DOCTORAL THESIS

The Effect of Cidermaking Practices on Ester Production by Yeast

Julia Rosend

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation, and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Julia Rosend

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List of Publications

These publications form the basis of this thesis and are reproduced in the appendix with permission from the publishers:

I Rosend, J., Kuldjärv, R., Rosenvald, R., Paalme, T. (2019) The effect of apple variety, ripening stage, and yeast strain on the volatile composition of apple cider. Heliyon 5, e01953. doi:10.1016/j.heliyon.2019.e01953

II Rosend, J., Kuldjärv, R., Arju, G., Nisamedtinov, I. (2019) Yeast performance characterisation in different cider fermentation matrices. Agronomy Research 17, 2040-2053. doi:10.15159/AR.19.178

III Rosend, J., Kaleda, A., Kuldjärv, R., Arju, G., Nisamedtinov, I. (2020) The effect of apple juice concentration on cider fermentation and properties of the final product. Foods 9, 1401. doi:10.3390/foods9101401

Author's Contribution to the Publications

I The author performed the analysis of volatile compounds by GC-MS, interpreted the results and wrote the manuscript.

II The author was responsible for the study design, conducted the experiments and analyses, interpreted the results and wrote the manuscript.

III The author was responsible for the study design, conducted the experiments and analyses, interpreted the results and wrote the manuscript.

Introduction

Cider is an alcoholic beverage obtained by fermentation of apple juice. Its history goes back to the first century AD when it appeared in the citations of the Roman writer Pliny (Lea & Drilleu, 2003). From the Roman empire, cider consumption has moved north to appear in France and England in the 9th century (Lea & Drilleu, 2003). By the 18th century, cider production had established itself as a commercial product on the market (Lea & Drilleu, 2003). Nowadays, the global cider market is valued at more than $10 billion and assumed to reach $16 billion value by 2023 (Allied Market Research, 2018) with the UK being the biggest producer (European Cider and Fruit Wine Association, 2019).

The specific cider regulations are country dependent. In countries with stricter legislation, the raw materials used for cider production as well as cider's quality properties are relatively well determined, whereas in other countries the legislation is less defined. For example, in the UK, cider should be based on at least 35% of apple juice and the alcohol content should remain 1.2% - 8.5 %vol (HM Revenue and Customs, 2020). According to French cider production policy, on the other hand, cider must be based on fresh apple juice without any other sugar-containing adjuncts (Légifrance, 2020). Notably, the juice may be partially obtained from the concentrate only if the latter does not exceed 50% by volume (Légifrance, 2020). In addition to the alcohol content (1.5% - 5%vol), the French ciders are more clearly defined in terms of other quality parameters, including volatile acidity, mineral content, maximum allowed ethanal content, maximum allowed sulfur dioxide content (Légifrance, 2020).

Depending on the country of origin and the production technology used, ciders can vary to a great extent in sensory style. Thus, French ciders can be described as robust, full-bodied and fruity, tannic with strong bittersweet apple characteristics (European Cider and Fruit Wine Association, 2019) while British ciders are typically medium-bodied and refreshing (European Cider and Fruit Wine Association, 2019). German ciders, on the contrary, are more wine-like – dry, sharp and moderately fruity (European Cider and Fruit Wine Association, 2019). Scandinavian ciders can range from dry to very sweet with pronounced fruitiness (European Cider and Fruit Wine Association, 2019).

Sensory properties play a primary role in the consumer acceptance of a product and, in the case of fermented beverages, largely depend on yeast metabolites produced during fermentation. Similarly to other fermented beverages, the fruity nuances in cider provided by esters offer a competitive edge that helps to differentiate a specific product from others. The objective of this PhD thesis was to explore how different cidermaking conditions affect the quality of cider in terms of ester production by yeast. The results of this study can be used to advise cider producers on the optimization of fermentation to achieve desired properties of the final product.

Abbreviations

ANOVA	Analysis of variance
Asn	Asparagine
Car/PDMS	Carboxen/Polydimethylsiloxane
CFU	Colony forming units
DAP	$(NH_4)_2HPO_4$ Diammonium hydrogen phosphate
DNFB	Dinitrofluorobenzene
DVB/Car/PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane
FAN	Free amino nitrogen
GC-MS	Gas chromatography – mass-spectrometry
IS	Internal standard
K_m	Michaelis constant in Michaelis-Menten reaction kinetics equation The concentration of a substrate at which an enzyme reaction rate is at half-maximum
MCFA	Medium-chain fatty acids
MLF	Malolactic fermentation
PCA	Principal component analysis
PLS-DA	Partial least square discriminant analysis
TCA cycle	Tricarboxylic acid cycle
UPLC	Ultra-performance liquid chromatography
YAN	Yeast assimilable nitrogen
YPG medium	Yeast Extract-Peptone-Glucose medium

1 Literature Review

1.1 Technological aspects of cider production

1.1.1 Cider apples

Apples used for cider production can be either table apples, including those unqualified for commercial use, or cider apple varieties (Downing, 1989; Nogueira & Wosiacki, 2012). Apples unqualified for commercial use are those that can potentially be deemed unsuitable from the standpoint of a consumer (Nogueira & Wosiacki, 2012). Those are apples with morphological defects, uneven color, irregular shape, small size (Nogueira & Wosiacki, 2012).

Although the production of cider does not necessarily require strict use of cider apples, there is a certain advantage in preferring cider varieties. Cider apples have a high sugar content (up to 15%) and a wide range of acidity across different varieties (Bamforth & Cook, 2019). Their fibrous structure allows for more efficient pressing and, thus, higher juice yields (Bamforth & Cook, 2019). Cider apples are also more abundant in tannins which contribute to body/mouthfeel of the product (Bamforth & Cook, 2019).

Cider apples are classified according to their composition of sugars, acids and polyphenols. In Europe, cider apples are mainly classified based on either acid-tannin or acid-tannin-sugar content ratio (Merwin et al, 2008; McKay et al, 2011; Nogueira & Wosiacki, 2012; Jolicoeur, 2013) (Table 1). Classification of cider apples allows producers to pick appropriate varieties for a blend eventually resulting in desired properties of the final product (Nogueira & Wosiacki, 2012). For example, apples with medium to high sugar content and moderate acidity are suitable as-is for the production of good quality cider (Jolicoeur, 2013). On the other hand, apples that are high in acidity require blending to balance out the sourness. For that, apples with low acidity and low to high sugar content are used (Jolicoeur, 2013). Low-sugar apples are generally not recommended for cider and are typically used to produce vinegar (Jolicoeur, 2013).

Table 1. *Cider apple classification examples (Merwin et al, 2008; McKay et al, 2011; Nogueira & Wosiacki, 2012; Jolicoeur, 2013)*

Classification	Class	Acidity*, % w/v	Phenolic content**, % w/v	Sugar
Acid-tannin	Sharp	>0.45 (high)	<0.20 (low)	
	Bittersharp	>0.45 (high)	>0.20 (high)	
	Bittersweet	<0.45 (low)	>0.20 (high)	
	Sweet	<0.45 (low)	<0.20 (low)	
Acid-tannin-sugar	Sweet	<0.45 (low)	<0.20 (low)	High
	Bittersweet	<0.45 (low)	>0.20 (high)	High
	Bitter	<0.45 (low)	>0.20 (high)	Low
	Acidic	>0.45 (high)	<0.20 (low)	Low

*As malic acid equivalents

**As tannic acid equivalents

1.1.2 Pre-fermentation processing

1.1.2.1 Apple must preparation

Cider apples are harvested when ripe. The estimation of ripening is often based on the iodine starch test where a degree of apple flesh staining is assessed on an index scale from 0 (unripe) to 5 (ripe) (Travers et al, 2002). If full maturation on trees is not possible (e.g., due to regional climate limitations or weather conditions), the harvested apples are stored in a dark room for a few weeks to convert excess starch into glucose, softening the fruit and allowing the development of volatile compounds associated with ripening (e.g., alcohols, aldehydes, esters) (Sapers et al, 1977; McKay et al 2011; Granell & Rambla, 2015; Alberti et al, 2016). Apples are sorted to remove spoiled fruits or fruits with visible defects and washed to remove surface dirt and grime.

Collected apples are ground and mashed to obtain a thin pulp (apple mash) and ease the release of juice in pressing. Enzymes with pectinolytic and cellulolytic activity are often added during milling and mashing to increase the yield (Nogueira & Wosiacki, 2012; Joshi & Attri, 2017). Apple mash is then pressed to release apple must. The average yield of this process is up to 700 L of apple must per ton of apple mash (Nogueira & Wosiacki, 2012).

Freshly pressed apple must contains pectins that increase the viscosity which can affect further processing (Nogueira & Wosiacki, 2012). Pectins are removed from the must in a process called depectinization where pectinases are added to hydrolyze pectins into soluble components (i.e., galacturonic acid) (Nogueira & Wosiacki, 2012; Verma et al, 2018). Depectinization is followed by clarification to remove haze of the hydrolyzed pectin (Nogueira & Wosiacki, 2012). In Brittany and Normandy, a process called keeving is used where endogenous pectin acts as a clarifying agent. Keeving involves the addition of calcium chloride and pectin methylesterase to apple must (Beech, 1993; Le Quéré et al, 2006; McKay et al, 2011). As a result, calcium pectate gel forms a film that can be mechanically removed (Beech, 1992; Le Quéré et al, 2006; McKay et al, 2011).

Before fermentation, clarified apple must might require additional adjustments. These adjustments can include the addition of sugars (if allowed) and yeast nutrients (Lea & Drilleu, 2003; Joshi & Attri, 2017). Sugar can be added in the form of apple juice, apple juice concentrate, or glucose syrup (Lea & Drilleu, 2003; Joshi & Attri, 2017). The most conventional yeast nutrients are nitrogen source in the form of diammonium sulfate/hydrogen phosphate and vitamins (thiamine, pantothene, pyridoxine, biotin) (Lea & Drilleu, 2003; Joshi & Attri, 2017). After necessary adjustments, apple must can be treated with sulfur dioxide (added in the form of potassium metabisulfite) to inactivate undesirable microflora (e.g., spoilage bacteria) and is inoculated with a yeast starter culture (Downing, 1989; Nogueira & Wosiacki, 2012; Joshi & Attri, 2017). Wild fermentation with indigenous microflora of the must is, however, also often used, especially by small/artisanal cider manufacturers.

1.1.2.2 Apple must composition

Table 2 provides a general comparative overview of apple and grape must composition. The values for each component are given as estimates based on multiple different reports and studies (Drilleau, 1990; Kunkee & Bisson, 1993; Cruz et al, 2002; Eisele & Drake, 2005; Hutkins, 2006; Wu et al, 2007; Orak, 2009; Aranda et al, 2011; Bakker & Clarke, 2012; Nogueira & Wosiacki, 2012; Jackson, 2014; Joshi & Attri, 2017; Boudreau et al, 2018).

Carbohydrates account for the largest portion of soluble solids in the apple must (McKay et al, 2011). Almost all sugars in the apple must are comprised of mono- (glucose, fructose) and disaccharides (sucrose) with fructose accounting for 75% on average of total sugars content (McKay et al, 2011; Nogiera & Wosiacki, 2012; Joshi & Attri, 2017).

Soluble pectin and pectic substances (mostly in the form of anhydrogalacturonic acid units) can be present in freshly pressed and untreated apple must in concentrations up to 5 g L^{-1} (Nogueira & Wosiacki, 2012; Joshi & Attri, 2017). Most of these pectic substances, however, are removed in depectinization process before fermentation. Organic acids are represented in the must predominantly by malic acid (up to 1.2% w/v) (McKay et al, 2011). Citric, citramalic, quinic, galacturonic, glycolic, gluconic, shikimic, succinic, pyruvic, lactic can be found in trace amounts (McKay et al, 2011; Joshi & Attri, 2017).

Nitrogen compounds in the apple must can be divided into yeast non-assimilable nitrogen and yeast assimilable nitrogen (YAN). The latter is comprised of free amino nitrogen (FAN, from free amino acids) and ammonia (Santos et al, 2016; Boudreau et al, 2018). Asparagine, aspartic acid, serine and glutamic acid are the main amino acids that contribute to FAN content of the apple must, accounting for 85-95% of the total amount of free amino acids (Wu et al, 2007; Nogueira & Wosiacki, 2012; Joshi & Attri 2017). In addition to free amino acids, smaller peptides (up to 5 amino acids) can be consumed by yeast and are also considered as part of YAN, albeit difficult to quantify (Patterson & Ingledew, 1999; Lekkas et al, 2012; Kevvai et al, 2015; Laos, 2018).

Table 2. *Average composition of apple and grape musts (Drilleau, 1990; Kunkee & Bisson, 1993; Cruz et al, 2002; Eisele & Drake, 2005; Hutkins, 2006; Wu et al, 2007; Orak, 2009; Aranda et al, 2011; Bakker & Clarke, 2012; Nogueira & Wosiacki, 2012; Jackson, 2014; Joshi & Attri, 2017; Boudreau et al, 2018)*

Component	**Apple must**	**Grape must**
Water, %	80 – 95	70 – 85
Fructose, %	4 – 13	6 – 12
Glucose, %	0.5 – 3	6 – 12
Sucrose, %	1 – 5	< 1
Total sugars, %	9 – 15	ca 20
Organic acids, %	0.2 – 1.80	1 – 2.7
Primary organic acids	Malic acid	Malic acid Tartaric acid
pH	3.0 – 4.2	3.0 – 3.5
Total nitrogen, mg L^{-1}	27 – 574	200 – 500
YAN, mg·N L^{-1}	29 – 250	50 – 450
Primary free amino acids	Asparagine Aspartic acid Glutamic acid	Proline Arginine

1.1.3 Alcoholic fermentation

1.1.3.1 Yeasts

Yeasts and other microorganisms are naturally present on the surface of the apple fruits which is why they end up also in the apple must (McKay et al, 2011). Composition and quantity of indigenous microflora depend on geographical location, ripening stage of the fruit, presence of mechanical defects, climatic/weather conditions, and agricultural practices applied in an orchard (McKay et al, 2011; Rodríguez-Bencomo et al, 2012).

Yeasts colonizing the surface of the apples are responsible for spontaneous (also known as wild) fermentation of the must (McKay et al, 2011; Nogueira & Wosiacki, 2012; Rodríguez-Bencomo et al, 2012). The indigenous yeast population at the start of spontaneous fermentation can reach up to 10^6 CFU mL^{-1} and follows a succession of different yeast species as the fermentation progresses (Lea & Drilleau, 2003). Thus, initial phases of the fermentation are dominated by non-*Saccharomyces* yeasts with *Hanseniaspora, Kloeckera, Metschnikowia, Candida, Pichia*, *Torulopsis, Hansenula* reported as the main contributors to spontaneous cider fermentation (Fleet, 2003; Lea & Drilleau, 2003; Tamang & Fleet, 2009; Ugliano & Henschke, 2009; Matei & Kosseva, 2017). Due to low resistance to ethanol of these non-*Saccharomyces* yeasts, their growth rate decreases at around 2-4%vol of ethanol (Lea & Drilleau, 2003; Matei & Kosseva, 2017). At this point, *Saccharomyces* sp. (*S. cerevisiae, S. uvarum, S. bayanus)* take over the fermentation (Lea & Drilleau, 2003; Tamang & Fleet, 2009; Matei & Kosseva, 2017).

Even though wild fermentation by the indigenous microflora has been alleged to provide sensorial complexity, large manufacturers opt for inoculation with specific isolated strains of *Saccharomyces* sp. to control the progress of fermentation (inc. avoiding sluggish fermentation) and provide stable quality (McKay et al, 2011; Nogueira & Wosiacki, 2012). Inoculation with a pure yeast starter culture is also used in case of pasteurized apple must (McKay et al, 2011; Jackson, 2014). *S. cerevisiae* is a preferred starter culture for alcoholic fermentation since it is highly adapted to growing in the high-sugar environments, tolerant to high concentrations of alcohol, high acidity and low pH values, and can grow multiple generations in the absence of oxygen (Tamang & Fleet, 2009; McKay et al, 2011; Jackson, 2014).

Recently, application of commercial yeast starter cultures of non-*Saccharomyces* species, either by co- or sequential inoculation with *S. cerevisiae* has also gained popularity among wine and cider producers due to the ability of non-*Saccharomyces* yeasts (e.g., *Pichia kluyveri, Kazachstania gamospora*, *Metchnikowia pulcherrima, Hanseniaspora* sp.) to generate a variety of flavor active compounds, including ethyl and acetate esters (e.g., ethyl acetate, ethyl butyrate, isoamyl acetate) (Lea & Drilleau, 2003; Ugliano & Henschke, 2009; McKay et al, 2011; Chr Hansen AS, 2015; Rodriguez Madrera et al, 2015). However, the practical use of non-*Saccharomyces* yeasts only is not as widespread as *S. cerevisiae* due to their limited survival at higher alcohol concentrations (McKay et al, 2011).

In conclusion, suitable yeast strains for cider fermentation are usually selected based on several factors such as fermentation kinetics, ability to complete fermentation, as well as the production of sensorially favorable compounds (e.g., esters), and absence of off-flavors (Downing, 1989; Rodríguez-Bencomo et al, 2012; Jackson, 2014). The pitching culture can either be propagated in-house or obtained as a commercial (dry) yeast (Downing, 1989). Commercial dry yeast will require appropriate rehydration before inoculation, usually according to manufacturer's instructions. The recommended number of inoculated active cells for *S. cerevisiae* is 10^5-10^6 CFU mL^{-1} (Ribereau-Gayon et al, 2006; McKay et al, 2011; Jackson, 2014).

1.1.3.2 Sugar uptake and fermentation in apple must

S. cerevisiae is a facultatively anaerobic microorganism. Under conditions of excess fermentable sugars (mainly glucose) as well as oxygen deficiency, *S. cerevisiae* switches from respiration to fermentation (anaerobic sugar metabolism) (Deak, 2006; Hutkins, 2006; Zamora, 2006; McKay, 2011; Jackson, 2014).

Uptake of hexoses in *S. cerevisiae* occurs via facilitated diffusion mediated by specific transport proteins (Hxt1p-Hxt17p, Snf3p, Rgt2p); sucrose has to be hydrolyzed into glucose and fructose by cell wall located invertase (Suc2p) before the latter can be transported into the cell (Boles & Hollenberg, 1997; Hutkins, 2006; Berry & Slaughter, 2003; Rodríguez-Bencomo et al, 2012; Marques et al, 2016). The expression and function of transport proteins are regulated by extracellular hexose concentrations (Boles & Hollenberg, 1997; Peeters & Thevelein, 2014). The signaling pathway for protein transcription is then generated by two protein sensors Snf3p (low concentration sensor) and Rgt2p (high concentration sensor). At low concentrations, Snf3p mediates the transcriptional induction of high-affinity transport proteins Hxt2p-Hxt4p and Hxt6p-Hxt7p (Boles & Hollenberg, 1997; Peeters & Thevelein, 2014). At high concentrations, sensor protein Rgt2p induces the expression of low-affinity transport protein Hxt1p (Boles & Hollenberg, 1997; Peeters & Thevelein, 2014). Transport proteins Hxt5p, Hxt8p and Hxt9p have been reported to possess low transcription levels and a limited contribution to hexose uptake in comparison to the aforementioned transport proteins (Boles & Hollenberg, 1997; Peeters & Thevelein, 2014). The contribution of Hxt10p and Hxt12p-Hxt17p to hexose transport is yet to be properly explored (Boles & Hollenberg, 1997; Peeters & Thevelein, 2014).

Once taken up into the cell, glucose and fructose enter Embden-Meyerhof-Parnas pathway of glycolysis until pyruvate which further will be decarboxylated to acetaldehyde and then reduced to ethanol (Figure 1) (Hutkins, 2006; Ribereau-Gayon et al, 2006; Jackson, 2014; Kampen, 2014; Rupasinghe et al, 2017). While the maximum theoretical yield of ethanol is 0.51 g ethanol/g used glucose/fructose according to stoichiometry of ethanolic fermentation, on average in practice the consumption of 17 g L^{-1} of simple sugars is required to raise alcohol content by 1%vol (Rodríguez-Bencomo et al, 2012).

Notably, fermentative metabolism in *Saccharomyces* sp. can also take place in the presence of oxygen, triggered by high concentrations of glucose (at least 9-10 g L^{-1}) in the must (Crabtree effect) (Ribereau-Gayon et al, 2006; Rupasinghe et al, 2017). Also known as catabolite repression, the process involves the suppression of respiratory and stress response genes (Geladé et al, 2003; Verstrepen et al, 2004; Dickinson & Kruckeberg, 2006; Rodrigues et al, 2006; Diaz-Ruiz et al, 2008; Zaman et al, 2008; McKay et al, 2011; Golzalez et al, 2011). To sustain metabolism and growth, the yeast cell is required to maintain redox homeostasis that is determined by NADH/NAD^+ ratio (Vemuri et al, 2007; Pfeiffer & Morley, 2014). High glycolytic flux during respiration leads to metabolic overflow where the conversion of glucose to pyruvate creates NADH excess (Vemuri et al, 2007; Pfeiffer & Morley, 2014). To achieve redox homeostasis, NADH is then oxidized back to NAD^+ through pyruvate decarboxylase pathway (ethanol formation pathway) (Vemuri et al, 2007; Pfeiffer & Morley, 2014).

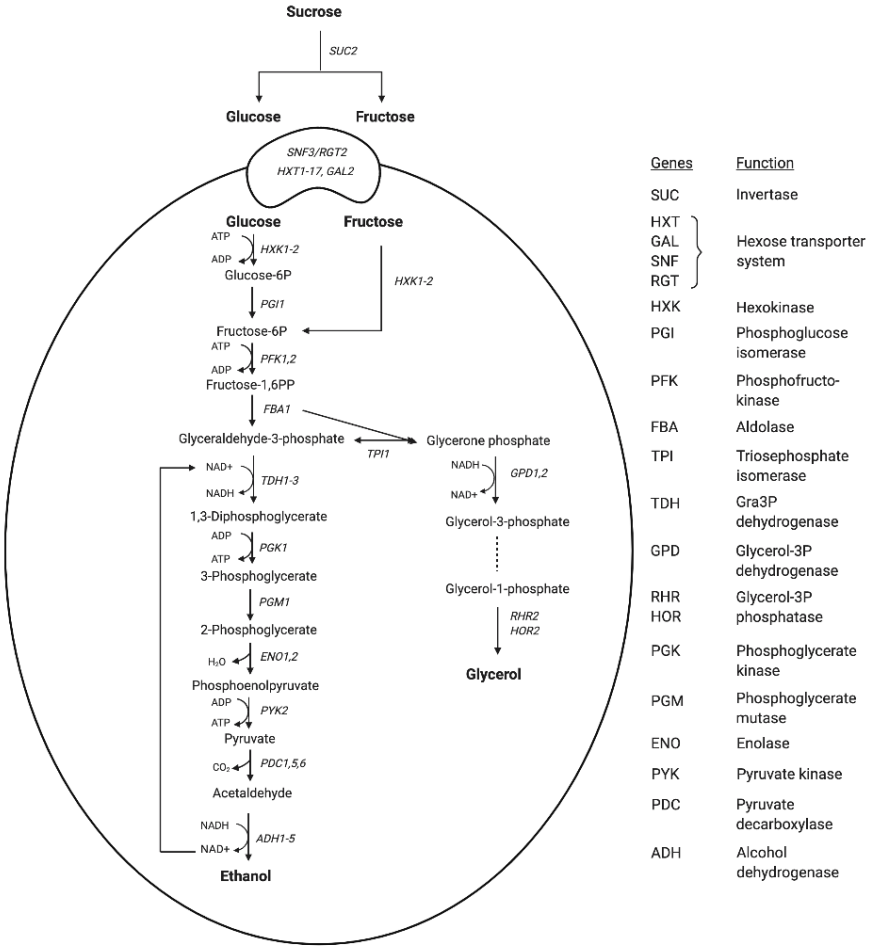


Figure 1. *Metabolic pathway of alcoholic fermentation (adapted from Boles & Hollenberg (1997) with permission of John Wiley & Sons, Pritchard & Kell (2002) with permission of John Wiley & Sons, Ciani et al (2008) with permission of Elsevier, Saccharomyces Genome Database (Stanford University; Stanford, CA, USA))*

1.1.3.3 Nutritional requirements of yeast

For rapid and complete alcoholic fermentation, an adequate composition of nutrients is important. Carbohydrates are a primary energy source for yeast; S. cerevisiae can ferment carbohydrates in the form of hexoses (glucose, fructose) and oligosaccharides (sucrose, maltose, maltotriose) (Deak, 2006; Hutkins, 2006; Ribereau-Gayon et al, 2006; Jackson, 2014). In the case of most *S. cerevisiae* oenological strains, fermentation activity usually remains stable until sugar concentrations of 200 g L^{-1} while higher sugar concentrations (above 30%) have been noted to retard the onset of fermentation and/or heighten the risk of sluggish fermentation due to high osmotic pressure (Ribereau-Gayon et al, 2006; Jackson, 2014; Mattei & Kosseva, 2017).

Next to carbohydrates, assimilable nitrogen compounds are the second most important group of nutrients for the yeast as they fulfill a row of metabolic and enzymatic

functions (McKay et al, 2011; Rupasinghe et al, 2017). The generic term for assimilable nitrogen compounds used by brewers and winemakers is called Yeast Assimilable Nitrogen (YAN) and this consists of easily available ammonium, free amino acids and small peptides (Hutkins, 2006; McKay et al, 2011). In comparison to free amino acids, ammonium is reported as a preferred source of nitrogen and consumed first as it can be easily converted to glutamic acid that acts as a primary starting metabolite for the synthesis of other amino acids (Berry & Slaughter, 2003; Dequin et al, 2003; Ribereau-Gayon et al, 2006; McKay et al, 2011; Jackson, 2014; Rupasinghe et al, 2017). However, co-consumption of ammonia, amino acids and peptides by *S. cerevisiae* was observed by Kevvai *et al* (2015) in synthetic media.

