in the later stages of fermentation.

The results imply that modifying the concentrations of wort amino acids has only a slight effect on the concentrations of aroma compounds in the beer. The concentrations of higher alcohols, and esters derived from these alcohols, produced in the yeast via the transamination of amino acids were increased in almost all cases when the concentration of the relevant amino acid precursor was increased, suggesting a positive correlation especially between the uptake rate of branched-chain amino acids and the higher alcohols produced from them. The concentrations of the higher alcohols remained below or around the flavour threshold though in all cases (Meilgaard 1982; Siebert 1988), suggesting that any changes in higher alcohol concentrations caused by altering wort amino acid concentrations on beer quality will be minor. The concentration of 3-methylbutylacetate was

above the flavour threshold for all fermentations, even the controls, so large changes in wort leucine concentration could affect the flavour impact of this ester on the beer. The concentrations of the examined ethyl esters were around or slightly below their flavour threshold in all fermentations, and the concentrations either remained unaffected or even decreased with the supplementation of amino acids. Ester-derived flavours and aroma are only desired in small amounts in lager beers (Verstrepen et al. 2003), so the minor impact of amino acid supplementation on ester concentrations is a positive result.

The results agree in that the diacetyl concentration of fermenting wort can be decreased by modifying its initial amino acid profile, and particularly the concentrations of valine and the other branched-chain amino acids, without any effect on fermentation performance. Consequently, the maturation time of the beer can potentially be decreased as well. The results from the experimental work suggest that the uptake rate of amino acids and their intracellular effect on the metabolic flux through the valine and isoleucine biosynthesis pathway are vital for understanding their relationship with diacetyl and 2,3-pentanedione production. Further research into modifying the wort amino acid profile, e.g. by altering mashing conditions (Schwarz et al. 2012), could yield valuable techniques for reducing diacetyl without the use of GM strains.

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