The average YAN content in the apple must is reported at 120 mg·N L^{-1}, but it can, for example, also be as low as 30 mg·N L^{-1} (Drilleau, 1990; Cruz et al, 2002). Low YAN values are associated with sluggish or even stuck fermentation and the risk of off-flavor formation (Hutkins, 2006; Alberti et al, 2011; McKay et al, 2011; Nogueira & Wosiacki, 2012; Jackson, 2014; Rupasinghe et al, 2017; Boudreau et al, 2018). On the other hand, too high YAN values promote excessive cell multiplication and decrease the efficiency of sugar conversion to ethanol as sugars are converted mainly into biomass (Aranda et al, 2011; McKay et al, 2011; Jackson, 2014). *S. cerevisiae* strains can differ significantly in their YAN requirement to achieve efficient fermentation and can be selected according to the YAN content of the must (Ribereau-Gayon et al, 2006). Additional YAN can also be supplemented before fermentation to meet the requirement of yeast. Most of the time, supplementary YAN is added in the form of ammonium salts (e.g., diammonium hydrogen phosphate) (Hutkins, 2006; Aranda et al, 2011; McKay et al, 2011; Rupasinghe et al, 2017).

B group vitamins (primarily biotin, thiamine, and nicotinic acid) are utilized by *S. cerevisiae* to fulfil catalytic functions as cofactors and their precursors (Aranda et al, 2011; McKay et al 2011; Jackson, 2014; Kampen, 2014; Paalme et al, 2014). Mineral elements (e.g., zinc, magnesium, potassium) also fulfil a crucial role in active sites of enzymatic reactions, as well as maintaining the pH and ionic balance of the cell (Jackson, 2014). In general, the content of vitamins and mineral elements in apple juice is considered sufficient in fulfilling the yeast requirements; however, thiamine can be added as a part of nutritional supplementation to further enhance yeast performance (Nogueira & Wosiacki, 2012; Paalme et al, 2014).

Despite anoxic conditions during most of the fermentation yeast still require some oxygen (ca 10 mg L^{-1}) at the start of fermentation to ensure vitality and viability of the cells in later stages of fermentation at increased ethanol concentration (Dequin et al, 2003; Aranda et al, 2011; Nogueira & Wosiacki, 2012). More specifically, the presence of oxygen at the onset of fermentation is necessary for the synthesis of sterols and unsaturated fatty acids of the plasma membrane and nicotinic acid (Dequin et al, 2003; Nogueira & Wosiacki, 2012; Jackson, 2014; Mattei & Kosseva, 2017). Sufficient aeration of the must improves the growth of biomass and fermentation speed (Dequin et al, 2003). When nutrient limitations do not exist, the beneficial effects of oxygen can be observed even at lower concentrations (up to 2 mg L^{-1}) (Nogueira & Wosiacki, 2012).

1.1.3.4 The effect of fermentation temperature on yeast metabolism

Yeasts are mesophilic microorganisms with a growth temperature range of 10-35°C (Salvadó et al, 2011). As a rule, the fermentative activity of yeasts will increase with the increase of temperature (Deak, 2006; Ribereau-Gayon et al, 2006; Kasemets et al, 2007;

Salvadó et al, 2011; Mattei & Kosseva, 2017). For example, a twofold increase in yeast cell division rate has been observed when increasing the temperature from 20°C to 30°C (Ribereau-Gayon et al, 2006; Jackson, 2014). The use of low and high temperatures for fermentation, however, has been shown to possess several advantages and disadvantages. Thus, lower fermentation temperatures prolong the duration of the lag phase and overall fermentation (Jackson, 2014). The vitality of yeast cells has been reported to increase at lower temperatures as the toxic effect of ethanol and other fermentation by-products on the yeast cell are less pronounced (Jackson, 2014; Mattei & Kosseva, 2017). Also, the increased diversity of volatile metabolic byproducts (e.g., ethyl esters) has been noted at lower temperatures which, in turn, affects the complexity in sensory properties of the final product (Hutkins, 2006). With higher temperatures which improve the fermentation rate the requirements for the nitrogen sources may increase (Ribereau-Gayon et al, 2006). The application of either too low or too high temperatures may both result in an increased risk of sluggish fermentation (Ribereau-Gayon et al, 2006). As fermentation is an exothermic process, part of the energy produced by yeast during anaerobic carbon metabolism is released in the form of heat. On average, yeast release about 580 kJ of energy per kg of fermentable sugars (Kunze, 2019) which is potentially enough to raise the ambient temperature by at least 15°C. Hence, temperature control is highly recommended in larger-scale processes.

1.1.4 Post-fermentation processes in cider production

Depending on the acidity levels in cider, malolactic fermentation (MLF) might be required during which malic acid is converted into lactic acid by malolactic bacteria present in the must either as part of indigenous microflora or added as a starter culture (McKay et al, 2011; Nogueira & Wosiacki, 2012). The bacteria carrying out MLF belong to *Oenococcus, Lactobacillus, Pediococcus* and *Leuconostoc* genera (Lerm et al, 2010). *Oenococcus oeni* in particular is the most adapted to grow in the conditions that are associated with the end of alcoholic fermentation (high ethanol content, low pH) (Lerm et al, 2010).

MLF involves enzymatic conversion of dicarboxylic malic acid into monocarboxylic lactic acid. From the physiological standpoint, MLF is used by malolactic bacteria to achieve a proton motive force for the generation of ATP (Lerm et al, 2010). Three distinct enzymatic pathways have been described. The first possible pathway involves direct decarboxylation of malic acid into lactic acid by malate decarboxylase (Lerm et al, 2010). The second pathway uses malate decarboxylase to first convert malic acid into pyruvic acid which is then reduced to lactic acid by lactate dehydrogenase (Lerm et al, 2010). In the third pathway, malic acid is reduced to oxaloacetate by malate dehydrogenase, decarboxylated to pyruvate by oxaloacetate decarboxylase and then reduced to lactic acid by lactate dehydrogenase (Lerm et al, 2010). The direct decarboxylation of malic acid into lactic acid, however, is the most common enzymatic pathway that is utilized by malolactic bacteria in wine (Lerm et al, 2010). MLF process is relatively slow and can take on average 20 to 35 days, depending on the population of malolactic bacteria and environmental factors that might affect its vitality (temperature, high ethanol content, low pH and sulfur dioxide pre-treatment of the must) (Nogueira & Wosiacki, 2012).

As a result of MLF, the acidity of cider is reduced improving the sensory attributes of cider. Besides reducing the acidity, malolactic bacteria can also provide additional diversity in the composition of volatile compounds (Lonvaud-Funel, 2010; McKay et al, 2011). Diacetyl is one of the primary odor-active compounds produced by malolactic bacteria that imparts “butterscotch” aroma to the beverage. However, at concentrations

above 5 mg L^{-1}, diacetyl can become a source of off-flavor described as “rancid butter” (Krieger-Weber et al, 2010; McKay et al, 2011). Other compounds like higher alcohols (isoamylic alcohols), aldehydes (hexanal, hexenal), lactones, succinic acid esters (ethyl monosuccinate, diethyl succinate), norisoprenoids (damascenone), terpenes (terpineol, linalool, geraniol), and phenols (vanillin) have been noted to increase as a result of MLF in wines and cider (Maicas et al, 1999; Izquierdo Canas et al, 2008; McKay et al, 2011; Zhao et al, 2014; Chen & Liu, 2016).

After fermentation (either with or without MLF), the yeast usually flocculates to the bottom of the fermentation tank and is then separated through either decantation, centrifugation or filtration. However, more traditional approaches to cidermaking usually include maturation of cider on yeast lees (*sur lie*) for up to several weeks to achieve additional flavor development (Bamforth & Cook, 2019). Yeast lees refer to precipitated and partially autolyzing yeast biomass. During maturation on lees, the cell contents released after autolysis of yeast into cider affect texture and flavor of the beverage (“nutty”, “toasty” notes) (McKay et al, 2011; Bakker & Clarke, 2012). The products of yeast autolysis include amino acids, peptides (including antioxidant compound glutathione), mannoproteins, fatty acids, and volatile compounds (alcohols, aldehydes, ethyl esters, lactones, terpenes) (Guilloux-Benatier & Chassagne, 2003; Ribereau-Gayon et al, 2006; Perez-Serradilla et al, 2008; Peinado & Mauricio, 2009; Liberatore et al, 2010; McKay et al, 2011; Loira et al, 2013; Joshi et al, 2017).

After separation of yeast, cider is usually subjected to additional clarification by the addition of fining agents (gelatin, bentonite, isinglass, chitosan), sedimentation, and/or centrifugation (McKay et al, 2011; Bamforth & Cook, 2019). Fining agents are chosen based on the desired effect. Thus, gelatin forms flocs when binding to tannins and precipitates them reducing astringency (McKay et al, 2011; Berry & Slaughter, 2003; Lea & Drilleau, 2003). Bentonite can be used to remove unstable protein fractions that cause clouding, in particular at higher temperatures; gelatin/bentonite combination is noted to be very effective in terms of clarification speed and efficiency (McKay et al, 2011; Berry & Slaughter, 2003; Lea & Drilleau, 2003). Isinglass is a gelatin obtained from dried swim bladders of fish. It forms a jelly-like substance with enhanced separation of yeast cells and/or tannins; however, excess isinglass can give a “fishy” taste to the product (McKay et al, 2011; Berry & Slaughter, 2003; Lea & Drilleau, 2003). Chitosan also binds to polyphenols, proteins, and yeast, but is considered as most suitable for low-tannin ciders (McKay et al, 2011; Berry & Slaughter, 2003; Lea & Drilleau, 2003).

With few exceptions (e.g. *Guillevic* apples), cider is rarely made from a single apple variety due to difficulties and limitations in finding a perfect balance of sweetness, acidity, and tannins (Lea & Drilleau, 2003). Most of the time, certain sensory properties according to the cider style, regional traditions, cidermaker’s vision or market demands are achieved and balanced through the blending of ciders from multiple apple varieties (Downing, 1989; McKay et al, 2011; Nogueira & Wosiacki, 2012; Bamforth & Cook, 2019). Notably, blending can occur at many different points in the cidermaking process (e.g, during juice preparation, after juice preparation, before fermentation, after fermentation) (McKay et al, 2011). If regulations allow, several properties (alcohol content, sweetness, acidity) may also be adjusted manually in post-fermentation processing. If the alcohol content is too high, the product can be thinned with water or pasteurized clarified apple juice to reduce the alcohol content to desired levels (Downing, 1989; McKay et al, 2011; Nogueira & Wosiacki, 2012; Bamforth & Cook, 2019). Additional sweetness can be achieved with sugar syrup, apple juice, apple juice concentrate, or

artificial sweeteners (Downing, 1989; McKay et al, 2011; Nogueira & Wosiacki, 2012; Bamforth & Cook, 2019). Apple juice, malic acid, and/or citric acid can be used to adjust the acidity (Downing, 1989; McKay et al, 2011; Nogueira & Wosiacki, 2012; Bamforth & Cook, 2019). A preservative in the form of potassium metabisulfite can also be added if deemed necessary and allowed by regional laws and regulations (McKay et al, 2011; Nogueira & Wosiacki, 2012). After the necessary adjustments described above, ciders can be filtered, pasteurized, carbonated, and bottled (McKay et al 2011; Nogueira & Wosiacki, 2012). Carbonation can be achieved either by secondary fermentation (*méthode traditionnelle*) or artificial gasification with pressurized carbon dioxide (Ribereau-Gayon et al, 2006).

1.2 Sensory properties of cider

The sensory properties of any given food product are a primary deciding factor in terms of consumer acceptance and overall quality (Muñoz et al, 1996). There are no clear definitions available on the acceptable sensory properties of cider. The properties can vary significantly based on regional differences in production. This can be explained by the fact that properties like color, sweetness, and bitterness depend on the varieties of apples used (Santos et al, 2016). The residual sweetness of the product also depends on the fermentation activity of yeast and duration of fermentation (Rodríguez-Bencomo et al, 2012).

Most compounds that define the perceived properties of cider are formed as yeast metabolites. Volatile compounds synthesized by yeasts are of particular importance as they constitute odor and flavor perception of the final product (Meilgaard et al, 2007; Duizer & Field, 2015). In addition to the main secreted metabolites of alcoholic fermentation – ethanol and glycerol, the compounds formed as a result of alcoholic fermentation can include but are not limited to organic acids (e.g., butanoic acid, octanoic acid), various carbonyl compounds (e.g., 3-octanone, phenylacetaldehyde), higher alcohols (e.g., 3-methyl-1-butanol, 2-phenylethanol), sulfur compounds (e.g., hydrogen sulfide, dimethyl sulfide), and esters (e.g., ethyl acetate, ethyl butanoate) (Ugliano & Henschke, 2009; Liu, 2012; Rodríguez-Bencomo et al, 2012; Joshi & Attri, 2017; Rupasinghe et al, 2017). The latter constitute the focus of this thesis and their synthesis by yeast is described in detail in the following chapters.

1.3 Ester synthesis in yeast

Esters impart fruity and floral characteristics to sensory properties of fermented beverages (Ugliano & Henschke, 2009; Saerens et al 2010; Aranda et al, 2011; Jackson, 2014; Darriet & Pons, 2017; Rupasinghe et al, 2017). They are produced by yeast during fermentation and can be divided into two distinct groups – acetate esters and medium-chain fatty acid (MCFA) ethyl esters – depending on their synthesis pathways (Figure 2, Table 3) (Saerens et al, 2010).

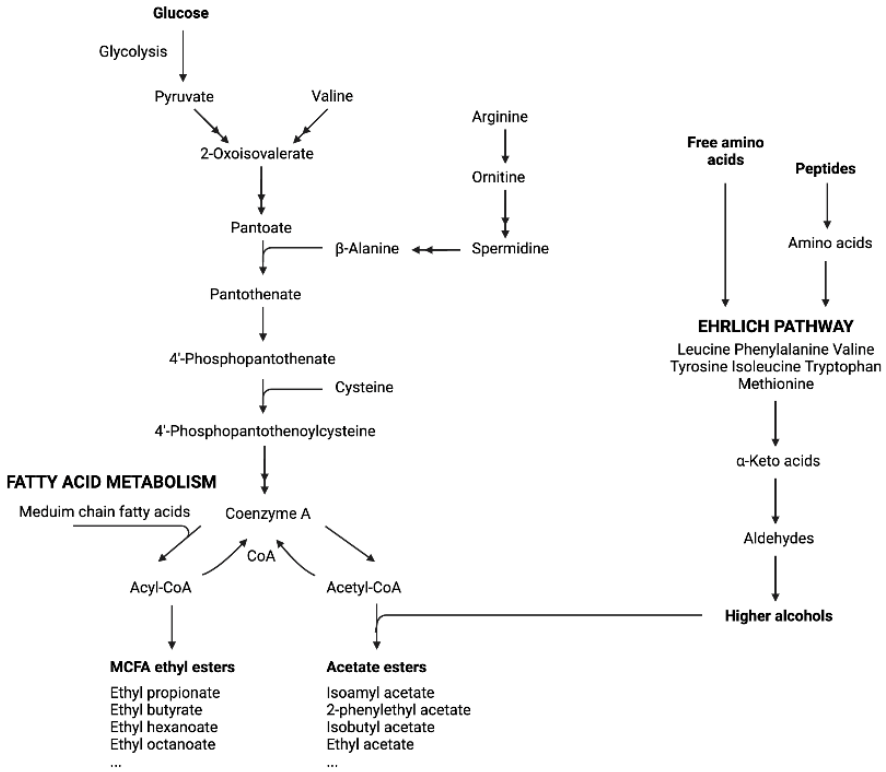


Figure 2. *Metabolic pathways of esters synthesis in a yeast cell (adapted from Boss et al (2015) with permission of MDPI)*

Fatty acid ethyl esters are formed by enzymatic esterification (medium-chain fatty acid ethyl ester synthases/esterases Eht1p and Eeb1p) of various medium-chain fatty acids (e.g., butyric acid, hexanoic acid, octanoic acid) with ethanol (Ugliano & Henschke, 2009; Bakker & Clarke, 2012; Carballo, 2012; Jackson, 2014; Rupasinghe et al, 2017). Fatty acids can originate from the extracellular supply, protein/lipid turnover or synthesized *de novo* (Tehlivets et al, 2007). Fatty acids are synthesized by the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (Acc1) (Tehlivets et al, 2007; Aranda et al, 2011; Carballo, 2012; Jackson, 2014). Malony-CoA then enters a series of reactions regulated by fatty acid synthases (Fas1 and Fas2), fatty acid acyl-CoA synthases (Faa1, Faa4) and elongases (ketoacyl-CoA synthases; Elo1, Elo2, Elo3) (Tehlivets et al, 2007; Aranda et al, 2011; Carballo, 2012; Jackson, 2014). A metabolic pathway of fatty acid ethyl ester formation with ethyl butanoate as an example is provided in Figure 3.

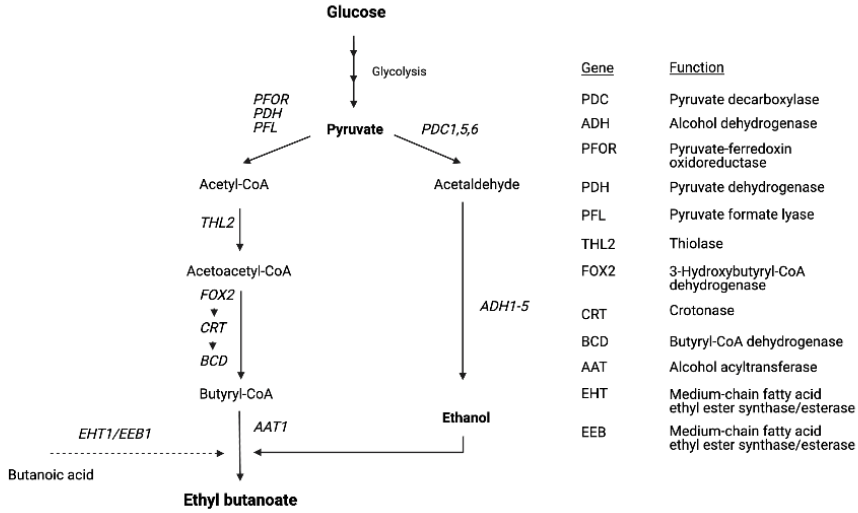


Figure 3. *Complete metabolic pathway of ethyl butanoate synthesis (adapted from Xu et al (2020) with permission of John Wiley & Sons)*

Acetyl esters are formed in the reaction of acetyl-CoA with alcohols (ethanol and higher alcohols) catalysed by acetyltransferases Atf1p and Atf2p (Lambrechts & Pretorius, 2000; Berry & Slaughter, 2003; Abbas, 2006; Ugliano & Henschke, 2009; Bakker & Clarke, 2012; Carballo, 2012; Rupasinghe et al, 2017; Molita et al, 2017). Higher alcohols originate either from extracellular environment or from the conversion of amino acids (Ehrlich pathway, Figure 4) or synthesized *de novo* from sugars (Aranda et al, 2011). Ehrlich pathway involves catabolism of amino acids (leucine, isoleucine, valine, phenylalanine, methionine, tyrosine, tryptophan) through three enzymatic reactions – transamination into corresponding α-keto acids, decarboxylation into corresponding aldehydes, and reduction of aldehydes into corresponding alcohols (Figure 4) (Messenguy et al, 2006; Ribereau-Gayon et al, 2006; Hazelwood et al, 2008). As mentioned above, higher alcohols can alternatively be synthesized with no involvement of amino acids, i.e. from keto acids that derive from glucose metabolism (Ribereau-Gayon et al, 2006). For example, isobutanol can be obtained from 2-ketoisovalerate instead of valine (Figure 5) (Chen et al, 2011; Kondo et al, 2012). A complete metabolic pathway of acetate ester formation with phenylethyl acetate as an example is provided in Figure 6.

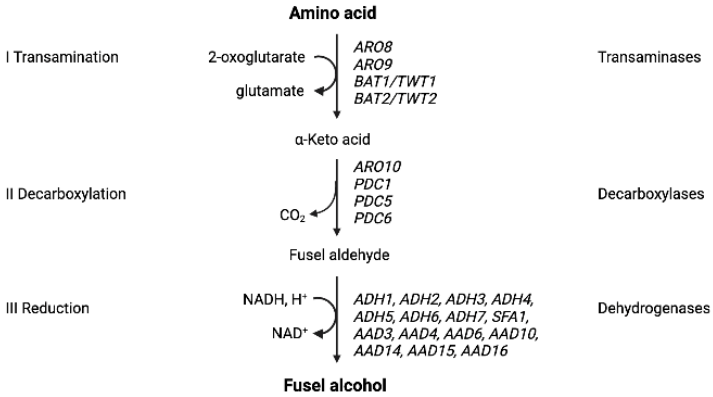
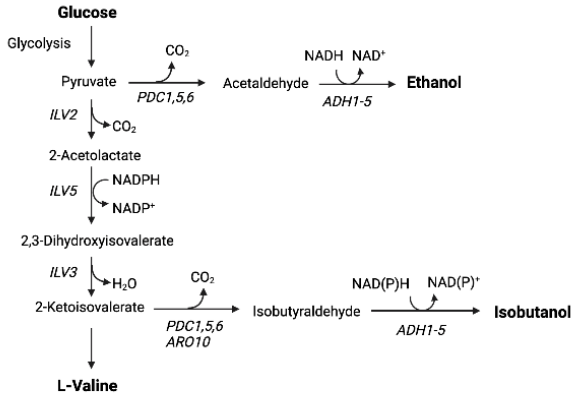


Figure 4. *Formation of higher alcohols in Ehrlich pathway (adapted from Hazelwood et al (2008) with permission of American Society of Microbiology)*



Gene	Function
ILV2	Acetolactate synthase
ILV5	Acetohydroxyacid reductoisomerase
ILV3	Dihydroxyacid dehydratase
PDC	Pyruvate decarboxylase
ARO10	Phenylpyruvate decarboxylase
ADH	Alcohol dehydrogenase

Figure 5. *Formation of isobutanol from pyruvate in S. cerevisiae (adapted from Kondo et al (2012) with permission of Elsevier and Wess et al (2019) with permission of Springer Nature)*

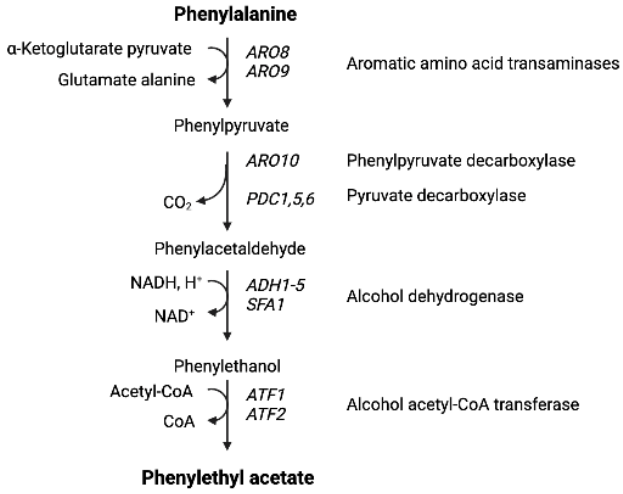


Figure 6. *Complete metabolic pathway of phenylethyl acetate synthesis (adapted from Kim et al (2013) with permission of John Wiley & Sons and Holt et al (2019) with permission of Oxford Academic Press)*

Table 3. *Examples of ethyl and acetate esters synthesized by yeast with their odor descriptions and metabolic precursors*

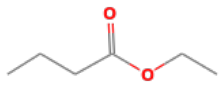
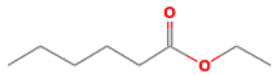
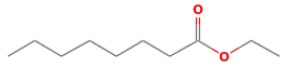
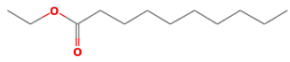
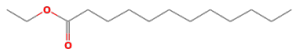
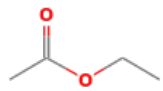
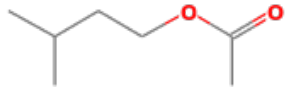
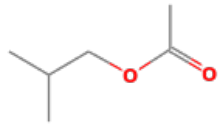
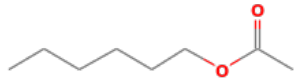
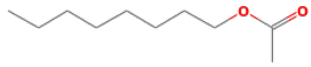
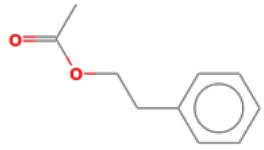
Ester	Structure[a]	Odor description[b]	Precursors
Ethyl esters			
Ethyl butyrate		Fruity, pineapple, cognac	Butanoic acid
Ethyl hexanoate		Fruity, pineapple, waxy, green	Hexanoic acid
Ethyl octanoate		Fruity, wine, apricot, banana	Octanoic acid
Ethyl decanoate		Waxy, fruity, apple, grape	Decanoic acid
Ethyl dodecanoate		Waxy, floral, soapy	Dodecanoic acid

Table 3 (cont.). *Examples of ethyl and acetate esters synthesized by yeast with their odor descriptions and metabolic precursors*

Acetate esters			
Ethyl acetate		Ethereal, fruity, green	Acetyl-CoA + Ethanol (Nordström, 1962)
Isoamyl acetate		Fruity, banana	Isoamyl alcohol, leucine, valine (Eden, et al, 1996; Plata et al., 2003)
Isobutyl acetate		Fruity, ethereal, banana	Isobutanol, valine (Nordström, 1964; Stribny et al, 2015)
Hexyl acetate		Fruity, green apple, banana	C6 alcohols/aldehydes (Dennis et al, 2012)
Octyl acetate		Green, earthy, mushroom	Octanol (Dennis et al, 2012)
2-Phenylethyl acetate		Floral, rose, honey	Phenylethyl alcohol, phenylalanine (Yoshioka & Hashimoto, 1981; Scharpf et al, 1986; Dickinson et al, 2003)

[a]NIST database (US Department of Commerce; Gaithersburg, MD, USA)
[b]based on www.goodscentscompany.com database

1.4 Ester synthesis management during cider production

The choice of a yeast strain can have a significant impact on the volatile composition of cider. *Saccharomyces* sp. are known as good producers of esters and higher alcohols during fermentation (Ugliano & Henschke, 2009; McKay et al, 2011). The concentration of these compounds, however, depends on the particular strain used in the fermentation (Ugliano & Henschke, 2009; McKay et al, 2011). For example, a strain of *S. bayanus* was shown to produce higher concentrations of higher alcohols (isoamyl alcohols, isobutanol,

2-methyl-1-butanol) and various esters (ethyl 2-methyl butyrate, ethyl 3-methyl butyrate, isoamyl acetate, ethyl hexanoate) while the strains of *S. cerevisiae* synthesized more of ethyl octanoate and ethyl hexanoate in icewines (Synos et al, 2015).

In addition to *Saccharomyces* sp., the strains of non-*Saccharomyces* species can contribute substantially to the sensory properties of cider. For example, *Kazachstania gamospora* in wine fermentation produced a much higher concentration of esters compared to *Saccharomyces cerevisiae* (Becker Whitener *et al* 2014). Similarly, the strains of *Hanseniaspora* sp. produced high amounts of acetic acid, acetate esters, and acetoin (Ugliano & Henschke, 2009; McKay et al, 2011; Rodriguez Madrera et al, 2015). *Pichia kluyveri* has been patented as a yeast which imparts "banana" and "pear" characteristics to fermented beverages as a result of isobutyl acetate, isoamyl acetate, hexyl acetate, ethyl propanoate, and ethyl valerate formation (Chr Hansen AS, 2015). *Metchnikowia pulcherrima* produces high concentrations of ethyl acetate, ethyl octanoate, 2-phenylethyl alcohol, and diacetyl which contribute to "fruity", "floral", and "creamy" notes (Ugliano & Henschke, 2009).

Apart from yeast strain, YAN content and composition is a major factor that has an impact on ester production by yeasts. YAN supplementation into the nitrogen-deficient grape and apple musts has proven to be efficient in supporting esters' production in multiple studies. Seguinot *et al* (2018) have found that nitrogen supplied in the form of diammonium hydrogen phosphate (DAP) was efficient in inducing acetate esters' production in cider. A similar effect was described by Garde-Cerdan *et al* (2008), Ugliano *et al* (2010), and Torrea *et al* (2011) in wine fermentation. The combined supplementation of apple must with some of the amino acids (glutamate, aspartate) in the study by Santos *et al* (2016) significantly increased the amount of both ethyl and acetate esters produced during fermentation. Linear correlation between added amino acid precursors (leucine, isoleucine, phenylalanine, threonine) and the corresponding esters (isoamyl acetate, 2-phenylethyl acetate, ethyl acetate) was demonstrated by Fairbairn *et al* (2017) in a synthetic grape must. The linear relationship, however, lost its predictability once the individual amino acids used in the study were supplemented in a mix (Fairbairn et al, 2017).

The effects of timing of YAN supplementation on esters' composition have also been explored. Seguinot *et al* (2018) compared the effect of supplementation of DAP and a mix of 19 amino acids either at the start of alcoholic fermentation or the onset of the stationary phase in fermentations with synthetic media. The results suggested that YAN supplementation was efficient regardless of the supplementation timing and source, with a more pronounced effect on acetate esters than ethyl esters. Similar conclusions were also reached by Beltran *et al* (2005).

Supplementation with fatty acids has also proven to be beneficial for the improvement of esters' synthesis. Thus, supplementation of synthetic grape must with unsaturated fatty acids (linoleic, oleic and linolenic) was shown by Duan *et al* (2015) to improve overall yeast vitality and fermentative activity. A positive correlation of supplemented unsaturated fatty acids with MCFA esters (ethyl hexanoate, ethyl octanoate) and some acetate esters (2-phenylethyl acetate, isoamyl acetate) was also found (Duan et al, 2015). A similar effect on ethyl hexanoate and ethyl octanoate was noted in supplementation of synthetic must with respective medium-chain fatty acids (hexanoic acid, octanoic acid) (Kong et al, 2021). The supplementation with unsaturated fatty acids was further explored by Liu *et al* (2019) in red wine fermentations. There, however, the efficiency of supplementation was shown to depend on the concentrations

used – concentrations 120 mg L^{-1} and higher had an inhibitory effect on both yeast performance and ester synthesis (Liu et al, 2019).

The dissolved oxygen content of the must has also been shown to affect esters' production by yeast. Thus, aeration of synthetic growth media suppressed the formation of acetate esters in the studies conducted by Fujii *et al* (1997) and Rojas *et al* (2001). This can be related to repression of genes encoding acetyltransferases (*ATF1*, *ATF2*) (Fujii et al, 1997; Plata et al, 2005). On the other hand, lack of aeration has been related to a decrease in fatty acid esters production due to inhibited cellular growth (Bardi et al, 1999; Valero et al, 2002).

Fermentation temperature has also been noted to affect both qualitatively and quantitatively esters' composition in fermented beverages. Lower temperatures reduce fermentation rate and prolong the viability of the yeast culture, allowing formed esters to accumulate in the must during fermentation (Downing, 1989; Torija et al, 2003; Jackson, 2014; Joshi & Attri, 2017). Lower temperatures have been reported to favorably impact the formation of ethyl esters (ethyl butanoate, ethyl hexanoate) (Molina et al, 2007; Beltran et al, 2008; Reddy & Reddy, 2011). The difference is said to be attributed to the higher expression of *EHT1* gene as well as the improved activity of the esterases (Eht1p, Eeb1p) related to ethyl ester production in yeast (Molina et al, 2007). On several occasions, nevertheless, it has been reported that higher fermentation temperatures favor the formation of certain long-chain fatty acid esters (ethyl octanoate, ethyl decanoate) (Killian & Ough, 1979; Molina et al, 2007; Saerens et al, 2008b). Higher fermentation temperatures, however, can also be associated with evaporation losses of volatile metabolic byproducts (Killian & Ough, 1979). Higher temperatures might also induce hydrolysis of synthesized esters reducing their accumulation in the product (Ramey & Ough, 1980; Jackson, 2014; Molina et al, 2017).

Technological advances in cider production have largely relied on the innovations from other fermented beverage industries, in particular wine industry. However, as seen in Table 2, apple juice and grape juice are significantly different in terms of their composition. Thus, the technological procedures currently available to improve the production of esters in wines may not be that easily transferred to cider fermentation. Additional insight needs to be obtained into the impact of cider fermentation practices to expand our knowledge on the development of esters with characteristics of apple juice in mind.

2 The Aims of this Dissertation

The main objective of this thesis was to explore how different cidermaking conditions affect the quality of cider in terms of ester production by yeast.

The specific goals were as follows:

- to study the effect of apple variety, ripening stage, and yeast strain on the volatile composition of cider (Publication I);
- to assess yeast performance in esters' production with YAN supplementation as one of the main tools for ester synthesis management (Publications II and Supplementary study);
- to determine the effects of juice pre-treatment (clarification and concentration) on cider fermentation and the composition of volatile esters (Publication III);
- to observe simultaneous effects of asparagine supplementation and oxygen availability on the production of higher alcohols and esters by yeast (Supplementary study).

3 Materials and Methods

The following sections are provided to make the materials and methods easily accessible to the readers. The information on each method employed and the materials used is also available in individual publications (Appendix 1, Appendix 2, Appendix 3).

3.1 Cider fermentation experiments

In Publications I-III, small-scale cider fermentations were performed. For that, the juice (400 mL) was distributed into sterile 500 mL fermentation bottles. Each bottle was inoculated (5 x 10^6 CFU mL^{-1}) with a chosen rehydrated yeast starter culture. An overview of the starter cultures used in the studies is provided in Table 4. An overview of the apple cultivars used in Publication I is provided in Table 5. An overview of the apple juices used in Publications II and III is provided in Table 6.

Table 4. *Yeast strains used in Publications I-III for cider fermentation*

Yeast strains	**Description**	**Study**
Torulaspora delbrueckii	non-*Saccharomyces* yeast for white and red wines	as Biodiva (BD) in Publication I as Y1 in Publication II
Saccharomyces bayanus	Sparkling and dessert wine yeast	as C1108 in Publication I
S. bayanus	White wine yeast	as QA23 Publication I
S. bayanus	Wine yeast for difficult fermentation conditions	as Y3 in in Publication II as Y1 in Publication III
S. bayanus	Sparkling wine yeast	as Y4 in Publication II as Y3 in Publication III
Saccharomyces cerevisiae	Champagne yeast	as EC1118 in Publication I
S. cerevisiae	Wine yeast for a broad range of fermentation conditions	as OKAY in Publication I
S. cerevisiae	Wine yeast with killer factor	as OPALE in Publication I Y5 in Publication III
S. cerevisiae	White wine yeast selected through directed breeding	as Y2 in Publication II as Y6 in Publication III
S. cerevisiae	Wine yeast selected through evolutionary adaptation	as Y5 in Publication II as Y4 in Publication III
S. cerevisiae	White wine yeast	as Y2 in Publication III

Table 5. *Apple varieties used in Publication I for cider fermentation*

	Fructose, %	**Glucose, %**	**Malic acid, %**
Unripe Melba	4.5 ± 0.1	0.5 ± 0.1	1.2 ± 0.1
Ripe Melba	5.3 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
Overripe Melba	6.3 ± 0.1	1.2 ± 0.1	0.8 ± 0.1
Unripe Antei	5.9 ± 0.1	1.8 ± 0.1	0.8 ± 0.1
Ripe Antei	6.5 ± 0.1	1.8 ± 0.1	0.6 ± 0.5
Overripe Antei	6.9 ± 0.1	2.2 ± 0.1	0.5 ± 0.1
Unripe Orlovski Sinap	5.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1
Ripe Orlovski Sinap	6.4 ± 0.1	1.7 ± 0.1	0.8 ± 0.1
Overripe Orlovski Sinap	6.9 ± 0.1	2.0 ± 0.1	0.5 ± 0.1
Unripe Kulikovskoye	4.9 ± 0.1	1.1 ± 0.1	0.7 ± 0.1
Ripe Kulikovskoye	6.4 ± 0.1	1.3 ± 0.1	0.5 ± 0.1
Overripe Kulikovskoye	6.5 ± 0.1	1.6 ± 0.1	0.4 ± 0.1

Table 6. *Apple juices used in Publications II and III for cider fermentation*

	ºBrix	**pH**	**TA*, g L^{-1}**	**YAN, mg·N L^{-1}**
Commercial juice 1 (UK)	12.8	3.28	6.7 ± 0.1	69.48 ± 2.00
Commercial juice 2 (Germany)	11.7	3.18	3.8 ± 0.1	51.21 ± 1.84
Ripe Antei (freshly pressed)	9.8	3.38	3.93 ± 0.1	76.13 ± 7.29**
Ripe Antei (pre-treated)	10	3.45	3.97 ± 0.1	87.91 ± 9.62**

*As malic acid equivalents

**Free amino nitrogen only

In Publication II where nitrogen supplementation was studied, yeast assimilable nitrogen (YAN) content in both commercial apple juice concentrates used for fermentation was brought to a similar content of 80 ± 2 mg·N L^{-1} by using inactive yeast based fermentation nutrient Fermaid K (Lallemand Inc.). In Publication III where the effects of juice pre-treatment (clarification and concentration) on cider fermentation were studied, three nutritional strategies were applied: i) w/o nutritional supplementation (control); ii) 9 g hL^{-1} of DAP as inorganic nitrogen supplement, and iii) 40 g hL^{-1} of inactive yeast based fermentation nutrient Fermaid O (Lallemand Inc, Montreal, Canada).

Inoculated bottles were sealed using screw caps with septums pierced with syringe needles (19G x 1 ½, 1.1 x 40 mm; Terumo Medical Corporation) coupled with microfilters to vent carbon dioxide. Fermentations were carried out at 18 ± 1 °C (Publications I, II, and III) and 30 ± 1 °C (Publication III) by following carbon dioxide dissipation (mass loss) every 24h. Fermentations were considered completed when the weight of fermentation bottles remained constant for three consecutive days. All fermentation experiments were conducted in duplicates.

In the supplementary study, larger-scale cider fermentations were performed to study simultaneous effects of asparagine supplementation and oxygen availability on the production of higher alcohols and esters. For that, a method previously described by Guichard *et al* (2019) was adapted. Homemade reactors (16 L of total volume) under inert atmosphere of nitrogen (Linde gas, Saint-Priest, France) were used. Each reactor was filled with 10 L of a blend of apple musts from local orchards of Brittany, France (ca 80 mg·N L^{-1} FAN). Three nutritional strategies were used: i) no additional asparagine

supplementation (control), ii) supplementation with asparagine (Sigma Aldrich; Saint Louis, MO, USA) to 140 mg·N L^{-1} FAN, and iii) supplementation with asparagine to 200 mg·N L^{-1} FAN. Two dissolved oxygen levels in the must were used at the start of fermentation: i) no dissolved oxygen in the juice (0 mg L^{-1}) achieved with N_2 flushing and ii) saturation with air to 12 mg L^{-1} of dissolved oxygen. The strain of *Saccharomyces uvarum* isolated from French ciders by INRA (French National Institute for Agronomical Research) was used. The pre-culture (10^8 CFU mL^{-1}) was grown in stirred flasks with YPG medium (12.5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 20 g L^{-1} glucose) for 72 h at 20 °C. The yeast was then centrifuged, rinsed with apple juice and resuspended for inoculation. The yeast was inoculated at 5 x 10^5 CFU mL^{-1}. The fermentations were carried out at 10 °C by following the change in density measurements. The fermentations were terminated once the density had reached 1010 g L^{-1} from the initial 1060 g L^{-1}. Depending on the fermentation rate, sampling was done either every day or every other day. All fermentation experiments were performed in triplicates.

3.2 Apple juice clarification and concentration

In Publication III, a concentrate was prepared from apple juice to assess the effects of juice pre-treatment (clarification and concentration) on cider fermentation kinetics and the production of volatile esters by yeast. Apple juice from ripe Antei apples (150 L) (9.80 °Brix; pH 3.38; titratable acidity 3.93 g L^{-1} in malic acid equivalent) was used. Half of the juice (75 L) was clarified and concentrated. Clarification was based on the method described by Grampp *et al* (1978). The juice was heated to 40 °C and 5 g hL^{-1} of commercial pectinase (Rapidase; Oenobrands SAS, France) and 5 g hL^{-1} of glucoamylase (AMG 300L; Novozymes, Denmark) were added. After 30 minutes at 40 °C, 100 mL hL^{-1} of silica sol (Baykisol® 30; E. Begerow GmbH & Co., Germany) and 10 g hL^{-1} of dissolved gelatin (SIHA Clarifying Gelatine; E. Begerow GmbH & Co., Germany) were added as flocculants. Flocculation occurred within 1.5 hours at 40 °C. Clarified juice was filtered and concentrated using a vacuum concentration system (Didacta Italy; Fruit juice and syrup line, code 640022). The following pressure and temperature programs were used: 60 °C at -60 cmHg (beginning), 40 °C at -70 cmHg (mid-point) and 30°C at -70 cmHg (end). Due to volume restrains, the process was performed in three batches of 25 L. Each batch was concentrated separately from 9.8 to 30 °Brix. The batches were then pooled and concentrated to the final value of 67.8 °Brix. For fermentations, the concentrate was diluted with distilled water to 10 °Brix (pH 3.45; titratable acidity 3.97 g L^{-1} in malic acid equivalents).

3.3 Analytical procedures

3.3.1 Yeast assimilable nitrogen (YAN) content

In Publication II, the YAN content at different stages of fermentation was measured using Dinitrofluorobenzene (DNFB) method to assess the rate of nitrogen consumption by yeast. The method is based on the reaction of amino groups of free amino acids and small peptides with 2,4-dinitrofluotobenzene. The reaction products are measured by spectrophotometry at 420 nm. Standard solutions of glycine with known nitrogen content were used to obtain calibration curve. Results were expressed as mg·N L^{-1}. Three analytical replicates were used for each sampling point.

3.3.2 Amino acid content

In Publication III, free amino acids were measured in the juice before and after concentration. In the supplementary study, free amino acids were measured before and after fermentation to assess their consumption by yeast. The measurements were done with UPLC (Acquity UPLC; Waters Corp., Milford, MA, USA) equipped with AccQ·Tag Ultra column and a UV detector. Before analysis, the samples were diluted 1:2 with MilliQ, filtered (Whatman Spartan 13; Dassel, Germany) and derivatized using AccQ·Tag reagent. AccQ·Tag Ultra eluent B (linear gradient from 0% to 100%) was used as mobile phase at a flow rate of 0.3 mL min^{-1}. Three analytical replicates were measured for each sampling spoint.

3.3.3 GC-MS analysis of volatile compounds

In Publications I-III, volatile compounds were measured across the samples by GC-MS. In Publication I, a full volatile profiling was performed; in Publications II-III and in the supplementary study, only selected esters and their alcohol precursors were analyzed.

In Publications I-III, the samples were diluted 1:19 with distilled water into a 20 mL vial; 2-chloro-6-methylphenol (100 µg L^{-1}) was added as an internal standard (IS) for semi-quantitation. Volatile compounds were extracted by using solid-phase microextraction (DVB/Car/PDMS 30/50 µm Stableflex, 2 cm; Supelco, Bellefonte, PA, USA) at 45 °C for 20 minutes. Quantification of volatile compounds was performed using gas chromatography system (6890N; Agilent Technologies, Santa Clara, CA, USA) equipped with mass spectrometer (GCT Premier TOF; Waters, Milford, MA, USA) and a DB5-MS column (30 m × 0.25 mm × 1.0 µm; J&W Scientific, Folsom, CA, USA). Helium was used as a carrier gas with 1.0 mL min^{-1} flow. The oven was programmed to ramp up from 40 °C at a rate of 7.5 °C min^{-1} to a final temperature of 280 °C with an additional holding time of three minutes (total run time 35 min). Mass spectra were obtained at ionization energy of 70 eV within a mass scan range of 35 to 350 m/z. Three analytical replicates were used for each sample. In Publications II and III, analytical standards were used for the accurate identification of selected esters. The concentrations were expressed as µg L^{-1} in IS equivalents.

In the supplementary study, a method previously used by Guichard *et al (2019)* was adapted for the measurements. 10 mL of cider sample was gathered into a 20 mL vial pre-flushed with carbon dioxide and containing 3 g of NaCl; 2-ethylhexanol (20 mg L^{-1}) was added as an internal standard. Volatile compounds were extracted by using solid-phase microextraction (Car/PDMS 85 µm Stableflex, 2 cm; Supelco, Bellefonte, PA, USA) at 35 °C for 20 minutes. The volatiles of interest were measured using GC-MS QP2010S system (Shimadzu; Kyoto, Japan). The system was equipped with a BP20 polar capillary column (30 m × 0.25 mm × 0.50 µm; VWR International, Radnor, PA, USA). Helium was used as a carrier as with 1.0 flow. Samples were injected in split mode with a 1:30 ratio. The oven was programmed to ramp up from 30 °C to 40 °C at a rate of 4 °C min^{-1}, from 40 °C to 180 °C at a rate of 8 °C min^{-}1, from 180 °C to a final temperature of 250 °C at a rate of 15 °C min^{-1} with an additional holding time of ten minutes (total run time 34.67 min). Mass spectra were obtained at ionization energy of 70 eV within a mass scan range of 30 to 300 m/z. Correct identification was performed using analytical standards and reference ions for each compound of interest. Three analytical replicates were used for each sampling point. The results of quantitation were expressed in absolute concentrations as mg L^{-1}.

3.3.4 Cell count and yeast viability

In the supplementary study, growth of yeast population and its viability was monitored during cider fermentation by using methods previously described by Guichard *et al* (2019). Total cell count was measured in a flow cytometer (CyFlow Cube 6; Sysmex Partec GmbH, Goerlitz, Germany). Yeast viability was assessed by comparing plate count on agar media with total cell count. For *Saccharomyces uvarum*, a following selective agar media was used: 1.7 g L^{-1} of Difco™ Yeast Nitrogen Base + 0.94 g L^{-1} of L-asparagine + 20 g L^{-1} of D-(+)-galactose + 20 g L^{-1} of Bacto™ Agar.

3.3.5 Sensory analysis

In Publication II, descriptive analysis was used to assess the sensory properties of the cider samples. A sensory panel of 8 assessors with previous experience in working with cider samples carried out the analysis. There was no pre-session to familiarize assessors with the samples. The working linear scale was established at 0-15, and relative intensities were used. A complete list of assessed attributes and their definitions is provided in Table 7. Samples were assessed independently by each assessor. Before the assessment, all samples were adjusted for sweetness to balance out the sour taste since the secondary malolactic fermentation was not carried out. For that, 3% of diluted sucrose was added. All samples were encoded with a randomized three-digit numerical key.

Table 7. *A complete list of sensory attributes and their definitions*

Attribute	**Description**
Odour	
Overall intensity	Overall strength of the perceived odour
Fruity	Strength of all fruity odours (excluding apple)
Cooked apple	Strength of odours characteristic to cooked apples
Apple-like	Strength of odours characteristic to fresh apples
Sweet	Strength of all sweet odours
Sour	Strength of all sour odours
Taste	
Fruity	Strength of overall sensation characteristic to fruits (excluding apples)
Cooked apple	Strength of overall sensation characteristic to cooked apples
Apple-like	Strength of overall sensation characteristic to fresh apples
Sweet	Strength of overall sweet sensation
Sour	Strength of overall sour sensation
Bitter	Strength of overall bitter sensation
Astringency	Strength of overall drying sensation

3.3.6 Data processing and statistical analysis

In Publication I, the results of GC-TOF-MS analysis were statistically evaluated by partial least square discriminant analysis (PLS-DA) (mixOmics package, R software 3.4.0; Boston, MA, USA). The correlation of cider samples with identified volatile compounds was observed using principal component analysis (PCA) (factoextra package, R software 3.4.0; Boston, MA, USA). Before the application of PCA and PLS-DA, the quantitation results

were autoscaled. The analysis of variance (ANOVA) was performed using R software 3.4.0 (Boston, MA, USA), and $p<0.05$ was considered statistically significant.

In Publication II, the results of chemical analysis were averaged across biological and analytical replicates. The analysis of variance (ANOVA) was performed using R software 3.4.0 (Boston, MA, USA), and $p<0.05$ was considered statistically significant. The results of sensory analysis were statistically evaluated by PCA (OriginPro software; OriginLab, Northampton, MA, USA). Prior to the application of PCA, the results were autoscaled.

In Publication III, the results of chemical analysis were averaged across biological and analytical replicates. ANOVA was performed using R software 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria) and $p < 0.05$ was considered statistically significant. The results of GC-MS analysis were evaluated by PLS-DA using R package 'mixOmics' 6.11.33 and presented as biplots. Response variable was constructed from a combination of juice type and nutritional supplement. Prior to the application of PLS-DA, the quantitation results were autoscaled.

In the supplementary study, the results of chemical analysis were averaged across analytical replicates. R software 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria) was used for data visualization.

4 Results and Discussion

A summary of the results is presented in this section. More detailed discussions on these results can be reached in publications' transcripts in Appendix 1, Appendix 2, and Appendix 3.

4.1 The effects of apple variety, ripening stage, and yeast strain on the volatile composition of apple cider

Publication I examined the degree of influence of apple variety, apple ripening stage, and yeast strain on the volatile composition of apple cider. Four apple varieties grown in South Estonia in a private orchard in Valgjärve (Valga County) were selected for the study – Antei, Melba, Kulikovskoye, and Orlovski Sinap. The apples were first harvested at the unripe stage (0 weeks, starch index 1) of their maturity and left to ripen at +4 °C. The ripe apples (starch index 3) were collected after 2-8 weeks (depending on the variety: Melba – 2 weeks, Kulikovskoye – 3 weeks, Antei – 6 weeks, and Orlovski Sinap – 8 weeks) in storage, and the overripe (starch index 5) – after 6-12 weeks (approx. one month after the ripe stage: 6, 8, 10 and 12 weeks, respectively). The must from the apples at various stages of ripening (unripe, ripe, overripe) underwent alcoholic fermentation using commercially available yeast strains (Table 4). Gas chromatography – mass-spectrometry was employed to assess the differences in volatile composition between the samples.

In total, 37 volatile compounds were identified in the cider samples (Table 6). Partial least square-discriminant analysis (PLS-DA) was applied to evaluate the influence of each treatment (apple variety, maturity level, and yeast strain) on the volatile composition of the samples. For that, one treatment at a time was taken as a predicted variable with the other two acting as replicates. The best visual separation of the samples was achieved when using apple variety as a predicted variable (Figure 7).

Based on the grouping according to the apple variety, ciders produced with Melba apples were found to have similar volatile profiles due to the proximity of the samples on the plot. This may indicate either a specific dominant aroma profile of Melba variety making it difficult to influence the cider by apple maturity level and different yeast strains or a lack of certain nutrients (e.g., amino acids) acting as metabolic precursors of the analyzed volatile compounds. By contrast to the ciders made with Melba variety, Antei variety showed a wide spread of the samples on the plot allowing additional subclustering based on the maturity level of apples – the samples made with unripe apples formed a clear subcluster. The clusters formed by the samples made with Kulikovskoye and Orlovski Sinap varieties overlapped due to similarities in volatile composition.

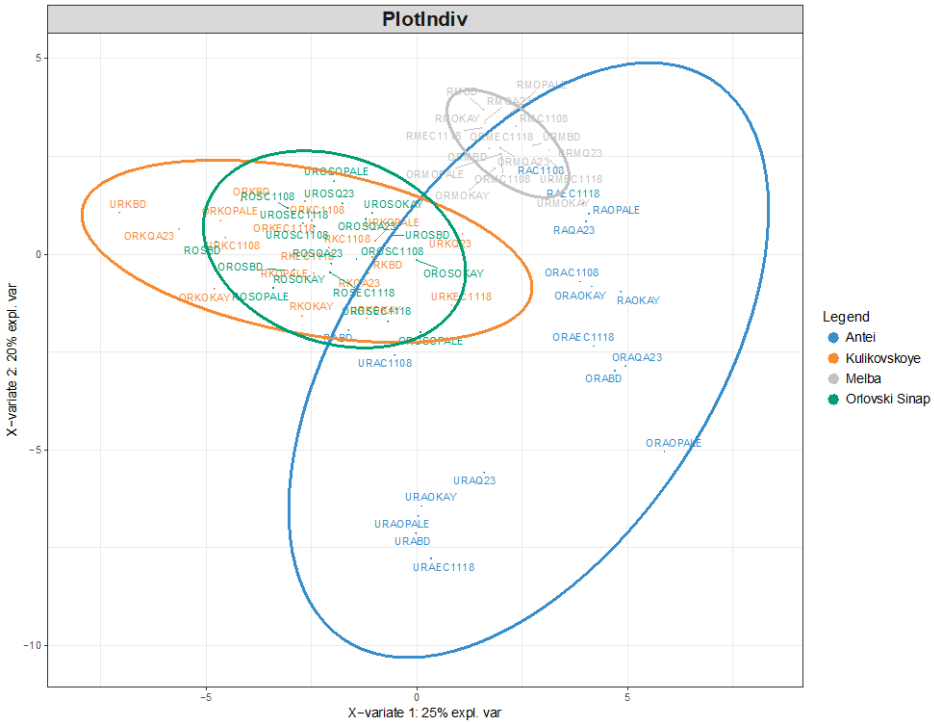


Figure 7. *PLS-DA plot for the cider samples. Samples made with Antei apples are represented with color blue, Kulikovskoye – orange, Melba – gray, Orlovski Sinap – green. Samples are coded according to the preparation: maturity level (UR – Unripe, R – Ripe, OR – Overripe) – apple cultivar (A – Antei, M – Melba, OS – Orlovski Sinap, K – Kulikovskoye) – yeast strain. Thus, sample coded ROSOKAY, for example, represents a cider made with ripe Orlovski Sinap apples and fermented with OKAY commercial yeast strain*

To evaluate the correlations between volatile compounds and apple variety, principal component analysis (PCA) was carried out (Figure 8). According to the PCA biplot, ciders from Melba apple variety had the least diverse volatile composition; the samples formed a tight cluster with a substantial similarity in the volatile profiles which corresponds to the results obtained using PLS-DA approach. Ciders made with Kulikovskoye, and Orlovski Sinap varieties had similar but the most diverse volatile composition with different alcohols, aldehydes, and esters contributing to the volatile composition. As per PLS-DA results, subclustering of the samples made with unripe Antei apples was also observed on the upper left corner of Figure 6. These samples had higher contents of acetate esters (isoamyl acetate, hexyl acetate, and butyl acetate), ethyl hexanoate, octanal, 1-octen-3-ol, and 1-octanol. The influence of apple variety, ripening stage, and yeast on the relative content of identified volatile compounds was further observed using p-values (Table 8). According to the results, most of the compounds that distinguished the samples from one another were associated with apple variety, which corresponded to the conclusions made based on the results of PLS-DA. Most of the alcohols identified in the samples, some esters (butyl acetate, ethyl-3-methylbutanoate, and isoamyl acetate), 3-methylbutanoic acid, 3-octanone, benzaldehyde, and phenylacetaldehyde were associated with the maturity stage of the apples used in processing. The mentioned compounds could have either originated from the biochemical changes during ripening or formed from specific precursors developed during ripening (e.g., 3-methylbutanol can

be utilized as a precursor in the formation of isoamyl acetate) (Eden, et al., 1996; Osorio et al., 2013).

The results of this study indicate that the apple variety can have a significant influence on cider's volatile composition. It is also obvious that the extent to which yeast could potentially influence the volatile composition and concentration in the final product will depend on the apple variety used.

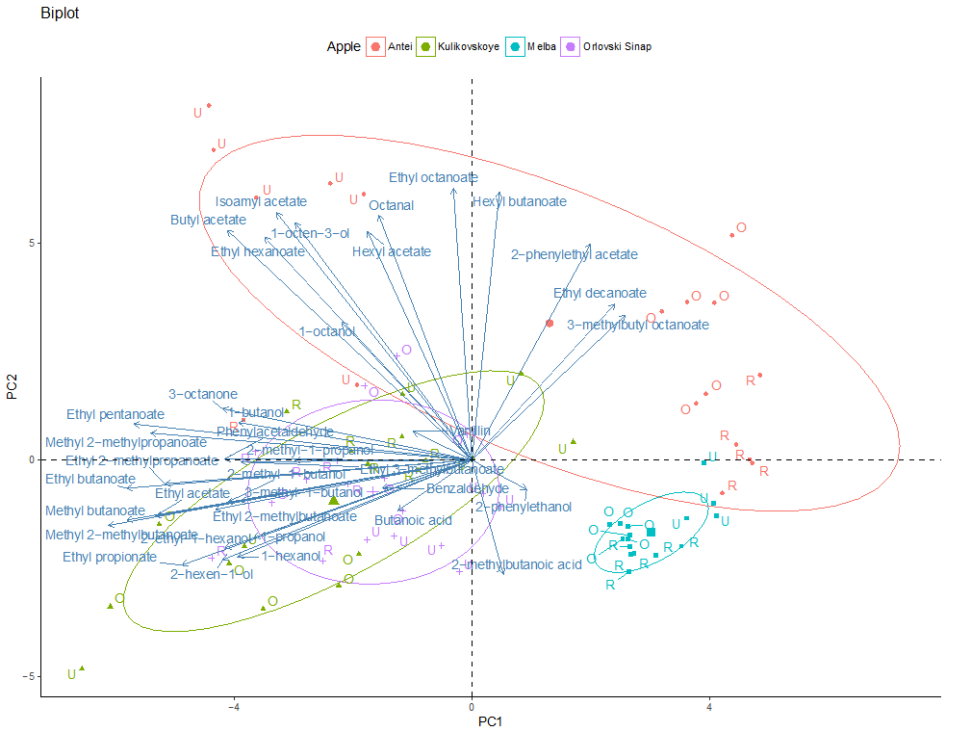


Figure 8. *Principal component analysis biplot. Samples made with Antei apples are represented with color red, Kulikovskoye apples – color green, Melba apples – color blue, Orlovski Sinap apples – color purple. Letter U represents samples made with unripe apples, letter R – ripe apples, letter O – overripe apples*

Table 8. *Identified volatile compounds, their relative concentrations and influence (p-value) of apple variety, ripening stage, and yeast strain*

Compound	**Odor description[b]**	**Concentration range, μg L^{-1} in 2-chloro-6-methylphenol equivalents**	**p-value**		
			Variety	**Maturity**	**Yeast**
1-propanol	Fermented, fruity, apple, pear	1.67 – 91.47	**0.000**	**0.002**	0.625
2-methyl-1-propanol	Wine, whiskey	0.00 – 233.15	**0.049**	**0.004**	**0.019**
1-butanol	Balsamic	20.24 – 411.62	**0.000**	0.434	0.776
3-methyl-1-butanol	Cognac, banana, fruity	0.00 – 3631.49	**0.000**	**0.006**	0.700
2-methyl-1-butanol	Wine, fruity	319.31 – 3541.74	0.000	0.002	0.850
2-hexen-1-ol, (E)	Green, leafy	12.61 – 124.72	0.021	0.068	0.309
1-hexanol	Green, pungent	283.49 – 4659.29	0.029	0.007	0.390
1-octen-3-ol	Earthy, vegetative, mushroom	0.00 – 83.00	0.000	0.003	0.677
2-ethyl-1-hexanol	Citrus, floral	0.01 – 51.97	0.000	0.020	0.720
1-octanol	Citrus, floral, fatty	0.00 – 2.87	0.000	0.184	0.541
2-phenylethanol	Floral, rose	0.00 – 598.83	0.000	0.000	0.583
Ethyl acetate	Fruity, green	10.27 – 713.71	0.000	0.094	0.165
Methyl-2-methylpropanoate	Fruity, ether	0.06 – 0.92	0.000	0.084	0.237
Ethyl propionate	Fruity, grape, pineapple, rum	1.55 – 109.10	0.000	0.589	0.004
Methyl butanoate	Pungent, fermented	1.19 – 19.85	0.000	0.221	0.949
Ethyl-2-methylpropanoate	Ether, pungent, fruity	0.49 – 24.63	0.000	0.038	0.028

Table 8 (cont). *Identified volatile compounds, their relative concentrations and influence (p-value) of apple variety, ripening stage, and yeast strain*

Methyl-2-methylbutanoate	Fruity, ripe, fatty	0.53 – 6.35	**0.000**	0.170	0.899
Ethyl butanoate	Pineapple, cognac	3.24 – 150.82	**0.000**	0.186	0.991
Butyl acetate	Solvent, banana	0.17 – 12.26	**0.000**	**0.034**	0.678
Ethyl-3-methylbutanoate	Fruity, pineapple, apple, orange	0.12 – 4.90	**0.000**	**0.014**	**0.001**
Isoamyl acetate	Banana, pear	3.91 – 252.74	**0.001**	**0.009**	0.546
Ethyl-2-methylbutanoate	Fruity, apple	0.22 – 5.98	**0.000**	0.619	0.999
Ethyl pentanoate	Fruity, berry, tropical	0.07 – 2.50	**0.000**	0.481	0.447
Ethyl hexanoate	Fruity, pineapple, banana	10.34 – 254.60	**0.001**	0.100	0.747
Hexyl acetate	Fruity, green apple, banana	0.50 – 165.57	**0.001**	0.122	0.557
Hexyl butanoate	Green, fruity, vegetative	0.00 – 51.86	**0.000**	0.470	0.825
Ethyl octanoate	Fruity, wine, banana, brandy	1.41 – 837.17	**0.000**	0.184	0.798
2-phenylethyl acetate	Honey, rose	0.00 – 24.34	**0.000**	0.125	0.385
Ethyl decanoate	Waxy, fruity, apple, grape	0.01 – 174.10	**0.000**	0.054	0.620
3-methylbutyl octanoate	Waxy, fruity, pineapple, coconut	0.00 – 2.11	0.000	0.237	0.314
Butanoic acid	Cheesy	0.00 – 27.24	0.236	0.109	0.001
2-methylbutanoic acid	Cheesy, fermented	0.00 – 13.71	0.002	0.016	0.009
Benzaldehyde	Almond, cherry	0.00 – 11.78	0.008	0.039	0.890
3-octanone	Herbal, lavender, mushroom	0.00 – 0.91	0.015	0.000	0.882

Table 8 (cont). *Identified volatile compounds, their relative concentrations and influence (p-value) of apple variety, ripening stage, and yeast strain*

Octanal	Waxy, citrus	0.07 – 150.04	**0.000**	0.102	0.446
Phenylacetaldehyde	Honey, rose	0.00 – 2.99	**0.000**	**0.006**	0.838
Vanillin	Vanilla	0.00 – 0.05	0.236	0.189	0.670

[a] – approximate average value according to NIST database (US Department of Commerce, Gaithersburg, MD, USA)

[b] – according to www.thegoodscentscompany.com

4.2 The effect of nitrogen supplementation on fermentation kinetics and ester formation during cider fermentation

Adjustment of Yeast Assimilable Nitrogen (YAN) content before fermentation is often used in cider production to compensate for nitrogen deficiency and avoid sluggish fermentations. In addition to enhanced fermentation rates, the YAN supplementation may have an impact on the flavor characteristics of cider. Publication II aimed to assess yeast performance in two different commercially available musts with similar non-limiting YAN content. For that, two different apple musts were fermented: M1 (Aspall, Suffolk, United Kingdom; 12.8 °Brix, pH 3.28, titratable acidity 6.7 ± 0.1 g L^{-1} in malic acid equivalents, initial YAN 69.48 ± 2.00 mg·N L^{-1}) and M2 (Döhler, Darmstadt, Germany; 11.7 °Brix, pH 3.18, titratable acidity 3.8 ± 0.1 g L^{-1} in malic acid equivalents, initial YAN 51.21 ± 1.84 mg·N L^{-1}). YAN content in both matrices was brought to a similar content of 80 ± 2 mg·N L^{-1} by using inactive yeast based fermentation nutrient (Fermaid K; Lallemand Inc.). Fermentation kinetics, nitrogen uptake, volatile ester production, and sensory properties of the final products were evaluated.

The results showed that fermentation kinetics (fermentation duration, maximum CO_2 production rate) with different yeasts (Figure 9; A, B) did not seem to be dependent on the apple must, at least under conditions of sufficient YAN content.

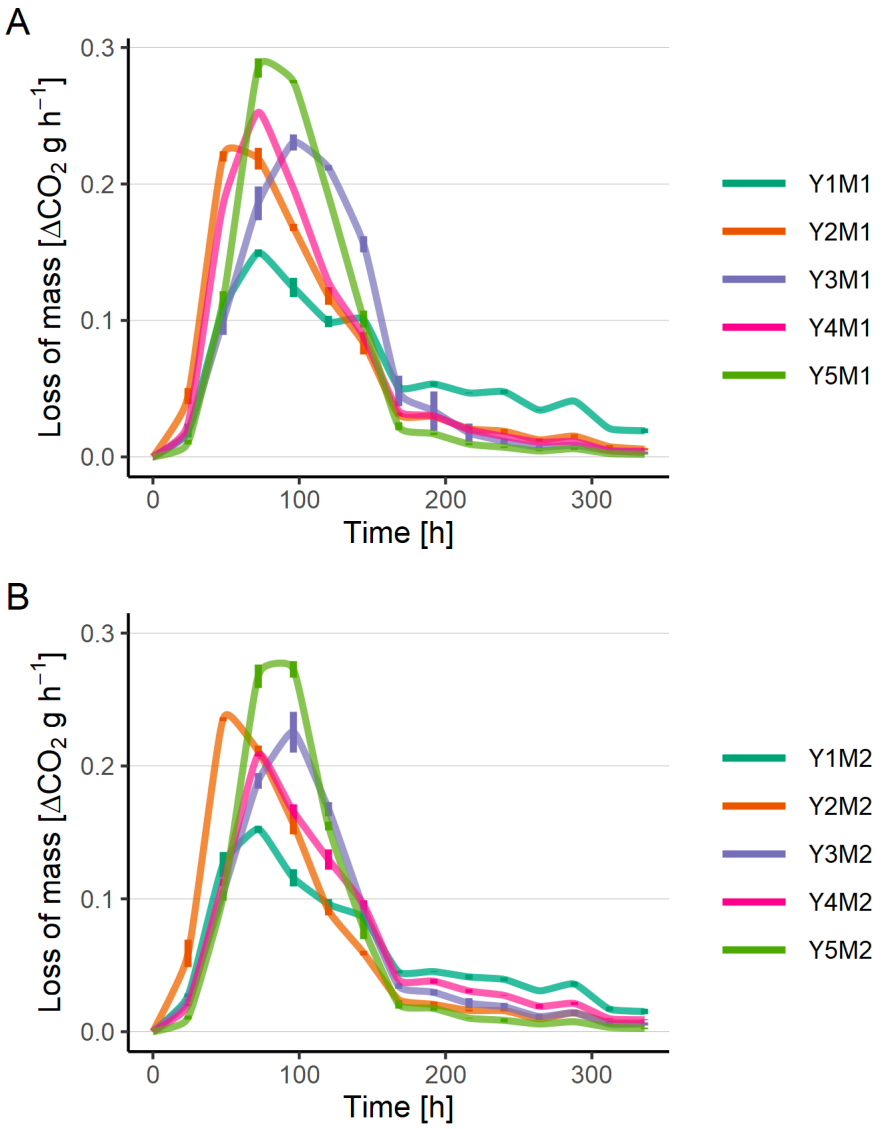


***Figure 9**. Fermentation kinetics with different yeasts (Y1-5) in musts M1 (A) and M2 (B)*

The assimilation kinetics of YAN by the yeast strains used in this study is shown in Figure 10. The intense YAN consumption occurred in the case of all strains within 2-4 days. The residual concentration of YAN at the end of fermentation was similar regardless of the yeast and must used.

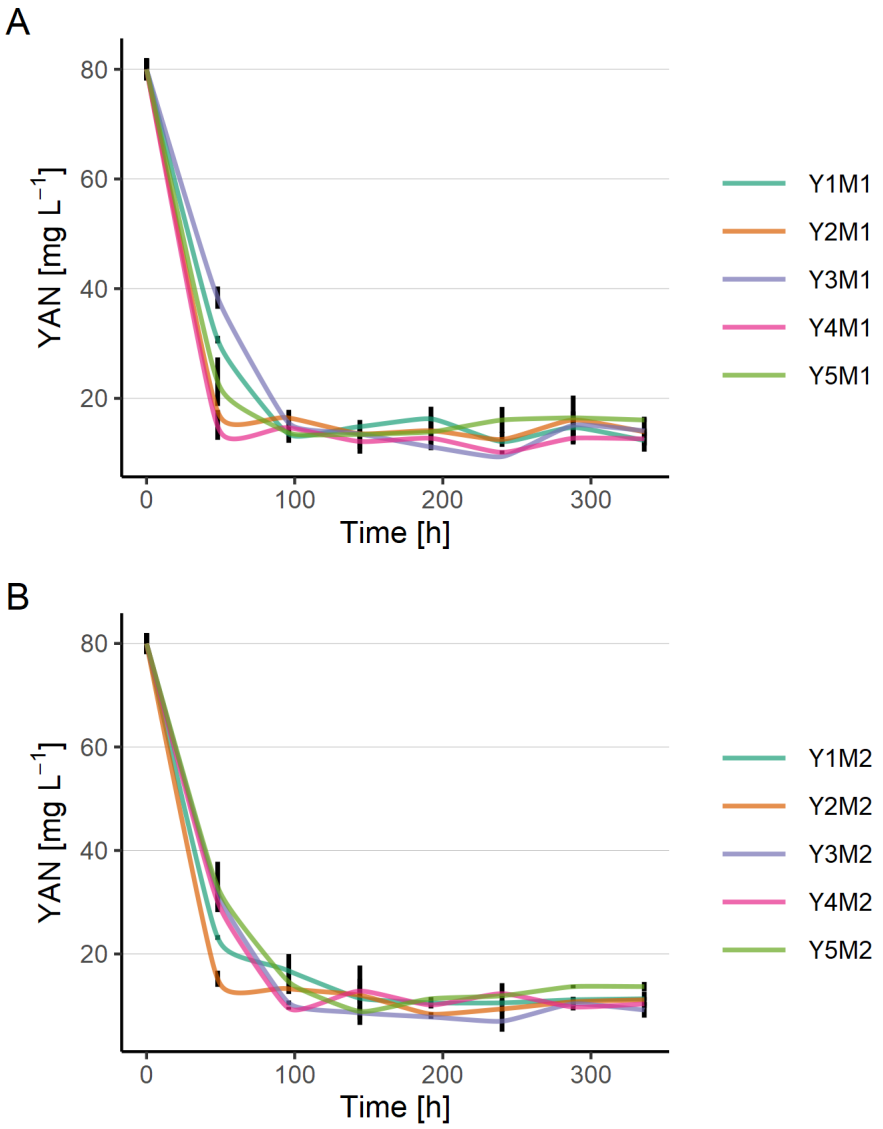


***Figure 10**. YAN consumption by yeasts (Y1-5) in the musts M1 (A) and M2 (B)*

In total, the concentration of 10 ethyl and acetate esters was monitored in the cider samples produced with five different yeasts and two commercial apple musts. From the results, it could be concluded that the formation of three acetate esters – ethyl acetate, isoamyl acetate, hexyl acetate depended on the must used (Figure 11). Thus, both ethyl acetate and isoamyl acetate were produced in considerably (up to 7 and 2 times, respectively) higher concentrations by most strains in the must M2 (11.7 °Brix pH 3.18, titratable acidity 3.8 g L^{-1} in malic acid equivalents). Hexyl acetate, on the other hand, had higher relative concentrations in the samples made with the must M1 (12.8 °Brix, pH 3.28, titratable acidity 6.7 g L^{-1} in malic acid equivalents). The amount of other

esters depended on the particular yeast-must combination. The production of the aforementioned esters could be tied to a potential difference in the availability of certain precursors in the must. For example, ethyl acetate is formed in anaerobic glucose metabolism from acetyl-CoA with glutamate, methionine and cysteine precursors in its synthesis pathway (Nordström, 1962). Isoamyl acetate can either be created from amino acids (leucine, valine) or *de novo* synthesised from isoamyl alcohol (Eden, et al, 1996; Plata et al., 2003). Hexyl acetate originates from C6 alcohols and aldehydes (e.g., hexanol, 2-hexenol, 2-hexenal) (Dennis et al. 2012). Thus, in terms of volatile composition development of the final product, other intrinsic properties of the fermentation matrix than the YAN content should also be considered.

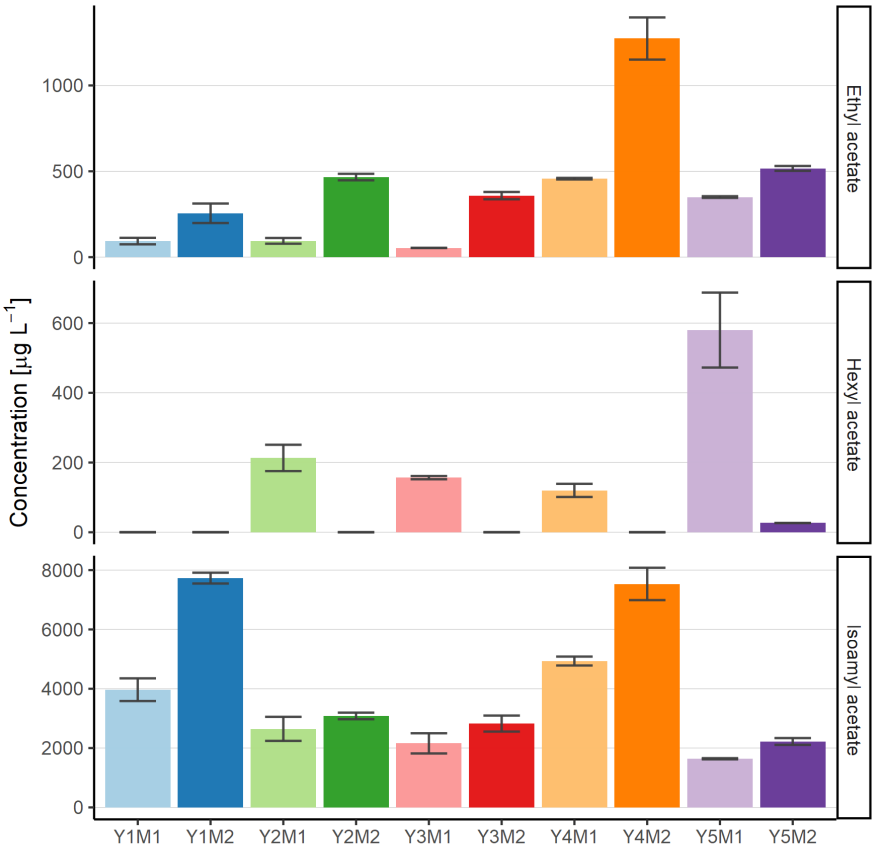


Figure 11. *Relative concentration (in µg L^{-1} of 2-chloro-6-methylphenol equivalents) of ethyl acetate, hexyl acetate and isoamyl acetate at the end of fermentation with different yeasts (Y1-5) and apple musts (M1 and M2) ($p < 0.05$)*

Among the sensory attributes assessed in this study, statistically significant differences ($p < 0.05$) were obtained for most of them with the exception of overall odour intensity ($p = 0.61$), 'apple-like' in odour ($p = 0.08$), 'apple-like' in taste ($p = 0.10$), sourness in odour ($p = 0.19$), and astringency ($p = 0.98$). The results of sensory analysis with the exception of statistically insignificant parameters were then subjected to principal component analysis (PCA). The obtained PCA biplot is presented in Figure 12.

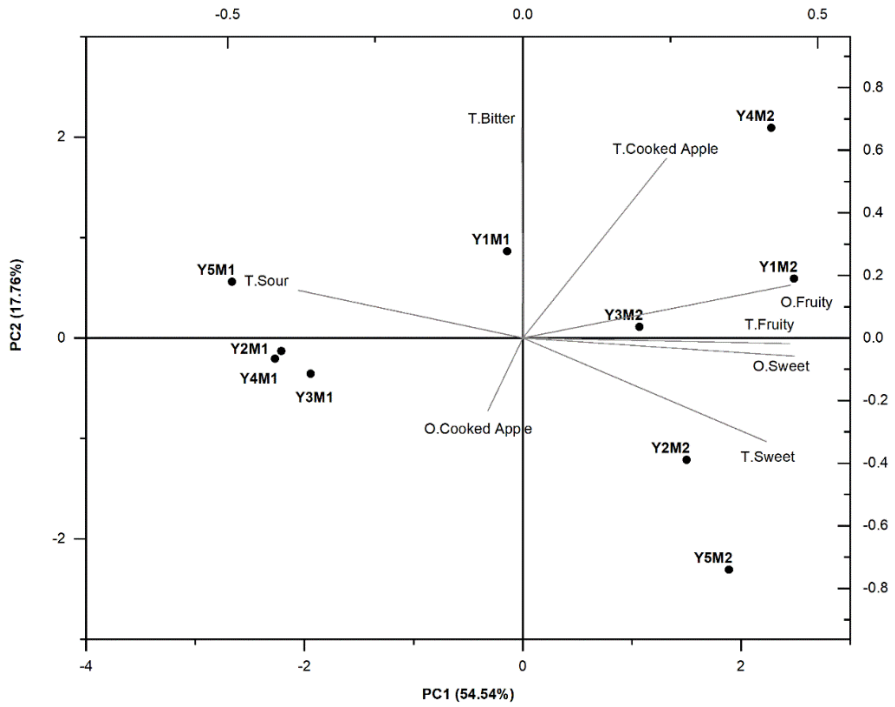


Figure 12. *Grouping of the samples on the PCA biplot according to statistically significant ($p < 0.05$) sensory properties perceived at the end of fermentation with each combination of yeast (Y1-5) and must (M1 and M2)*

Based on the biplot, cider mainly gathered on the separate sides of the biplot based on the must. Samples produced with the apple must M2 possessed strong correlation with fruitiness and sweetness, which is well in line with higher concentration of ethyl acetate and isoamyl acetate in these samples. The ciders made with the must M1 strongly correlated with sourness, which corresponds well with higher titratable acidity and higher malic acid content in these ciders.

The results of this study demonstrated that apple must can have significant effect on the concentration of some key esters in cider. As similar level of initial YAN content was used in the musts, the substantial differences in some esters' concentrations observed in my experiments suggest that other factors can play role in formation of esters (e.g., specific amino acid composition of the must and availability of free fatty acids and/or their *de novo* synthesis capability by yeasts). Further insight into specific factors or combination of these factors in cider would be required.

4.3 The effect of apple juice concentration on cider fermentation kinetics and composition of volatile esters

The number of studies on cider properties and their development during fermentation has recently increased to a considerable extent. However, there are no studies available on the comparison of freshly pressed apple juice and the concentrate made from the same juice pressing batch. Publication III aimed to apply freshly pressed juice and juice

concentrate made from the same apple variety (Antei; private orchard in Valgjärve, South Estonia) as a substrate for cider fermentation at 18 °C and 30 °C. Different nutritional strategies (DAP, inactive yeast based nutrient and control with no supplementation) were also applied to assess their efficiency in fermentation kinetics and development of volatile esters between apple juice and the concentrate.

First, the concentration of individual free amino acids (FAA) was measured in the juice and the diluted apple juice concentrate before the start of fermentation and free amino nitrogen content (FAN) content was calculated (Table 9). Knowing the amount of added fermentation nutrients and their YAN/NH_4^+ content, the approximate amount of yeast assimilable nitrogen (YAN) at the start of fermentation could be determined.

Table 9. *Free amino acid (FAA) composition, free amino nitrogen (FAN) content before and yeast assimilable nitrogen (YAN) content after supplementation. Standard deviation is shown (n = 3)*

FAA, mg L^{-1}	**Juice, 9.8 °Brix**	**Concentrate, 10 °Brix**
His	1.71 ± 0.32	2.27 ± 0.44
Asn	269.60 ± 24.48	315.03 ± 32.70
Ser	6.28 ± 0.93	9.21 ± 0.82
Gln	3.26 ± 0.60	2.87 ± 0.26
Arg	21.68 ± 1.10	19.36 ± 3.63
Gly	0.32 ± 0.04	1.19 ± 0.21
Asp	38.10 ± 5.26	44.42 ± 4.46
Glu	32.43 ± 3.54	38.15 ± 3.67
Thr	2.75 ± 0.45	3.62 ± 0.33
Ala	8.85 ± 1.56	10.91 ± 1.01
Pro	2.32 ± 0.37	3.22 ± 0.33
Orn	0.27 ± 0.02	0.51 ± 0.10
Cys-cys	0.30 ± 0.03	0.34 ± 0.03
Lys	0.82 ± 0.09	1.93 ± 0.08
Tyr	0.88 ± 0.06	1.04 ± 0.22
Met	1.06 ± 0.16	1.27 ± 0.12
Val	3.93 ± 0.73	4.41 ± 0.46
Ile	2.34 ± 0.45	2.90 ± 0.33
Leu	1.18 ± 0.23	3.21 ± 0.39
Phe	1.97 ± 0.16	1.98 ± 0.21
Trp	0.57 ± 0.10	0.41 ± 0.03
TOTAL, mg L^{-1}	400.62 ± 40.68	468.25 ± 49.83
FAN, mg·N L^{-1}	76.13 ± 7.29	87.91 ± 9.62
+ DAP YAN, mg·N L^{-1}	95.03 ± 7.29[1]	106.81 ± 9.62[1]
+ Fermaid O YAN, mg·N L^{-1}	93.33 ± 7.29[2]	105.11 ± 9.62[2]

[1] Taking into consideration that DAP contains 21% of nitrogen

[2] According to manufacturer's instructions, 40 g hL^{-1} dose of Fermaid O is equivalent to 17.2 mg·N L^{-1} of YAN

The most abundant free amino acids in the apple juice used in the study were asparagine, aspartic acid, and glutamic acid. This correlates with what has been shown previously in other studies (Wu et al, 2007; Nogueira & Wosiacki, 2012). Overall statistical significance according to the analysis of variance showed that the juice and diluted concentrate to the same Brix value of the same juice can be considered as different

($p < 0.05$), the latter containing much more free amino acids. Thus, the concentration of serine, glycine, threonine, ornithine, lysine, tyrosine, isoleucine, and leucine was deemed statistically significantly higher ($p < 0.05$) and, hence, attributed to the process of juice concentration. The conventional industrial concentration of juices is carried out by a high-pressure boiling process that has been reported to possess several effects on the juice like thermal and evaporation losses (Maccarone etl al, 1996; Gunko et al, 2006; Kozák et al, 2009; Juliana et al, 2020). The increase of certain amino acids' concentration in the diluted concentrate could also be attributed to partial peptidolysis/proteolysis that may have led to the release of individual amino acids. Protease activity has been noted in the apple fruit during ripening (Koak et al, 2011). The activity of apple fruit proteases is yet to be properly explored; however, the activity range for other plant-derived proteases like papain (found in papaia), bromelain (found in pineapple), and ficin (found in figs) have been reported at 45-80 °C (Arshad et al, 2016). Since the temperatures used during juice concentration process (30-60 °C) overlap with the active temperatures of plant-derived proteases, the proteolytic activity of apple proteases might have contributed to the increase in free amino acids. Finally, the increase of free amino acids concentration could also be attributed to a shift in proportions of the components of the juice matrix during dilution, e.g. the removal of haze (pectins) in clarification pre-treatment shifts the proportions in favor of amino acids when the water is added back according to °Brix.

The fermentation kinetics of ciders prepared from either fresh juice or juice concentrate, fermented with different yeasts at two different temperatures with or without nutrients are provided in Figure 13. In all samples, fermentations were completed with no signs of sluggish fermentation.

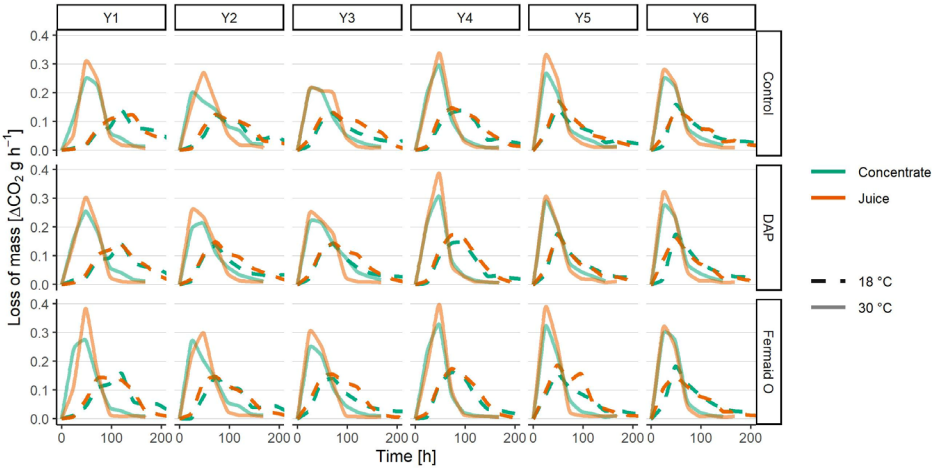


***Figure 13**. Fermentation kinetics of cider samples prepared from fresh juice or juice concentrate of the same apple cultivar with different yeasts at 18 or 30 °C. The samples fermented with the juice from diluted concentrate are represented by a green line and those with the fresh juice by an orange line. The samples fermented at 18 °C are represented by a dashed line of appropriate color; by a solid line at 30 °C*

Expectedly, the temperature was the main factor affecting yeast fermentative activity while some differences between yeast strains could also be observed. In general, the fermentations were at least two times faster at 30 °C. No particular difference in

fermentation activity could be observed between the juice and the concentrate at 18 °C. However, at 30 °C, the fermentation activity on diluted concentrate was slightly inferior to that of the fresh juice. A loss of certain nutrients (e.g. some heat-sensitive B complex vitamins) during concentration process might have affected the ability of yeast to cope with higher temperatures but that was likely not drastic enough to significantly impact the fermentation kinetics.

The presence of 8 esters was monitored across the samples. PLS-DA was employed as a statistical approach to observe the differences in ester production (Figure 14). The best representation of the results was achieved when viewing the samples according to the yeast species – *S. bayanus* strains Y1 and Y3 (Figure 14A) were compared to *S. cerevisiae* strains Y2, Y4, Y5 and Y6 (Figure 14B). According to the biplots, all ciders fermented with *S. cerevisiae* strains clustered closely together showing more consistency across the samples in volatile esters composition than in case the of *S. bayanus* strains.

The cider produced from the concentrate had higher concentration of isoamyl acetate (up to 6700 ± 230 μg L^{-1} in the concentrate; up to 5100 ± 410 μg L^{-1} in the juice). Isoamyl acetate can be synthesized by yeast either from amino acids leucine and valine or from isoamyl alcohol (Eden et al, 1996; Plata et al, 2003). As was noted previously, the diluted concentrate had elevated amounts of most amino acids, including leucine. Higher content of leucine might have been successfully utilized by the strains used in this study for isoamyl acetate production. *S. cerevisiae* yeasts used in the study were shown to produce more of isoamyl acetate (up to 6700 ± 230 μg L^{-1}) than *S. bayanus* strains (isoamyl acetate concentration up to 5300 ± 420 μg L^{-1}).

The use of a juice concentrate in cider fermentation also resulted in higher production of certain long-chain fatty acid esters (up to 20 000 ± 1420 μg L^{-1} of ethyl decanoate, up to 10 000 ± 1480 μg L^{-1} ethyl dodecanoate) in comparison to fresh apple juice (up to 6 000 ± 860 μg L^{-1} of ethyl decanoate, up to 6 000 ± 580 μg L^{-1} ethyl dodecanoate). The intensity of the production, however, varied depending on the yeast strain and was more pronounced with *S. bayanus* species. It should be noted that long-chain fatty acid esters have been previously tied to the onset of cell death (Alexandre & Benatier, 2006; Saerens et al, 2008a; Wang et al, 2018). Due to their low permeability through cell membrane, the long-chain fatty acid esters are released along with other compounds (e.g., amino acids, fatty acids, lipids, glycoproteins, mannoproteins) into the environment during cell autolysis (Alexandre & Benatier, 2006). The correlation of long-chain fatty acids esters with the cider samples produced using concentrate could thus signify higher cell death rate in comparison to the samples prepared with apple juice.

The nutritional supplementation approaches used in this study did not possess any significant influence on the production of volatile esters by *S. cerevisiae* strains. On the other hand, inactive yeast based nutrient supplementation increased the production of medium-chain fatty acid ethyl esters (ethyl butyrate, ethyl hexanoate, ethyl octanoate) in *S. bayanus* strains and reduced the formation of long-chain fatty acid esters. Supplementation of nitrogen with organic sources such as inactive yeast/yeast autolysate has proven to be efficient at stimulating ethyl ester production in multiple previous studies (Torrea et al, 2011; Santos et al, 2016; Hu et al, 2018).

In conclusion, this study suggests that the process of clarification and concentration might affect the amount of free amino acids in juice produced from the respective concentrate. In terms of fermentation kinetics, the juice diluted from concentrate was shown to be slightly inferior at higher temperatures to that of the fresh apple juice, most likely due to the partial loss of certain nutrients. The production of volatile esters was

also affected by the use of the juice concentrate. When using the concentrate, the yeasts strains under study showed increased production of long-chain fatty acid esters (ethyl decanoate, ethyl dodecanoate). This effect was more evident with *S. bayanus* strains. Increased synthesis of isoamyl acetate was also noted in the samples fermented with the juice recovered from concentrate which could be attributed to the higher concentration of leucine in it, which is a precursor for isoamyl acetate synthesis. However, this effect was more pronounced in *S. cerevisiae* strains.

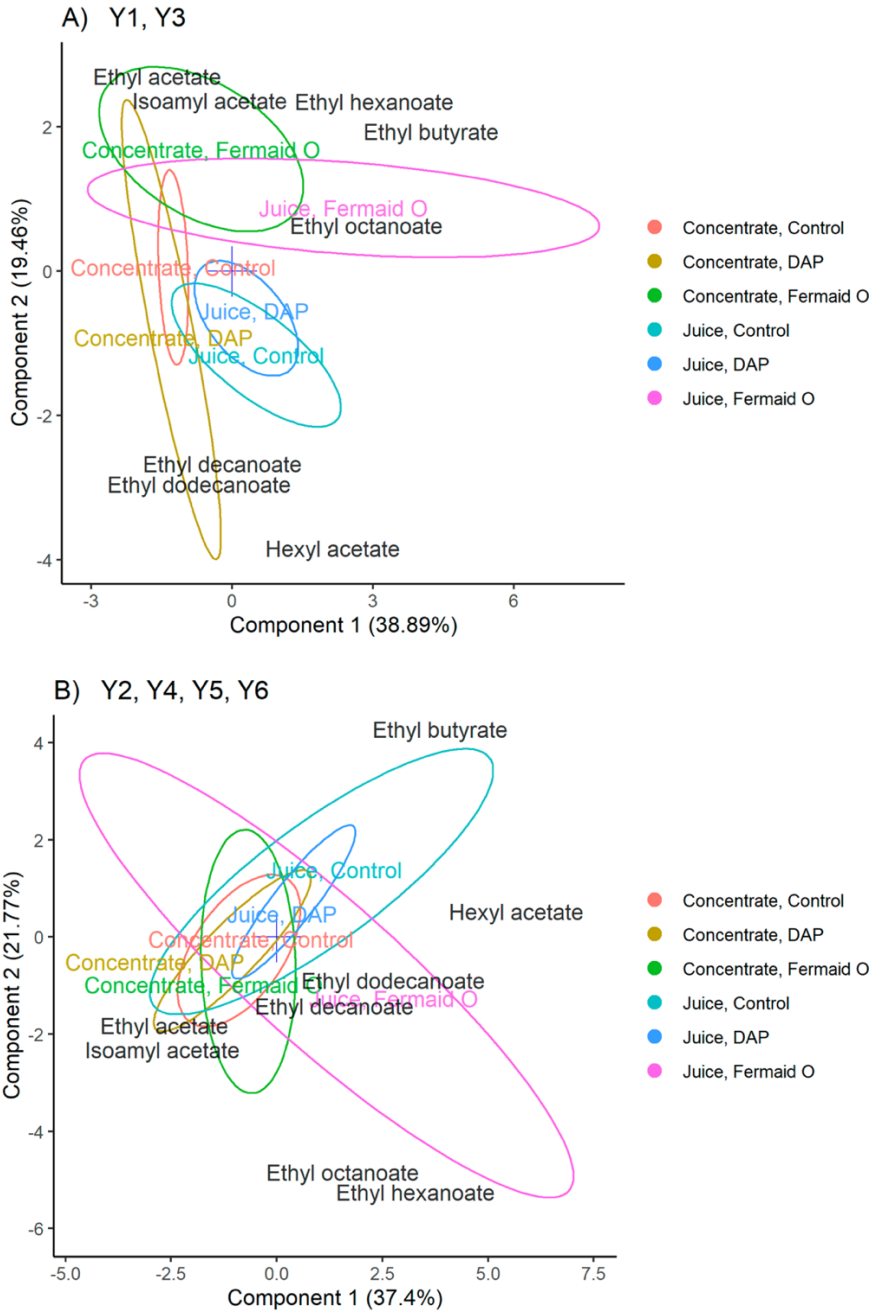


Figure 14. *PLS-DA biplots of volatile ester production in cider samples. A) S. bayanus strains; B) S. cerevisiae strains. Colors correspond to the combinations of juice type and nutritional supplement. Ellipses indicate confidence region at 0.95 level*

4.4 The effect of asparagine and oxygen availability on production of higher alcohols and esters by yeast

The effects of apple must supplementation with some amino acids both individually or in a mixture have been explored in the few studies with cider and wine fermentations (Santos et al, 2016; Fairbairn et al, 2017; Seguinot et al 2018). However, these studies only made use of the amino acids known as the precursors for higher alcohols and esters. Even though asparagine constitutes the majority of free amino nitrogen in apple must (Table 7), its effects on the yeast performance and the production of volatile compounds in cider are yet to be properly explored. The supplementary study to this PhD thesis aimed to assess the effect of asparagine (as a single additional YAN source) and oxygen availability on the production of higher alcohols and esters by yeast during cider fermentation. For that, larger scale fermentations (10 L) were conducted with *Saccharomyces uvarum* strain under different conditions. Three nutritional strategies were used: i) control with no additional supplementation (80 mg·N L^{-1} FAN), ii) supplementation with Asn to 140 mg·N L^{-1} of FAN, and iii) supplementation with Asn to 200 mg·N L^{-1} of FAN. Each of these nutritional strategies was tested under conditions of oxygen depletion and oxygen saturation at the start of fermentation. Yeast performance under these different fermentation conditions was assessed in terms of fermentation kinetics, population growth, cell viability, amino acid consumption, and production of higher alcohols and esters.

Fermentation kinetics were expressed as a rate of decline in density. All fermentations were stopped at the density of 1010 g L^{-1}. The fermentation rates were shown to depend on the availability of oxygen as they proceeded faster in the experiments saturated with dissolved oxygen (Figure 15). Thus, the fermentations with dissolved oxygen saturation reached the target density in 13 to 24 days, depending on nutritional strategy, compared to 29 to 64 days in experiments with no oxygen at the start of the fermentation. Similar effects of oxygen availability on fermentation kinetics were noted previously by Giovanelli *et al* (1996), Rosenfeld *et al* (2003), Morales *et al* (2015), Ochando *et al* (2017), Englezos *et al* (2018) in their studies on wine. Oxygen availability in the wort and must is widely known to affect the biomass yield and viability of the yeast population during fermentation which directly translates to the fermentation rates (Rosenfeld et al, 2003). Some oxygen at the start of the fermentation is required by yeast for the synthesis of sterols and unsaturated fatty acids for the maintenance of the yeast growth during fermentation and eventually resist the toxicity of higher ethanol concentrations (Mauricio et al, 1998; Rosenfeld et al, 2003; Klug & Daum, 2014; Marbà-Ardébol et al, 2017).

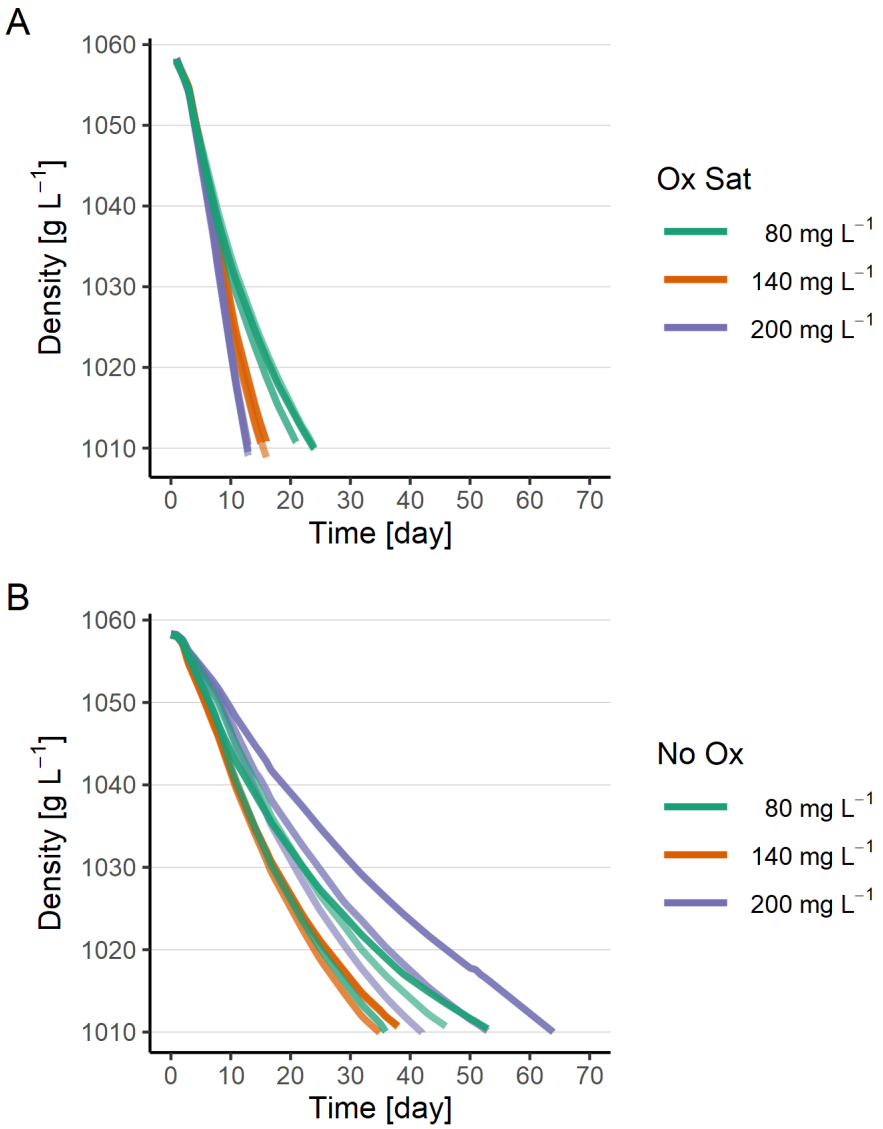


Figure 15. *Density loss in the fermentation vessels with oxygen (A) and with no oxygen (B) at different FAN levels adjusted by asparagine addition at the start of fermentation*

Limited yeast population growth and lower viability of the yeast population due to lack of oxygen was also noticed in this study (Figure 16, Table 10). Thus, the yeast population in the vessels saturated with dissolved oxygen reached up to 7 x 10^7 CFU mL^{-1} with almost 100% viability while in the vessels with no oxygen the population was up to 7 times lower with the viability of only 57-66%.

The effect of asparagine supplementation on fermentation kinetics depended on the availability of dissolved oxygen at the start of the fermentation (Figure 13). In the vessels saturated with dissolved oxygen, the fermentations proceeded faster as more asparagine was added while with no oxygen at the start of fermentation the beneficial effect of

asparagine addition was noted only to a limited extent. Increasing FAN level to 140 mg·N L^{-1} with asparagine under conditions of oxygen deficiency had a positive effect on the fermentation rate; however, with its further increase to 200 mg L^{-1} the effect turned out to be negative – the fermentation rate was similar or even slower than in the control. The similar trends can also be noted with the population growth rates (Figure 14).

Table 10. *Measured yeast population viability in the samples expressed as a percentage of a plate count to total cell count at the end of fermentation at different FAN (80, 140, and 200 mg·N L^{-1}) and aeration levels (No Ox – no oxygen; Ox Sat – oxygen saturation). Biological replicates for the fermentations are indicated as A, B, and C*

	Plate count, CFU mL^{-1}	**Total count, CFU mL^{-1}**	**Measured viability, %**
No Ox 80 A	8.39×10^6	1.64×10^7	51.18
No Ox 80 B	1.77×10^7	3.20×10^7	55.32
No Ox 80 C	1.13×10^7	1.73×10^7	65.53
No Ox 140 A	1.41×10^7	1.90×10^7	74.05
No Ox 140 B	1.29×10^7	2.17×10^7	59.45
No Ox 140 C	1.25×10^7	1.98×10^7	63.26
No Ox 200 A	8.20×10^6	1.30×10^7	63.09
No Ox 200 B	9.46×10^6	1.62×10^7	58.40
No Ox 200 C	1.40×10^7	2.20×10^7	63.42
Ox Sat 80 A	4.88×10^7	4.68×10^7	104.30
Ox Sat 80 B	5.50×10^7	4.99×10^7	110.24
Ox Sat 80 C	4.80×10^7	4.86×10^7	98.67
Ox Sat 140 A	4.48×10^7	4.43×10^7	101.05
Ox Sat 140 B	5.79×10^7	5.66×10^7	102.31
Ox Sat 140 C	5.69×10^7	5.34×10^7	106.51
Ox Sat 200 A	5.94×10^7	5.63×10^7	105.59
Ox Sat 200 B	5.62×10^7	5.22×10^7	107.67
Ox Sat 200 C	5.74×10^7	5.92×10^7	97.03

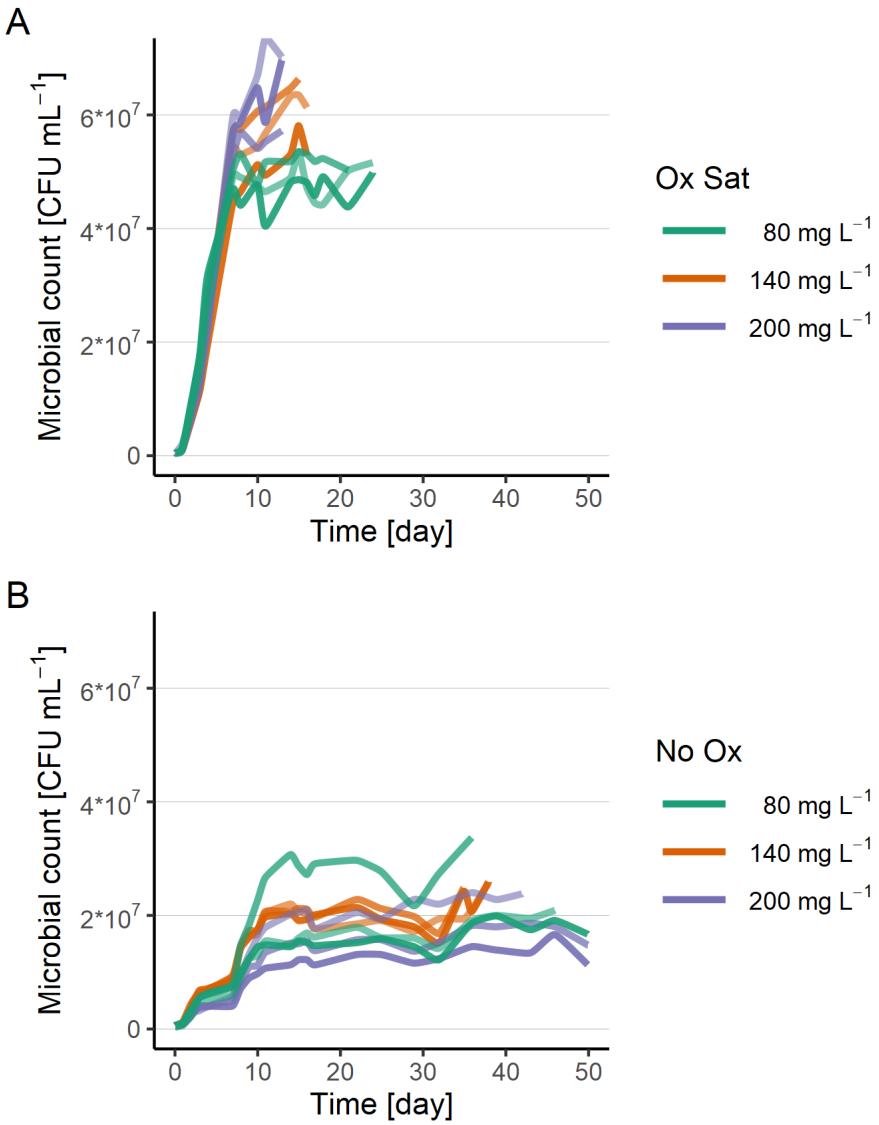


Figure 16. *Yeast growth dynamics in the fermentation vessels with oxygen (A) and with no oxygen (B) at different FAN levels adjusted by asparagine addition at the start of fermentation*

The concentration of nitrogen from free amino acids and asparagine was determined before and after fermentations (Figure 17). Most of free amino acids, including asparagine were consumed when oxygen was present in the environment at the start of fermentation; on the other hand, the addition of asparagine resulted in the increase of residual amino acids with no oxygen was present at the start of fermentation. Along with limited yeast population growth, this implies the limitations in the consumption of available nitrogen sources and, hence, the lower efficiency of nitrogen supplementation in the absence of oxygen in the fermentation environment.

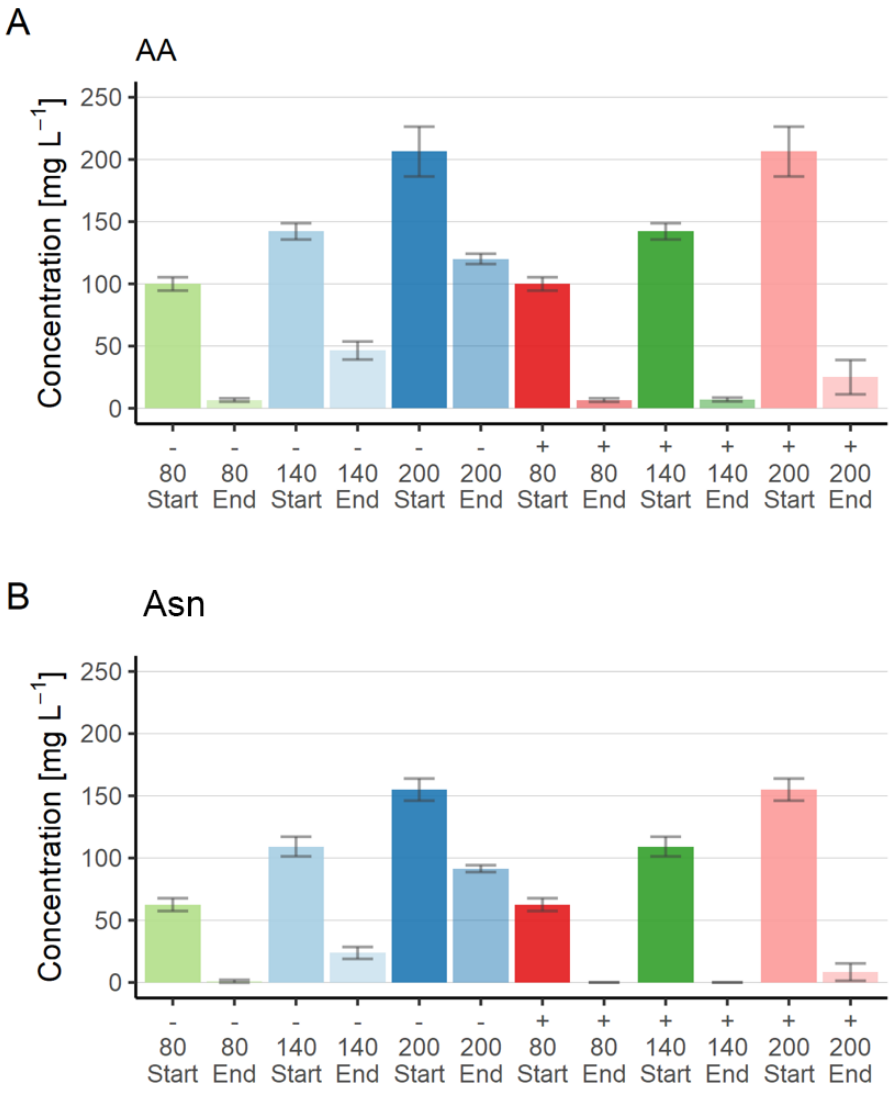


Figure 17. *The nitrogen content from free amino acids (A) and asparagine (B) before and after fermentation. The experiments with no oxygen are indicated by "-" sign; experiments saturated with oxygen by "+" sign*

The concentration of 5 higher alcohols and 10 esters was monitored in the same experiments (Table 11). As seen from the results, in the samples fermented with aerated must lower concentration of esters can be noted in comparison to the samples with no oxygen at the start of fermentation. The suppression of production of acetate esters by aeration has been previously noted by Fujii *et al* (1997) and Rojas et al (2001) with *Saccharomyces cerevisiae* strains and has been related to repression of genes encoding for acetyltransferases Atf1p and Atf2p (ATF1, ATF2) by oxygen (Fujii et al, 1997; Plata et al, 2005). Despite the positive effect of oxygen deficiency on esters' production, a remarkable

effect of asparagine supplementation could not be observed in the absence of oxygen. By contrast, the effect of asparagine supplementation on the composition of the volatile compounds in the samples fermented in the presence of oxygen at the start of fermentation was observed. Thus, the more asparagine was added the higher was the concentration of esters (Table 11), suggesting that the suppression of ester formation due to oxygen can be partially compensated for with an appropriate amount of asparagine supplementation. Even though asparagine itself is not reported as an amino acid that directly participates in ester formation pathways, it can still be utilized to stimulate the process. Notably, the addition of asparagine may suppress the formation of phenylethyl alcohol through uptake inhibition of phenylalanine – a precursor in phenylethyl alcohol biosynthesis by downregulation of amino acid permease Agp1p (Saenz et al, 2014; Dai et al, 2021).

In this study, the effect of FAN supplementation with asparagine under conditions of either oxygen saturation or deficiency at the start of fermentation was successfully explored. The efficiency of asparagine supplementation on the fermentative activity of yeast and the growth of yeast population was found to positively correlate with the presence of oxygen. On the other hand, it was shown that asparagine supplementation becomes inhibitory at higher concentrations (above 140 mg·N L^{-1} FAN) under complete anaerobic conditions at the start of fermentation. The presence of oxygen also affected the formation and composition of volatile compounds in cider, as aeration of the must correlated with higher production of fusel alcohols instead of esters. Additional studies are, however, required to understand whether the combination of other amino acids with different aeration conditions yields the same effect.

Table 11. *Concentrations (in mg L^{-1}) of acetate esters at the end of fermentation at different FAN (adjusted with asparagine) and aeration levels. The experiments with no oxygen are indicated as No Ox; experiments saturated with oxygen – as Ox Sat. The concentration of FAN in the samples in indicated as 80, 140, and 200 mg·N L^{-1}*

	No Ox 80	**No Ox 140**	**No Ox 200**	**Ox Sat 80**	**Ox Sat 140**	**Ox Sat 200**
Ethyl acetate	7.415 ± 0.826	10.175 ± 0.249	9.818 ± 0.869	3.704 ± 0.197	5.371 ± 0.032	8.701 ± 0.588
Isobutyl acetate	0.018 ± 0.001	0.022 ± 0.003	0.022 ± 0.003	0.017 ± 0.001	0.024 ± 0.001	0.041 ± 0.005
Ethyl butyrate	0.332 ± 0.032	0.347 ± 0.016	0.372 ± 0.021	0.146 ± 0.010	0.176 ± 0.009	0.214 ± 0.017
Butyl acetate	0.187 ± 0.022	0.237 ± 0.020	0.240 ± 0.006	0.138 ± 0.011	0.156 ± 0.012	0.247 ± 0.008
Isoamyl acetate	1.400 ± 0.222	1.747 ± 0.052	1.794 ± 0.059	0.423 ± 0.097	0.786 ± 0.044	1.396 ± 0.201
Ethyl hexanoate	0.719 ± 0.009	0.695 ± 0.097	0.768 ± 0.064	0.327 ± 0.044	0.709 ± 0.026	0.899 ± 0.061
Hexyl acetate	1.375 ± 0.188	1.495 ± 0.115	1.664 ± 0.147	0.542 ± 0.086	0.919 ± 0.015	1.187 ± 0.162
Ethyl octanoate	0.183 ± 0.033	0.123 ± 0.011	0.145 ± 0.039	0.018 ± 0.001	0.042 ± 0.006	0.145 ± 0.063
Ethyl decanoate	0.523 ± 0.073	0.257 ± 0.022	0.351 ± 0.031	0.025 ± 0.005	0.049 ± 0.001	0.071± 0.001
2-Phenylethyl acetate	0.132 ± 0.017	0.165 ± 0.016	0.155 ± 0.023	0.095 ± 0.012	0.046 ± 0.006	0.036 ± 0.002
Isobutanol	4.843 ± 0.123	6.803 ± 0.373	5.857 ± 0.979	9.95 ± 0.091	8.520 ± 0.173	13.260 ± 0.467
1-Butanol	2.775 ± 0.524	2.698 ± 0.493	3.039 ± 0.084	2.466 ± 0.102	2.707 ± 0.162	3.807 ± 0.177
3-Methyl-1-butanol	33.195 ± 4.959	44.196 ± 3.075	37.762 ± 2.934	43.863 ± 3.537	40.993 ± 2.048	51.642 ± 6.002
Hexanol	3.807 ± 0.621	3.699 ± 0.132	3.520 ± 0.103	4.555 ± 0.199	4.361 ± 0.272	5.784 ± 0.362
Phenylethyl Alcohol	12.443 ± 1.602	10.288 ± 1.715	9.850 ± 1.584	29.766 ± 2.786	6.809 ± 2.652	2.931 ± 0.013

5 Conclusions

The main objective of this thesis was to study the effect of different cidermaking conditions on fermentation kinetics and the production of esters by yeast. The work started from the general assessment of the statistical significance of raw materials such as apple variety, apple ripening stage, and yeast strain in the formation of volatile composition in cider and finished with an evaluation of the effects of individual and combined technological aspects (juice pre-processing, nitrogen availability, oxygen availability) on the production of selected volatile compounds.

The following conclusions can be drawn from the obtained results:

- Apple variety was the primary attribute influencing the yeast-derived volatile composition of apple cider.
- Additional YAN supplementation does not necessarily guarantee higher esters formation. Other specific factors or a combination of these factors should be considered for increasing the production of volatile esters.
- Oxygen availability at the start of fermentation seems to determine the efficiency of additionally supplemented YAN assimilation as well as of the formation of volatile compounds by yeast.
- Apple juice concentrate was successfully applied in cider fermentation resulting in rather similar yeast fermentative performance as in the case with fresh apple juice, albeit the effect of concentration on amino acids' content and the development of volatile esters was substantial.

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Abstract

The effect of cidermaking practices on ester production by yeast

Sensory properties play a primary role in the consumer acceptance of cider, depending on various factors, such as apple varieties, process technologies, and yeast strains used for fermentation. The current knowledge on the impact of these factors in cider production is still limited and often obtained from the studies made on grape wine. The objective of this PhD thesis was to explore how different technological conditions affect the quality of cider in terms of ester production by yeast. The impact of apple variety, ripening stage, and yeast strains were studied along with different process conditions: clarification and concentration of apple juice, oxygenation of the must, and nitrogen supplementation. The results showed that apple variety and ripening stage have a significant influence on both cider's overall volatile composition as well as on the formation of esters. The effect was shown to be independent of the yeast assimilable nitrogen (YAN) content in the tested apple musts, at least at concentrations ≥ 80 ppm when adjusted with diammonium hydrogen phosphate. However, the results indicate that free amino acids composition in the must might play a more important role in ester synthesis. Apple must pre-treatment by clarification and concentration was found to affect positively the concentration of free amino acids (relative to fermentable sugars), and the formation of isoamyl acetate and long-chain fatty acid esters (ethyl decanoate, ethyl dodecanoate) during fermentation. The increased synthesis of isoamyl acetate in pre-processed must could be attributed to the higher concentration of corresponding amino acid precursor (leucine). Furthermore, the synthesis of aforementioned esters differed between the yeast species under the same fermentation conditions. Thus, two *Saccharomyces bayanus* strains produced more long-chain fatty acid esters while four *Saccharomyces cerevisiae* strains produced more isoamyl acetate. The formation and composition of volatile compounds in cider was also significantly affected by oxygenation of the must, which caused higher accumulation of fusel alcohols (isobutanol, 1-butanol, 3-methyl-1-butanol, hexanol, phenylethyl alcohol).

Lühikokkuvõte

Siidri valmistamise tingimuste mõju estrite tootmisele pärmide poolt

Sensoorsed omadused omavad peamist rolli siidri aktsepteeritavuse väljakujunemisel tarbijate seas ning sõltuvad erinevatest teguritest nagu näiteks õunasort, tootmistehnoloogiad ja kääritamisel kasutatavad pärmitüved. Praegused teadmised nende tegurite mõju kohta siidritootmises on endiselt piiratud ning saadud sageli viinamarjaveini kohta tehtud uuringutest. Käesoleva doktoritöö eesmärgiks oli uurida, kuidas erinevad siidri valmistamise tehnoloogilised tingimused mõjutavad siidri kvaliteeti lähtuvalt fermentatsiooni käigus toimuvast estrite tootmisest pärmide poolt. Selleks uuriti õunasordi, küpsusastme ja pärmitüve mõju koos teiste tootmistingimustega: mahla eeltöötlus (selitamine ja kontsentreerimine), hapniku kättesaadavus ja lämmastiku lisamine. Tulemused näitasid, et õunasordil ja küpsusastmel on oluline mõju nii siidri üldisele lenduvate ühendite profiilile kui ka konkreetselt estrite koostisele. Mõju ei sõltunud uuritud mahlade pärmidele omastatava lämmastiku (YAN) sisaldusest, vähemalt diammooniumvesinikfosfaadiga korrigeeritud kontsentratsioonide ≥ 80 ppm juures. Tulemused näitasid, et mahla aminohappeline koostis võib estrite sünteesil mängida olulisest rolli. Leiti, et õunamahla eeltöötlus selitamise ja kontsentreerimise teel mõjutab positiivselt vabade aminohapete kontsentratsiooni (kääritavate suhkrute suhtes) ning isoamüülatsetaadi ja pika ahelaga rasvahepete estrite (etüüldekanoaat, etüüldodekanoaat) moodustumist. Suurenenud isoamüülatsetaadi sünteesi eeltöödeldud mahlas võib siduda nimetatud ühendi aminohappelise eelühendi (leutsiini) suurema kontsentratsiooniga. Lisaks erines ülalmainitud estrite süntees erinevates pärmiliikides samade kääritamistingimuste juures. Nii tootsid kaks *Saccharomyces bayanus* tüve rohkem pika ahelaga rasvhapete estreid, neli *Saccharomyces cerevisiae* tüve aga rohkem isoamüülatsetaati. Lenduvate ühendite moodustumist ja koostist siidris mõjutas oluliselt ka hapniku kättesaadavus fermentatsiooni alguses, mis põhjustas kõrgemat alkoholide sünteesi (isobutanool, 1-butanool, 3-metüül-1-butanool, heksanool, fenüületüülalkohol).

Appendix 1

Publication I

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The effects of apple variety, ripening stage, and yeast strain on the volatile composition of apple cider

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ABSTRACT

This work examined the degree of influence of apple variety, apple ripening stage, and yeast strain on the volatile composition of apple cider. Four apple varieties grown in Estonia were selected for the study – Antei, Melba, Kulikovskoye, and Orlovski Sinap. The must from the apples at various stages of ripening (unripe, ripe, overripe) underwent alcoholic fermentation using commercially available yeast strains. Gas chromatography - mass spectrometry was employed to assess the differences in volatile composition between the samples. Out of the variables analyzed in this work, apple variety turned out to be the primary attribute influencing the quality and aroma properties of apple cider. The effect of yeast strain and the maturity of the fruit was variety-specific where the volatile profiles of ciders made with Melba variety were the least influenced by the ripening stage of apples and yeast strains used for the fermentation.

1. Introduction

Cider is a fermented alcoholic beverage made from apples. Despite holding a smaller position on a global scale, cider production is common throughout Europe and has also spread to other Western markets (Northern America, Australia) (Nogueira and Wosiacki, 2012). Technological advances made in other parts of the fermented beverage industry strongly influence cider production. Because of that, a limited amount of published information exists regarding the aroma of cider and factors affecting it the most.

No conclusive information is available on the effect of different apple varieties on the volatile composition of cider. However, some apple varieties themselves have been distinguished from one another based on their chemical composition (El Hadi et al., 2013). As far as non-volatile composition is concerned, aside from apparent fluctuations in sugar and acid ratios, the primary varietal differences occur in the phenolic content (Blanco-Gomisa et al., 1998; Wu et al., 2007; Kalinowska et al., 2014). Phenolic compounds, in turn, have been reported to contribute to the odor profiles of alcoholic beverages such as beer, wine, sherry, and whiskey (Vanbeneden et al., 2008).

Some information is available on how the level of apple fruit ripeness impacts the aroma profile of cider. For example, one of the latest researches conducted on this subject by Alberti et al. (2016) has provided some insight into cider aroma based on the ripeness of the apples used. The cider made from senescent apples was 24–52 % (depending on the variety) more abundant in different volatile compounds than the counterparts made from unripe apples.

The yeast used in the production of fermented beverages contributes to the final aroma profile mainly by elevating the levels of higher alcohols and esters (McKay et al., 2011). The relative concentrations of the fermentation products, however, may depend on the strain. Many yeast strains have already been investigated in another fermented beverage – wine. The application of different yeasts in the wine industry and the impact on the sensory properties have been described by Henick-Kling et al. (1998), Soden et al. (2000), Cadez et al. (2002), Becker Whitener et al. (2014), and Synos et al. (2015), to name a few. For example, Synos et al. (2015) have demonstrated the influence of the yeast strain on the formation of aroma compounds in Cabernet icewines. The yeasts differed not only in the diversity of generated odor-active volatile compounds but also in the amounts generated. According to the results, yeast EC1118 displayed the highest amounts of various alcohols, esters, furfural, hexanoic acid, and β-damascenone. Becker Whitener et al. (2014) observed the fermentation of red and white grape musts with non-*Saccharomyces* yeasts. They found that the majority of the investigated non-*Saccharomyces* yeasts provided lower levels of alcohols, esters, and terpenes, except for *Kazachstania gamospora*, which produced more total

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Table 1
Malic acid content and sugar profile of apple musts obtained from different apple varieties at diferent stages of maturity.

Must	Fructose, %	Glucose, %	Disaccharides, %	Malic acid, %
Unripe Melba	4.5 ± 0.1	0.5 ± 0.1	3.8 ± 0.3	1.2 ± 0.1
Ripe Melba	5.3 ± 0.1	0.7 ± 0.1	4.9 ± 0.1	0.9 ± 0.1
Overripe Melba	6.3 ± 0.5	1.2 ± 0.1	5.0 ± 0.1	0.8 ± 0.1
Unripe Antei	5.9 ± 0.2	1.8 ± 0.1	1.3 ± 0.1	0.8 ± 0.1
Ripe Antei	6.5 ± 0.1	1.8 ± 0.1	2.8 ± 0.1	0.6 ± 0.1
Overripe Antei	6.9 ± 0.2	2.2 ± 0.1	2.3 ± 0.1	0.5 ± 0.1
Unripe Orlovski Sinap	5.2 ± 0.3	1.1 ± 0.1	2.4 ± 0.1	1.3 ± 0.1
Ripe Orlovski Sinap	6.4 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	0.8 ± 0.1
Overripe Orlovski Sinap	6.9 ± 0.2	2.0 ± 0.1	3.0 ± 0.1	0.5 ± 0.1
Unripe Kulikovskoye	4.9 ± 0.4	1.1 ± 0.1	1.6 ± 0.1	0.7 ± 0.1
Ripe Kulikovskoye	6.4 ± 0.2	1.3 ± 0.1	2.0 ± 0.1	0.5 ± 0.1
Overripe Kulikovskoye	6.5 ± 0.3	1.6 ± 0.1	1.8 ± 0.1	0.4 ± 0.1

esters than *Saccharomyces cerevisiae*. Less information is known on the effects of different yeast strains on aroma development in cider. The fermentation of industrial apple pomaces with three indigenous yeasts (*Saccharomyces cerevisiae, Hanseniaspora valbyensis,* and *Hanseniaspora uvarum*) was described by Rodríguez Madrera et al. (2015). Suárez Valles et al. (2005) have inoculated Asturian apple juice with three strains of cider making isolates (*S. cerevisiae* r. *cerevisiae* SSA, *S. cerevisiae* r. *uvarum* SSB, *S. cerevisiae* r. *cerevisiae* SSC) and a commercial wine yeast (*S. cerevisiae* r. *bayanus* UVA-PM). The compounds that were found to differentiate the strains were ethyl acetate, acetaldehyde, and isobutanol.

Additional insights need to be gained regarding the impact of cider production at the molecular level so that the industry can understand and control the character of the final product. Given the knowledge, it should be possible to maintain and expand the diversity in the cider market and influence the economics of cider production. The objective of this study was to examine the degree of influence of apple variety, apple ripeness, and yeast strain on the volatile composition of apple cider.

2. Materials and methods

2.1. Chemicals and reagents

Distilled water was obtained using Elix 5 UV Water Purification system (Merck Millipore, Billerica, MA, USA). 2-chloro-6-methylphenol, as an internal standard, was obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cider preparation in a laboratory environment

Four autumn or winter apple varieties, 'Antei', 'Kulikovskoye', 'Melba', and 'Orlovski Sinap' grown in South Estonia, at a private orchard in Valgjärve (58°8′ N, 26°66′ E) were used in the study. Apples were selected at three different stages of ripening: unripe, ripe, and overripe. The estimation of the ripening stage was based on the iodine starch test (Travers et al., 2002). All apples were first harvested at the 'unripe' stage (0 weeks, starch index 1) of their maturity and left to ripen at +4 °C. This is a common practice in Northern-Europe region because some of the

Table 2
Identified volatile compounds.

Compound name	Odor description[b]	RT	RI_{exp}	RI_{lit}[a]	Concentration range in the samples, ppb in IS equivalents
1-propanol	Fermented, fruity, apple, pear	2.60	589	595	1.67–91.47
2-methyl-1-propanol	Wine, whiskey	3.10	627	635	0.00–233.15
1-butanol	Balsamic	3.54	657	660	20.24–411.62
3-methyl-1-butanol	Cognac, banana, fruity	4.85	735	730	0.00–3631.49
2-methyl-1-butanol	Wine, fruity	5.01	743	740	319.31–3541.74
2-hexen-1-ol, (E)	Green, leafy	7.51	861	865	12.61–124.72
1-hexanol	Green, pungent	7.67	868	865	283.49–4659.29
1-octen-3-ol	Earthy, vegetative, mushroom	10.48	990	1000	0.00–83.00
2-ethyl-1-hexanol	Citrus, floral	11.38	1030	1030	0.01–51.97
1-octanol	Citrus, floral, fatty	12.06	1061	1070	0.00–2.87
2-phenylethanol	Floral, rose	13.25	1115	1110	0.00–598.83
Ethyl acetate	Fruity, green	2.74	603	610	10.27–713.71
Methyl-2-methylpropanoate	Fruity, ether	4.02	690	684	0.06–0.92
Ethyl propionate	Fruity, grape, pineapple, rum	4.13	697	705	1.55–109.10
Methyl butanoate	Pungent, fermented	4.38	711	720	1.19–19.85
Ethyl-2-methylpropanoate	Ether, pungent, fruity	5.18	752	760	0.49–24.63
Methyl-2-methylbutanoate	Fruity, ripe, fatty	5.73	780	775	0.53–6.35
Ethyl butanoate	Pineapple, cognac	6.08	798	800	3.24–150.82
Butyl acetate	Solvent, banana	6.58	820	815	0.17–12.26
Ethyl-3-methylbutanoate	Fruity, pineapple, apple, orange	7.37	855	855	0.12–4.90
Isoamyl acetate	Banana, pear	7.76	872	875	3.91–252.74
Ethyl-2-methylbutanoate	Fruity, apple	8.91	891	895	0.22–5.98
Ethyl pentanoate	Fruity, berry, tropical	9.09	930	930	0.07–2.50
Ethyl hexanoate	Fruity, pineapple, banana	10.62	996	1000	10.34–254.60
Hexyl acetate	Fruity, green apple, banana	10.93	1010	1010	0.50–165.57
Hexyl butanoate	Green, fruity, vegetative	14.70	1084	1190	0.00–51.86
Ethyl octanoate	Fruity, wine, banana, brandy	14.80	1189	1190	1.41–837.17
2-phenylethyl acetate	Honey, rose	16.22	1260	1260	0.00–24.34
Ethyl decanoate	Waxy, fruity, apple, grape	18.54	1382	1380	0.01–174.10
3-methylbutyl octanoate	Waxy, fruity, pineapple, coconut	19.73	1449	1450	0.00–2.11
Butanoic acid	Cheesy	6.85	832	840	0.00–27.24
2-methylbutanoic acid	Cheesy, fermented	7.72	870	870	0.00–13.71
Benzaldehyde	Almond, cherry	9.79	960	955	0.00–11.78
3-octanone	Herbal, lavender, mushroom	10.20	978	980	0.00–0.91
Octanal	Waxy, citrus	10.75	1002	1005	0.07–150.04
Phenylacetaldehyde	Honey, rose	11.71	1045	1045	0.00–2.99
Vanillin	Vanilla	18.78	1395	1400	0.00–0.05

[a] Approximate average value according to NIST database (US Department of Commerce, Gaithersburg, MD, USA).
[b] According to www.thegoodscentscompany.com.

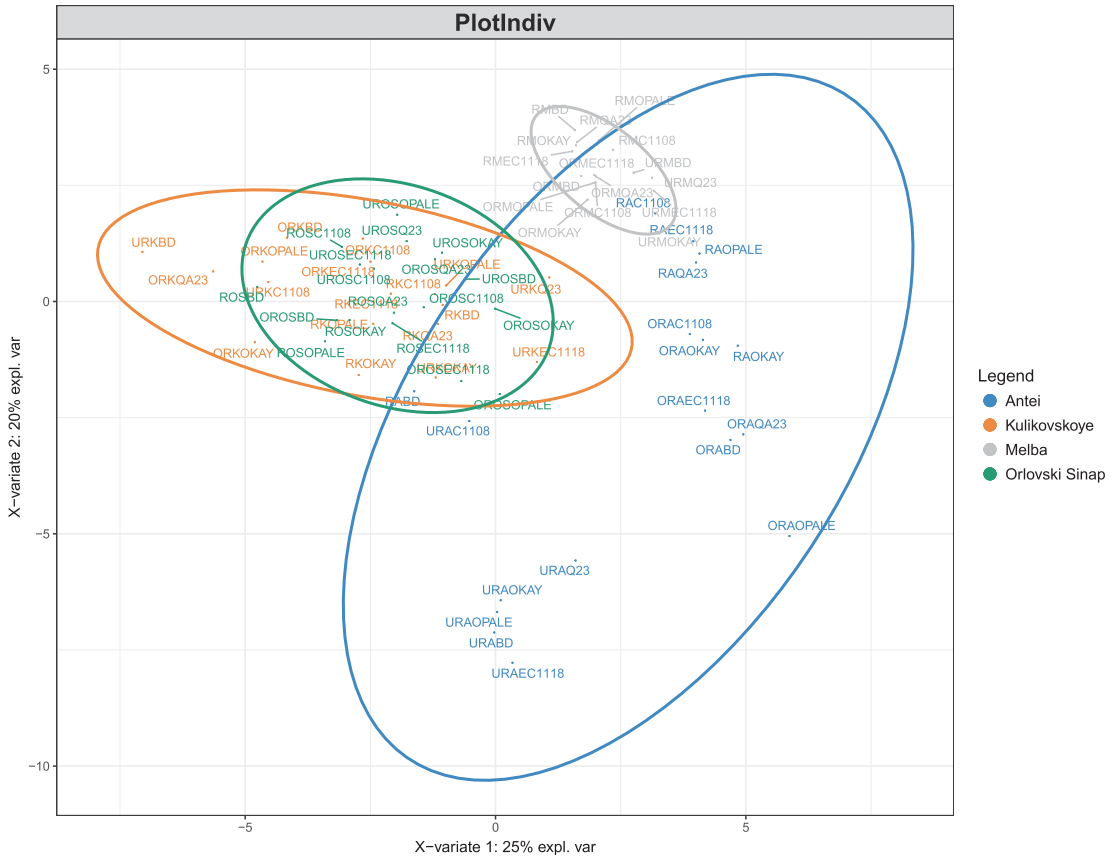

Fig. 1. PLS-DA plot for the cider samples. Samples made with Antei apples are represented with color blue, Kulikovskoye – orange, Melba – gray, Orlovski Sinap – green. Samples are coded according to the preparation: maturity level (UR – Unripe, R – Ripe, OR – Overripe) – apple cultivar (A – Antei, M – Melba, OS – Orlovski Sinap, K – Kulikovskoye) – yeast strain. Thus, sample coded ROSOKAY, for example, represents a cider made with ripe Orlovski Sinap apples and fermented with OKAY commercial yeast strain.

Table 3
Classification error rates of PLS-DA for different cider treatments (Mahalanobis distance, 10 times repetition).

No. of components	Maturity	Variety	Yeast
1 component	0.37	0.44	0.81
2 components	0.31	0.34	0.78
3 components	0.23	0.13	0.79
4 components	0.21	0.16	0.73
5 components	0.17	0.07	0.74

autumn and most winter varieties do not reach their maturity before the first frost. The ripe apples (starch index 3) were collected after 2–8 weeks (depending on the variety: 'Melba' 2 weeks, 'Kulikovskoye' 3 weeks, 'Antei' 6 weeks and 'Orlovski Sinap' 8 weeks) in storage, and the overripe (starch index 5) – after 6–12 weeks (approx. one month after the ripe stage: 6, 8, 10 and 12 weeks, respectively). Apples were washed with tap water and drained. Apples with visible defects (e.g., rotting, mold) were excluded; however, no further selection was made according to the size or appearance. Before pressing, 20 kg of apples of each variety were randomly selected at each ripening stage. The juice was prepared in two batches of 10 kg using a centrifugal juice press (Vita Pro-Active JE810; Kenwood). The resulting batches were first combined and then distributed immediately into 1 L bottles for fermentation. Malic acid content and sugar profile of the musts are provided in Table 1. The commercial starter cultures used in this study were as follows: Biodiva (*Torulaspora delbrueckii*), C1108 (*Saccharomyces bayanus*), EC1118 (*Saccharomyces cerevisiae*), OKAY (*Saccharomyces cerevisiae*), OPALE (*Saccharomyces cerevisiae*), and QA23 (*Saccharomyces bayanus*). The inoculation and fermentation steps were followed according to the procedure previously described by Laaksonen et al. (2017). The number of cider samples was 144 (4 varieties × 3 ripening stages × 6 yeasts × 2 replicates). A representative sample was taken of each cider and stored at −20 °C in 10 mL plastic tubes.

2.3. Extraction of cider volatiles

The extraction of cider volatiles was carried out using headspace – solid-phase microextraction (HS-SPME). 300 μL of the sample was measured into a 20 mL glass autosampler vial capped with a PTFE/silicone septum and diluted with 700 μL of distilled water. 7.5 ppb of 2-chloro-6-methylphenol was added as an internal standard. Vials were pre-incubated at 45 °C for 5 minutes. SPME fiber (30/50μm DVB/Car/PDMS Stableflex, length 2 cm; Supelco, Bellefonte, PA, USA) recommended by Villière et al. (2012) was used to extract the volatile compounds from the headspace for 20 minutes under stirring at 45 °C.

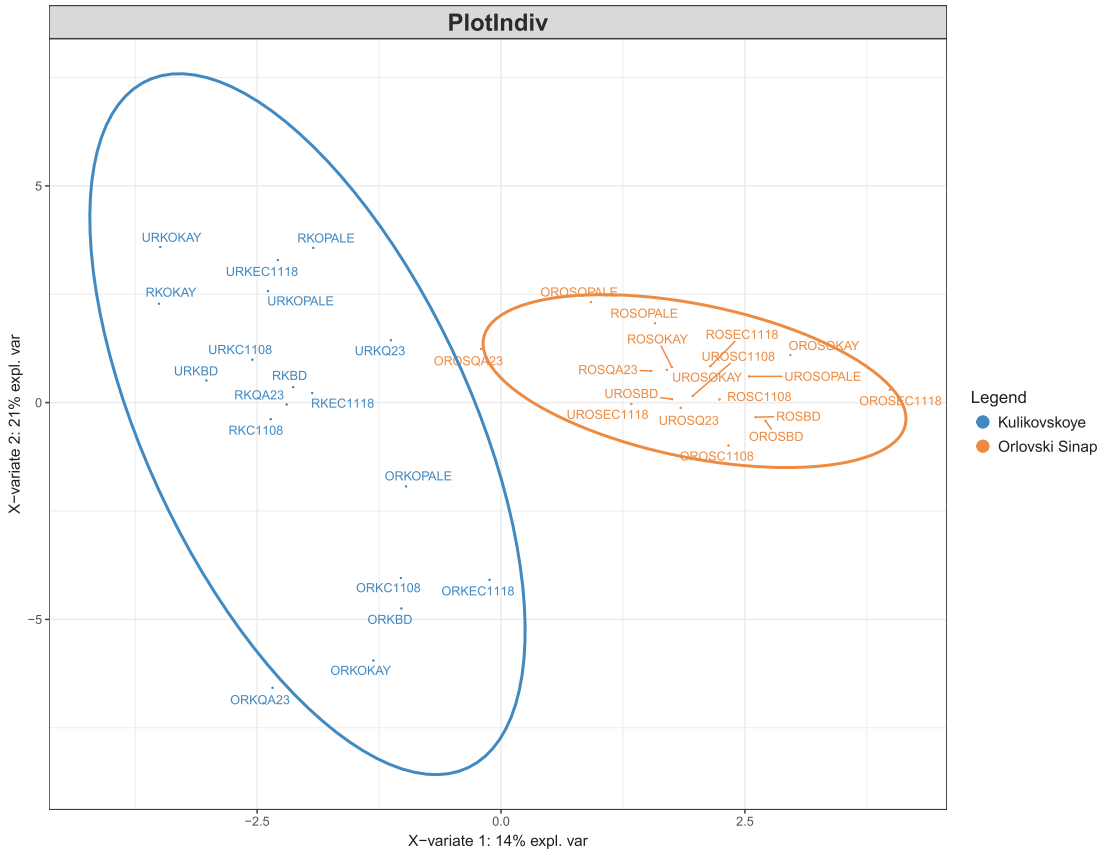

Fig. 2. PLS-DA plot for the cider samples made with Kulikovskoye (color blue) and Orlovski Sinap (color orange) apples. Samples are coded according to the preparation: maturity level (UR – Unripe, R – Ripe, OR – Overripe) – apple cultivar (A – Antei, M – Melba, OS – Orlovski Sinap, K – Kulikovskoye) – yeast strain. Thus, sample coded ROSOKAY, for example, represents a cider made with ripe Orlovski Sinap apples and fermented with OKAY commercial yeast strain.

2.4. GC-TOF-MS analysis of volatiles

Identification and relative quantitation of cider volatiles were performed using a Micromass GCT Premier gas chromatograph system (Waters, Milford, MA, USA) coupled with CombiPAL autosampler (CTC Analytics AG, Lake Elmo, MN, USA). After the SPME procedure, the volatile compounds were desorbed in splitless mode into a GC injection port equipped with a 0.75 mm internal diameter liner at 250 °C for 10 minutes. A DB5-MS column (30 m length × 0.25 mm i.d. × 1.0 μm film thickness; J&W Scientific, Folsom, CA, USA) was used with helium as a carrier gas at a flow rate of 1.0 mL min^{-1}. The oven was programmed to ramp up from 45 °C at a rate of 10 °C min^{-1} to a final temperature of 280 °C with an additional holding time of one minute (total run time 24.50 min). Mass spectra were obtained at ionization energy of 70 eV and a scan speed of 10 scans s^{-1}, with a mass range of 35–350. Each cider sample was analyzed in three analytical replicates.

Non-targeted identification of volatile compounds was carried out using ChromaLynx application (MassLynx software; Waters, Milford, MA, USA) and theoretical calculation of retention indices (RI). Theoretical retention indices were calculated using the retention times of the eluting compounds normalized to the retention times of adjacent n-alkanes. Accurate identification of the compounds was verified by comparing theoretical retention indices to the NIST database (US Department of Commerce, Gaithersburg, MD, USA). Semi-quantitative approach against an internal standard (2-chloro-6-methylphenol) was used for quantitation purposes – the amounts of identified volatile compounds were expressed in internal standard equivalents.

2.5. Statistical analysis and data processing

The results of GC-TOF-MS analysis were statistically evaluated by partial least square discriminant analysis (PLS-DA) (mixOmics package, R software 3.4.0; Boston, MA, USA). For evaluation of the classification power of each treatment, cross-validation of PLS-DA results was carried out by calculating classification error using Mahalanobis distance (10 times repetition) – the low numerical value of classification error signifies the statistical importance of the clustering seen on the plot.

The correlation of cider samples with identified volatile compounds was observed using principal component analysis (PCA) (factoextra package, R software 3.4.0; Boston, MA, USA). Each volatile compound is represented on the PCA biplot by a vector. The length of any given vector illustrates the level of correlation between the compounds and the samples – the longer the vector, the stronger the correlation. Before the application of PCA and PLS-DA, the quantitation results were autoscaled.

3. Results and discussion

In total, 37 volatile compounds were identified in the cider samples (Table 2). Partial least square-discriminant analysis (PLS-DA) was applied to evaluate the influence of each treatment (apple variety,

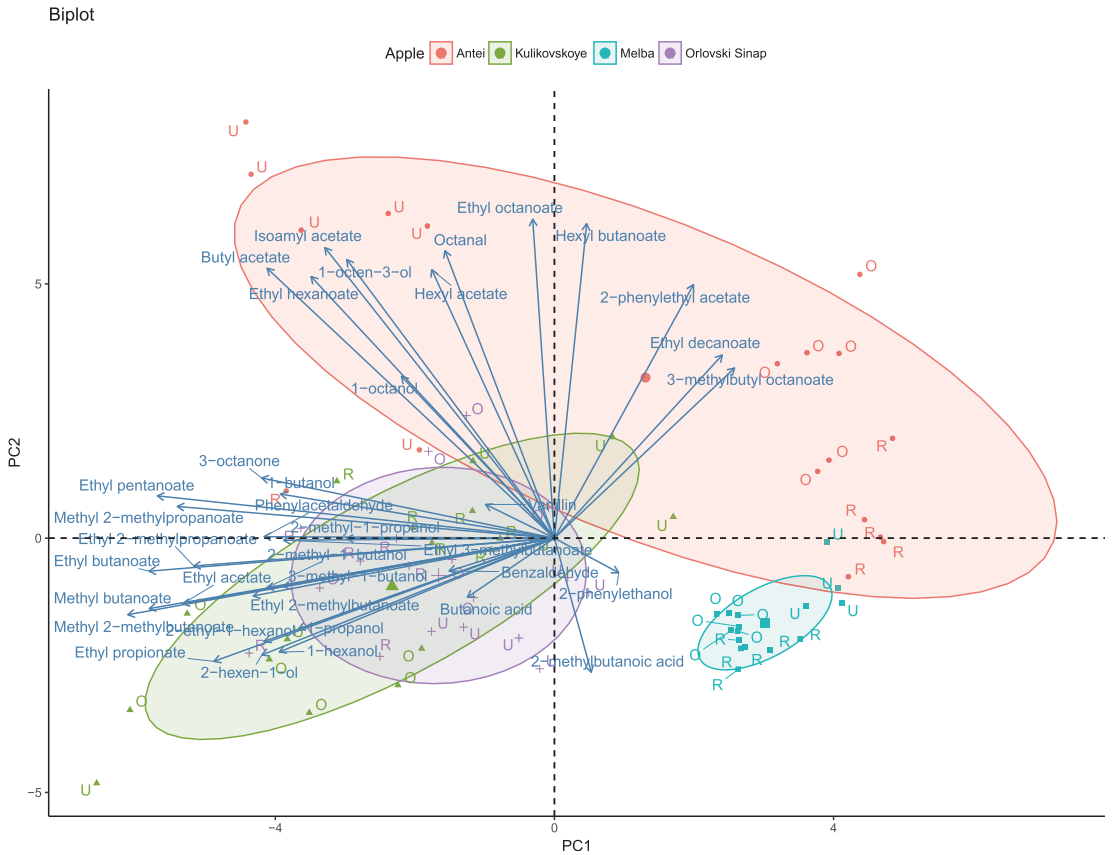

Fig. 3. Principal component analysis biplot. Samples made with Antei apples are represented with color red, Kulikovskoye apples – color green, Melba apples – color blue, Orlovski Sinap apples – color purple. Letter U represents samples made with underripe apples, letter R – ripe apples, letter O – overripe apples.

maturity level, and yeast strain) on the volatile composition of the samples. For that, one treatment at a time was taken as a predicted variable with the other two acting as replicates. The best visual separation of the samples was achieved when using apple variety as a predicted variable (Fig. 1). Statistical significance of the separation was observed using classification power of each treatment. Based on calculated PLS-DA classification error rates, apple variety also showed the best classification capacity followed by maturity and then yeast strain (Table 3). Since there was no clear grouping of the samples based on the maturity level of apples or yeast strains used for fermentation, no conclusive evidence could be drawn on their significance in terms of the volatile composition of the final product. Based on the grouping according to the apple variety, cider samples obtained with Melba apple variety were found to possess similar volatile profiles due to the proximity of the samples on the plot. Thus, the profiles of ciders made with Melba were influenced the least by apple maturity and yeast strain. This may indicate either a specific dominant aroma profile of Melba apple variety making it difficult to influence the cider by using different yeast strains or a lack of certain nutrients (e.g., amino acids) to be used as metabolic precursors in the formation of volatile compounds. As a contrast to the samples made with Melba variety, Antei variety showed a wide spread of the samples on the plot allowing additional subclustering based on the maturity level of apples. The samples made with unripe apples formed a clear subcluster, whereas the samples made with ripe and overripe samples were more similar to each other. The clusters formed by the samples made with Kulikovskoye and Orlovski Sinap varieties overlapped due to similarities in volatile composition. New PLS-DA analysis was carried out with these samples to get a better insight on clustering patterns (Fig. 2). As seen from Fig. 2, better separation of the samples on the plot was achieved. The samples made with Orlovski Sinap variety were relatively similar to each other. However, with the samples of Kulikovskoye variety, a specific clustering based on the maturity level of apples could be observed – the samples made with overripe apples were separate from the samples made with unripe and ripe apples. In terms of the effect of the maturity level of apples, the samples made with Kulikovskoye variety resulted differently to Antei variety where the samples from unripe apples were more different from the other stages of maturity.

According to overall results of PLS-DA analysis, ciders were grouped based on the apple variety first and only then based on the maturity level or yeast type. These results indicate that the apple variety has a significant influence on the technological aspects of cider production. As was shown with Melba variety, the extent to which any given yeast could potentially influence the volatile composition of the final product will depend on the apple variety used. Similarly, the degree of influence of the maturity stage of apples on the volatile composition of the final product is closely related to an apple variety picked for processing.

To evaluate the correlations between volatile compounds and apple variety, principal component analysis (PCA) was carried out (Fig. 3). According to the PCA biplot, ciders from Melba apple variety had the least diverse volatile composition; the samples formed a tight cluster with

Table 4
Statistical importance (p-values) of the identified volatile compounds in differentiating the sampling according to variety, maturity level, and yeast used. The compounds with significant statistical importance across each viewed variable are marked in **bold**.

Compound name	p-value		
	Maturity	Variety	Yeast
1-propanol	**0.001860**	**0.000050**	0.624665
2-methyl-1-propanol	**0.004188**	**0.048784**	**0.018511**
1-butanol	0.433735	**0.000000**	0.775817
3-methyl-1-butanol	**0.005499**	**0.000004**	0.700366
2-methyl-1-butanol	**0.001963**	**0.000000**	0.849864
2-hexen-1-ol, (E)	0.067941	**0.020647**	0.308530
1-hexanol	**0.007116**	**0.028643**	0.390312
1-octen-3-ol	**0.002924**	**0.000270**	0.676520
2-ethyl-1-hexanol	**0.020197**	**0.000000**	0.719560
1-octanol	0.183813	**0.000117**	0.541413
2-phenylethanol	**0.000015**	**0.000206**	0.583034
Ethyl acetate	0.093975	**0.000004**	0.165060
Methyl-2-methylpropanoate	0.083557	**0.000088**	0.237205
Ethyl propionate	0.589052	**0.000162**	**0.003749**
Methyl butanoate	0.220832	**0.000000**	0.948923
Ethyl-2-methylpropanoate	**0.038091**	**0.000005**	**0.027933**
Methyl-2-methylbutanoate	0.169946	**0.000000**	0.899196
Ethyl butanoate	0.185900	**0.000007**	0.991370
Butyl acetate	**0.034334**	**0.000061**	0.677873
Ethyl-3-methylbutanoate	**0.014082**	**0.000005**	**0.001300**
Isoamyl acetate	**0.009240**	**0.000662**	0.546437
Ethyl-2-methylbutanoate	0.618731	**0.000052**	0.999389
Ethyl pentanoate	0.481229	**0.000000**	0.446490
Ethyl hexanoate	0.099934	**0.000735**	0.747280
Hexyl acetate	0.122128	**0.001101**	0.556462
Hexyl butanoate	0.469554	**0.000000**	0.825437
Ethyl octanoate	0.183897	**0.000000**	0.797699
2-phenylethyl acetate	0.125416	**0.000008**	0.384849
Ethyl decanoate	0.054354	**0.000007**	0.620213
3-methylbutyl octanoate	0.237256	**0.000014**	0.313501
Butanoic acid	0.108986	0.236146	**0.000084**
2-methylbutanoic acid	**0.016010**	**0.001982**	**0.009141**
Benzaldehyde	**0.038750**	**0.007941**	0.890327
3-octanone	**0.000041**	**0.015216**	0.881535
Octanal	0.102392	**0.000253**	0.446443
Phenylacetaldehyde	**0.006131**	**0.000000**	0.837806
Vanillin	0.188694	0.235916	0.670147

a substantial similarity in the volatile profiles which corresponds to the results obtained using PLS-DA approach. Ciders made with Kulikovskoye, and Orlovski Sinap varieties had similar but the most diverse volatile compositions with different alcohols, aldehydes, and esters contributing to the aroma. As per PLS-DA results, subclustering of the samples made with unripe Antei apples was also observed on the upper left corner of Fig. 3. These samples had higher contents of different acetates like isoamyl acetate, hexyl acetate, and butyl acetate.

The influence of apple variety, maturity level, and yeast strain on the relative content of identified volatile compounds was observed using p-values (Table 4). According to the results, most of the compounds that distinguished the samples from one another were associated with apple variety, which corresponded to the conclusions made based on the results of PLS-DA. The apple variety and juice obtained from it can be viewed as a nutritional base for fermentation that directly affects the volatile composition and properties of cider. The difference between the apple varieties could, for example, come at the expense of initial nitrogen content. According to Santos et al. (2015), the initial content of nitrogen-containing compounds in apples is mainly composed of amino acids, especially aspartic acid, glutamic acid, asparagine, serine, and proline. The content of amino acids and yeast assimilable nitrogen can, in turn, be tied to the production of desired volatile compounds (e.g., esters) (Lambrechts and Pretorius, 2000; Bell and Henschke, 2005; Belda et al., 2017). Most of the alcohols identified in the samples, some esters (butyl acetate, ethyl-3-methylbutanoate, and isoamyl acetate), 3-methylbutanoic acid, 3-octanone, benzaldehyde, and phenylacetaldehyde were associated with the maturity stage of the apples used in processing. Some of the compounds (2-methylbutanol, 3-methylbutanol and hexanol) were previously detected by Sapers et al. (1977) in ripe McIntosh apples as indicators of ripeness. The mentioned compounds could have either originated from the biochemical changes during ripening or formed from specific precursors developed during ripening (e.g., 3-methylbutanol can be utilized as a precursor in the formation of isoamyl acetate) (Eden et al., 1996; Osorio and Fernie, 2013). The number of volatiles significantly influenced (significance level of more than 95%) by the yeast strain used for fermentation was the lowest when compared to the other treatments. Compounds 2-methyl-1-propanol, ethyl propionate, ethyl-2-methyl butanoate, ethyl-3-methyl butanoate, butanoic acid, and 2-methylbutanoic acid were found to be associated with the yeast strain used for fermentation and are products of metabolic activity. For example, 2-methyl-1-propanol is a product of amino acid (valine) catabolism (Bigelis et al., 1983). Acids (butanoic acid, 2-methylbutanoic acid) are formed as a by-product of either fatty acid metabolism or oxidation of intermediate compounds in amino acid catabolism (Alexandre and Charpentier, 1998). Fatty acid esters are reported to be the result of enzymatic activity during lipid biosynthesis (Suomalainen, 1981).

4. Conclusions

Apple variety was the primary attribute influencing the volatile composition of apple cider. The effect of yeast strains and the maturity of apples was highly variety-specific. Ripe and overripe apples imparted mostly similar aroma profiles; however, with Kulikovskoye variety, the cider from overripe apples differed the most from the others. The volatile profiles of the samples made with Melba variety were the least influenced by the maturity level of apples and yeast strains used for the fermentation.

Declarations

Author contribution statement

Julia Rosend: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Rain Kuldjärv: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Sirli Rosenvald: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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Appendix 2

Publication II

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Yeast performance characterisation in different cider fermentation matrices

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Abstract. Nitrogen content management before fermentation is often used in cider production to avoid sluggish fermentations. In addition to enhanced fermentation rates, the proper nitrogen content in the apple must may have an impact on the flavour characteristics of cider. This research aimed to assess yeast performance in two different commercially available musts with similar non-limiting yeast available nitrogen (YAN) content. In addition to fermentation kinetics, volatile ester production by yeast, and sensory properties of the final product were evaluated. The results showed that the fermentation rate and consumption of sugar and nitrogen sources by yeast did not vary between the two different musts. Yeasts consumed more malic acid in the environment of higher initial malic acid content. The content of volatile esters and sensory properties of the final products varied significantly. The occurrence of intense sulfur off-flavour was noted in one of the products.

Key words: cider, fermentation rate, gas chromatography, sensory analysis.

INTRODUCTION

Cider is a beverage made from apples through alcoholic fermentation, although in North-America, the term 'cider' generally refers to cloudy unpasteurized apple juice, and the term 'hard cider' – to a fermented product (Downing, 1995). The alcohol content of cider usually varies between 1.2 to 8.5% by volume (Lea & Piggott, 2003). However, the official alcohol limits are country-specific and defined by local laws. Notably, there are no definitive sensory characteristics for cider since the parameters like that colour, odour, sweetness, and bitterness vary significantly between the apple varieties and regions (Santos et al., 2016). Therefore, in order to obtain a cider with high quality, it is crucial to understand key parameters that contribute to the formation of desirable sensory characteristics during cider fermentation.

The main factors that influence the sensory quality of fermented beverages are the quality of must, the fermentation conditions, and yeast culture (Belda et al., 2017; Laaksonen et al., 2017). One of the primary parameters for successful fermentation is the proper yeast assimilable nitrogen (YAN) content of the must (Swiegers et al., 2008; Seguinot et al., 2018). In apple must, YAN is primarily composed of free amino acids (the so-called free amino nitrogen, FAN) and ammonium ions (Santos et al. 2016; Bourdeau et al., 2018). The contribution of amino acids to YAN can vary across different apple cultivars (Bourdeau et al., 2018).

Even though the average YAN content in apple juice is 120 mg L^{-1}, it can be as low as 30 mg L^{-1} (Drilleau, 1990; Cruz et al., 2002). The lack of initial nitrogen is strongly associated with slow fermentation or incomplete sugar utilisation, i.e. stuck fermentation (Alberti et al., 2014; Boudreau et al., 2018). Nitrogen can be a limiting nutrient in peptide/protein synthesis, sugar transport system, and fermentative activity during the initial stages of alcoholic fermentation (Santos et al., 2016). Initial nitrogen is also essential in terms of aroma generation during fermentation (Carrau et al., 2008; Barbosa et al., 2009; Seguinot et al., 2018) with amino acids acting as metabolic precursors for the biosynthesis of different volatile compounds, e.g. esters (Santos et al., 2016). The lack of nitrogen is often associated with hydrogen sulphide (H_2S) production, although the exact mechanisms of how YAN deficiency influences H_2S formation by yeast are still not clearly defined (Ugliano et al., 2007; Ugliano et al., 2010; Ugliano et al., 2011; Barbosa et al., 2012). Therefore, the management of H_2S in fermented beverages through nitrogen supplementation requires knowledge of both initial YAN and yeast H_2S production characteristics (Ugliano et al., 2011; Barbosa et al., 2012).

Nowadays, the selection of different yeasts (strains of *Saccharomyces cerevisiae* as well as non-*Saccharomyces* yeast species) for fermented beverages production is very diverse. Therefore, the information about yeast performance shared by yeast starter culture manufacturers is of great value for cider producers for choosing the right strain. Today, most yeast manufacturers provide information on the recommended fermentation conditions (e.g. temperature range and optimum temperature, alcohol tolerance), yeast nutrition requirements, expected fermentation duration, and possible sensory properties of the final product. With growing cider production and the increasing number of craft cider producers on the market (The European Cider and Fruit Wine Association, 2018) there is a need for product differentiation in order to gain a competitive advantage while maintaining stable quality. Thus, the proper selection of yeasts along with nutrition management of the fermentation process to ensure sufficient fermentation rate, complex aroma development and avoid off-aroma related defects (e.g., H_2S formation) is becoming more critical.

This study aims to explore and compare the performance of five different commercially available wine yeast strains in fermentation of different apple musts. The free amino nitrogen (FAN) content was brought to a similar level in the used apple musts to observe yeast behaviour in different matrices where free amino nitrogen is not a limiting factor. Yeast performance was observed in terms of fermentation efficiency, consumption of nutrients, production of ethyl and acetate esters, and overall sensory properties of the final product, including H_2S.

MATERIALS AND METHODS

Cider fermentation

This study made use of two different apple musts: M1 (Aspall, Suffolk, United Kingdom; Brix 12.8%, pH 3.28, titratable acidity 6.7 g L^{-1} in malic acid equivalents, initial FAN 69.48 ± 2.00 mg L^{-1}) and M2 (Döhler, Darmstadt, Germany; Brix 11.7%, pH 3.18, titratable acidity 3.8 g L^{-1} in malic acid equivalents, initial FAN 51.21 ± 1.84 mg L^{-1}). Free amino nitrogen content in both matrixes was brought to a similar content of 80 ± 2 mg L^{-1} by using organic yeast nutrient (Fermaid K; Lallemand Inc.). The musts (400 mL) were distributed into sterile 500 mL fermentation bottles.

Each bottle was inoculated with a chosen rehydrated yeast starter culture, according to the manufacturer's (Lallemand, Inc.) instructions. The starter cultures used in this study were as follows: Y1 (*Torulaspora delbrueckii*), Y2 (*Saccharomyces cerevisiae*; white wine yeast selected through directed breeding), Y3 (*Saccharomyces cerevisiae var. bayanus*; red and white wine yeast for demanding conditions), Y4 (*Saccharomyces cerevisiae var. bayanus*; sparkling wine yeast), and Y5 (*Saccharomyces cerevisiae*; red, rosé, and white wine yeast selected through evolutionary adaptation).

Inoculated bottles (5×10^6 CFU mL^{-1}) were sealed using screw caps with septums pierced with syringe needles (19G × 1 ½, 1.1 × 40 mm; Terumo Medical Corporation) and coupled with microfilters to vent carbon dioxide. Fermentations were carried out at 18 ± 1 °C by following carbon dioxide production by weighing of the fermentation bottles once per day on a daily basis. Fermentations were considered completed when the mass loss due to carbon dioxide dissipation could not be observed anymore (approx. 336 hours). Samples were withdrawn every second day for subsequent analysis. For each experiment, two parallel fermentations were performed.

Sugar and malic acid content

Sugars and malic acid content during the fermentation was analysed using high-performance liquid chromatograph (Alliance HPLC) equipped with BioRad HPX87H column, RI and UV detectors. Prior to analysis, the samples were diluted 1:10 with MilliQ water and filtered (Whatman Spartan 13; Dassel, Germany). 0.005 M H_2SO_4 solution was used as mobile phase with a flow rate of 0.6 mL min^{-1}. Standard solutions of fructose, glucose, sucrose, and malate were used for calibration curves.

FAN content

The FAN content at different stages of fermentation was assessed using DNFB (dinitrofluorobenzene) method which is based on the reaction of amino groups of free amino acids with 2,4-dinitrofluorobenzene. The reaction derivatives were subsequently measured by spectrophotometry at 420 nm. Standard solutions of glycine with known nitrogen content were used to obtain calibration curve. Results were expressed as mg L^{-1} in glycine equivalents. Three analytical replicates were used for each sample.

GC-TOF-MS analysis of volatile esters

Headspace – solid phase microextraction (HS-SPME) was used for the extraction of volatile compounds. For that, 50 µL of each sample was diluted with 950 µL of distilled water; 2-chloro-6-methylphenol (100 µg L^{-1}) was used as an internal standard for quantitation purposes. Vials were pre-incubated at 45 °C for 5 minutes. An SPME

fiber (30/50 µm DVB/Car/PDMS Stableflex, length 2 cm; Supelco, Bellefonte, PA, USA) was used to extract the volatile compounds from the headspace for 20 minutes.

GCT Premier 6890N gas chromatograph system (Agilent Technologies, Santa Clara, CA, USA) equipped with TOF mass spectrometer (Waters, Milford, MA, USA) and a DB5-MS column (30 m length × 0.25 mm i.d. × 1.0 µm film thickness; J&W Scientific, Folsom, CA, USA) was used with helium as a carrier gas at a flow rate of 1.0 mL min^{-1}. The oven was programmed to ramp up from 40 °C at a rate of 7.5 °C min^{-1} to a final temperature of 280 °C with an additional holding time of three minutes (total run time 35 min). Mass spectra were obtained at an ionization energy of 70 eV and a scan speed of 10 scans s^{-1}, with a mass scan range of 35 to 350 Da. Two analytical replicates were used for each sample.

A presence of selected ethyl (medium-chain fatty acid ethyl esters) and acetate esters was monitored across the samples. These two groups of esters are synthesized through different pathways and play a primary role in the perception of desired fruity attributes in fermented beverages (Saerens et al., 2010). A complete list of the compounds of interest and their odour descriptions is provided in Table 1. Accurate identification of the compounds was achieved using respective analytical standards. The concentrations were expressed in µg L^{-1} of internal standard equivalents.

Table 1. A list of selected volatile esters and their odour descriptions

Ester	CAS	Odour description*
Ethyl acetate	141-78-6	Ethereal, fruity, green
Isobutyl acetate	110-19-0	Fruity, ethereal, banana
Isoamyl acetate	123-92-2	Fruity, banana
Butyl acetate	123-86-4	Ethereal, fruity, banana
Hexyl acetate	142-92-7	Fruity, green apple, banana
Ethyl butanoate	105-54-4	Fruity, pineapple, cognac
Ethyl hexanoate	123-66-0	Fruity, pineapple, waxy, green
Ethyl octanoate	106-32-1	Fruity, wine, apricot, banana
Ethyl decanoate	110-38-3	Waxy, fruity, apple, grape
Ethyl dodecanoate	106-33-2	Waxy, floral, soapy

*According to www.thegoodscentscompany.com

Sensory analysis

Descriptive analysis was used in the study to assess the sensory properties of the cider samples. A local sensory panel of 8 highly trained assessors with previous experience in working with cider samples carried out the analysis. There was no pre-session to familiarize assessors with the samples. The working linear scale was established at 0–15, and relative intensities were used. A complete list of assessed attributes and their definitions is provided in Table 2. Additional commentary (e.g., the presence of off-flavours) was encouraged if necessary. Samples were assessed independently by each assessor. Prior to the assessment, all samples were adjusted for sweetness to balance out the sour taste since the secondary malolactic fermentation was not carried out. For that, 3% of diluted sucrose was added. All samples were encoded with a randomized three-digit numerical key.

Table 2. A complete list of sensory attributes and their definitions

Attribute	Description
Odour	
Overall intensity	Overall strength of the perceived odour
Fruity	Strength of all fruity odours (excluding apple)
Cooked apple	Strength of odours characteristic to cooked apples
Apple-like	Strength of odours characteristic to fresh apples
Sweet	Strength of all sweet odours
Sour	Strength of all sour odours
Taste	
Fruity	Strength of overall sensation characteristic to fruits (excluding apples)
Cooked apple	Strength of overall sensation characteristic to cooked apples
Apple-like	Strength of overall sensation characteristic to fresh apples
Sweet	Strength of overall sweet sensation
Sour	Strength of overall sour sensation
Bitter	Strength of overall bitter sensation
Astringency	Strength of overall drying sensation

Data processing

The results of chemical analysis were averaged across biological and analytical replicates. The analysis of variance was performed using ANOVA (R software 3.4.0; Boston, MA, USA), and $p < 0.05$ was considered statistically significant. The results of sensory analysis were statistically evaluated by principal component analysis (PCA) using OriginPro software (OriginLab; Northampton, MA, USA). Prior to the application of PCA, the results were autoscaled.

RESULTS AND DISCUSSION

Fermentation kinetics with different yeasts (Fig. 1a, 2b) did not seem to be dependent on the apple must. Each yeast strain showed similar performance (i.e, fermentation duration, maximum speed of carbon dioxide production) in both matrices. Various authors have previously linked fermentation activity to the nitrogen content of the must (Santos et al., 2016; Boudreau et al., 2017; Lemos Junior et al., 2017; Seguinot et al., 2018). In this study, the nitrogen content of both musts was adjusted to the same value (80 ppm). Thus, the same nitrogen content might be the reason behind the absence of visible differences in fermentation rates between the studied musts M1 and M2.

The composition of initial fermentable sugars in the musts and residual sugars in the samples after the fermentation is summed up in Table 3. Up to 60% of initial sugar content in both apple musts consisted of fructose. The main difference in residual sugar consumption between the musts fermented with different yeasts was due to the different ability of the studied yeasts to consume fructose. For example, residual fructose concentrations in fermentations with *T. delbrueckii* (Y1) were up to 7 times higher than with the other studied strains. In general, sugar consumption patterns for the same strains were quite similar in the two studied musts, and small differences in residual amounts could instead be attributed to the initial Brix%.

Comparing fermentation kinetics and fructose consumption (Fig. 1a, 2b, Table 3) it becomes evident that yeasts with more fructophilic characteristics conduct a more intense fermentation. Thus, intrinsic differences in yeast fructose uptake could be utilised in cider production for process optimisation.

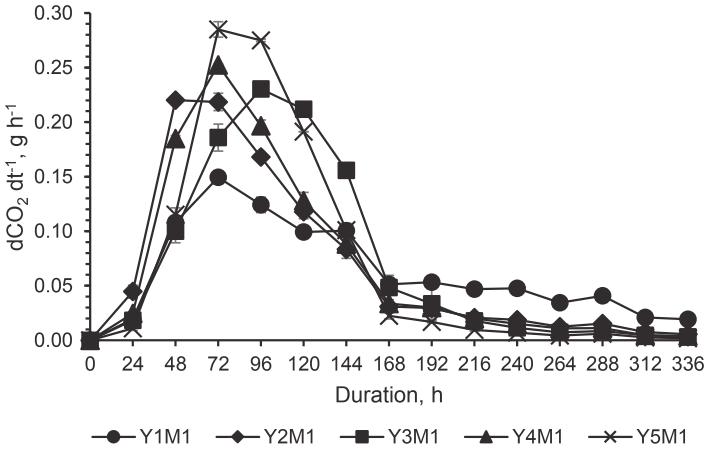

Figure 1a. Fermentation kinetics with different yeasts (Y1-5) in must M1.

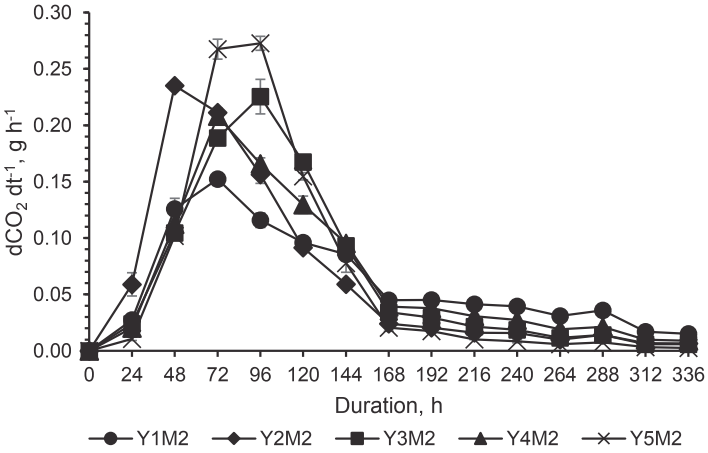

Figure 1b. Fermentation kinetics with different yeasts (Y1-5) in must M2.

Table 3. Composition of initial sugars in the musts (M) and residual sugars at the end of fermentation with different yeasts (YM) and musts ($p < 0.05$)

Amount, g L^{-1}	Glucose	Fructose	Sucrose	TOTAL
M1	25.61 ± 2.18	74.93 ± 2.88	20.35 ± 3.02	120.89 ± 8.08
M2	26.83 ± 3.65	70.69 ± 2.93	16.33 ± 3.94	113.85 ± 10.52
Y1M1	0.67 ± 0.03	8.85 ± 0.94	1.15 ± 0.31	10.67 ± 0.96
Y1M2	1.12 ± 0.33	8.74 ± 0.86	1.27 ± 0.39	11.13 ± 0.79
Y2M1	0.45 ± 0.02	1.81 ± 0.18	0.60 ± 0.06	2.86 ± 0.58
Y2M2	1.29 ± 0.20	3.12 ± 0.29	1.19 ± 0.23	5.60 ± 0.93
Y3M1	0.24 ± 0.05	1.22 ± 0.17	0.53 ± 0.08	1.99 ± 0.12
Y3M2	1.31 ± 0.19	2.17 ± 0.40	1.27 ± 0.27	4.75 ± 0.98
Y4M1	0.22 ± 0.04	2.07 ± 0.18	0.57 ± 0.15	2.86 ± 0.22
Y4M2	1.20 ± 0.25	3.72 ± 0.49	1.15 ± 0.14	6.07 ± 0.81
Y5M1	0.23 ± 0.02	1.11 ± 0.10	0.60 ± 0.03	1.94 ± 0.19
Y5M2	1.36 ± 0.12	1.16 ± 0.17	1.70 ± 0.18	4.22 ± 0.50

A decrease in malic acid concentration was observed to some extent as a result of the fermentation process regardless of the must used (Fig. 2). The consumption of malic acid by yeasts was significantly higher in the must M1 where the initial concentration was approximately two times bigger than in the must M2 (5.02 ± 0.09 g L^{-1} vs 3.03 ± 0.13 g L^{-1}). The most drastic decrease of almost 44% in the malic acid concentration was observed in the case of *S. cerevisiae var. bayanus* (Y3) in M1 must. It has been previously reported that up to 50% of extracellular malic acid can be metabolised by Saccharomyces sp. yeasts during alcoholic fermentation of wine (Barnett & Kornberg, 1960; Delfini & Formica, 2001). The effect of more significant consumption of malic acid at its higher concentration by yeast could potentially be attributed to otherwise low affinity to malic acid of yeast malic enzyme (Mae1p) responsible for its decarboxylation to pyruvate (Volschenk et al., 2003). The K_m of Mae1p enzyme for malic acid is reported to be 50 mM (Boles et al., 1998) or approximately 6.7 g L^{-1} which is close to the initial concentration in M1.

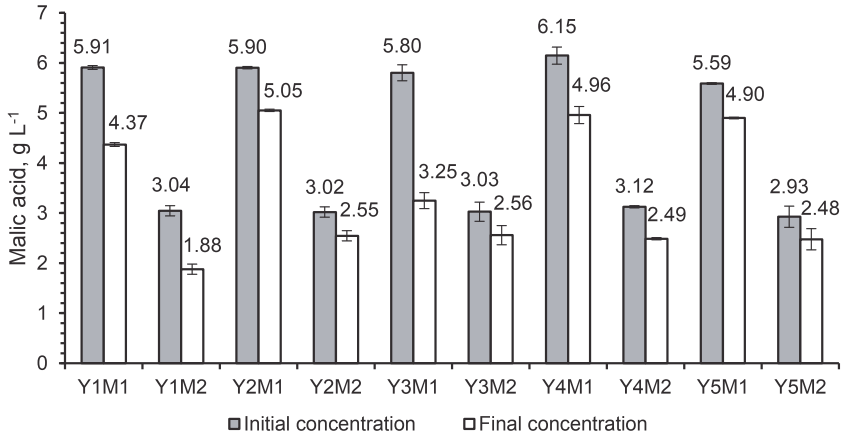

Figure 2. Malic acid consumption by different yeasts (Y1-5) in the two apple musts (M1 and M2) used in the study ($p < 0.05$).

The assimilation of free amino nitrogen (FAN) by the yeast strains used in this study is shown in Fig. 3a, 3b. Notably, complete depletion of FAN was not observed with any of the yeasts regardless of the fermentation matrix. The intense FAN consumption occurred in case of all strains within 2–4 days. The residual concentration of FAN at the end of fermentation was similar regardless of the yeast and must used although differences occurred in the FAN consumption rates. Notably, the consumption rates of FAN by yeasts used in this study did not always correlate with their fermentation activity. However, there might be a potential difference between yeasts with regards to their preference for specific amino acids for synthesis of volatile compounds (Lambrechts & Pretorius, 2000; Santos et al., 2015; Belda et al., 2017; Gobert et al., 2017; Fairbairn et al., 2017).

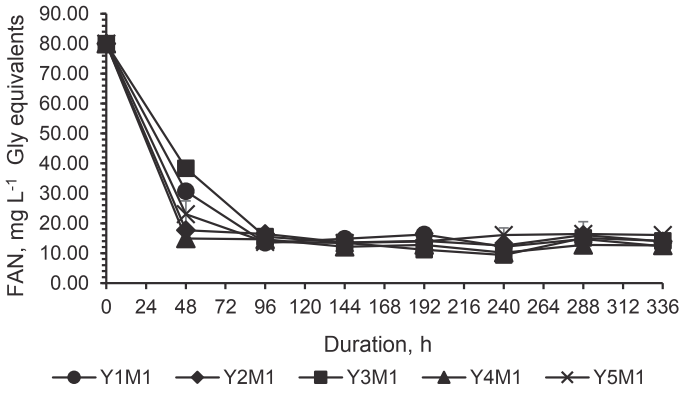

Figure 3a. Free amino nitrogen consumption by yeasts (Y1-5) during cider fermentation in the must M1.

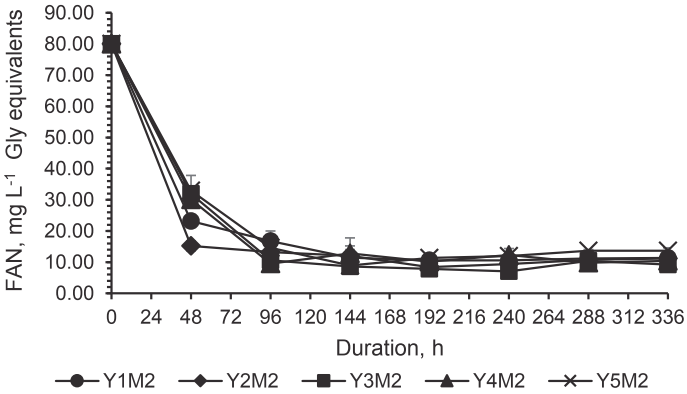

Figure 3b. Free amino nitrogen consumption by yeasts (Y1-5) during cider fermentation in the must M2.

In total, the concentration of 10 ethyl and acetate esters was monitored in the cider samples produced with five different yeasts and two musts (Table 4). Isobutyl acetate, butyl acetate, and ethyl dodecanoate were not detected in any of the analysed samples. *T. delbrueckii* (Y1) showed the lowest production of esters except isoamyl acetate. Others have previously noted significant production isoamyl acetate in *T.delbrueckii/S.cerevisiae* mixed inoculation during winemaking trials (Herraiz et al., 1990; Zhang et al., 2018). In addition to *T. delbrueckii*, *S. cerevisiae var. bayanus* (Y4) also produced considerably more (up to 3.5 times) isoamyl acetate in comparison to the others. The formation of three esters – ethyl acetate, isoamyl acetate, hexyl acetate depended on the matrix used. Both ethyl acetate and isoamyl acetate were produced in considerably (up to 7 and 2 times, respectively) higher concentrations by most strains in the must M2. Hexyl acetate, on the other hand, had higher relative concentrations in the samples made with the must M1. All three aforementioned esters are yeast metabolites produced during fermentation process. Ethyl acetate is formed in anaerobic glucose metabolism from acetyl-CoA with glutamate, methionine and cysteine precursors in its synthesis pathway (Nordström, 1962). Isoamyl acetate can either be created from amino acids (leucine, valine) or de novo synthesised from isoamyl alcohol (Eden, et al., 1996; Plata et al., 2003). Hexyl acetate originates from C6 alcohols and aldehydes (e.g., hexanol, 2-hexenol, 2-hexenal) (Dennis et al., 2012). Thus, in terms of volatile composition development of the final product, other intrinsic properties of the fermentation matrix than the YAN content should also be taken into account. The amount of ethyl esters depended on the particular yeast-must combination. For example, Y2 (*S.cerevisiae)* and Y4 (*S. cerevisiae var. bayanus)* favoured the production of ethyl decanoate in M1; Y3 (*S. cerevisiae var. bayanus)* and Y5 (*S. cerevisiae*)– in M2.

Table 4. Relative concentration (in μg L^{-1} of IS equivalent) of selected esters at the end of fermentation with different yeasts (Y1-5) and apple musts (M1 and M2) ($p < 0.05$)

	Y1M1	Y2M1	Y3M1	Y4M1	Y5M1
Ethyl acetate	93.33 ± 18.86	95.00 ± 16.50	53.33 ± 0.00	456.67 ± 4.71	350.00 ± 4.71
Isobutyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.
Isoamyl acetate	3,970.00 ± 381.84	2,646.67 ± 405,41	2,160.00 ± 341.50	4,933.33 ± 150.85	1,640.00 ± 18.86
Butyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.
Hexyl acetate	n.d.	213.33 ± 37.71	156.67 ± 4.71	120.00 ± 18.86	580.00 ± 107.50
Ethyl butanoate	115.56 ± 15.40	248.89 ± 30.79	253.33 ± 18.86	222.22 ± 30.79	120.00 ± 18.86
Ethyl hexanoate	253.33 ± 56.57	1,484.44 ± 253.45	1,564.44 ± 320.37	1,366.67 ± 250.51	2,862.22 ± 348.03
Ethyl octanoate	24.44 ± 3.85	1,733.33 ± 188.56	1,960.00 ± 320.56	1,066.67 ± 149.27	6,680.00 ± 358.27
Ethyl decanoate	n.d.	66.67 ± 18.86	186.67 ± 37.71	n.d.	328.89 ± 55.51
Ethyl dodecanoate	n.d.	n.d.	n.d.	n.d.	n.d.

Table 4 (continued)

	Y1M2	Y2M2	Y3M2	Y4M2	Y5M2
Ethyl acetate	255.56 ± 56.70	466.67 ± 18.86	358.33 ± 21.21	1,273.33 ± 122.57	516.67 ± 14.14
Isobutyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.
Isoamyl acetate	7,730.00 ± 183.85	3,084.44 ± 111.02	2,826.67 ± 270.64	7,533.33 ± 546.83	2,222.22 ± 114.96
Butyl acetate	n.d.	n.d.	n.d.	n.d.	n.d.
Hexyl acetate	n.d.	n.d.	n.d.	n.d.	26.67 ± 0.00
Ethyl butanoate	166.67 ± 24.04	266.67 ± 37.41	228.89 ± 19.25	226.67 ± 56.57	186.67 ± 37.71
Ethyl hexanoate	66.67 ± 18.86	1,048.89 ± 181.52	1,813.33 ± 226.27	4,160.00 ± 37.71	3,040.00 ± 263.99
Ethyl octanoate	n.d.	4,231.11 ± 858.46	2,106.67 ± 462.65	19,866.67 ± 1,395.36	7,680.00 ± 1,244.51
Ethyl decanoate	n.d.	106.67 ± 0.00	95.00 ± 16.50	623.33 ± 61.28	266.67 ± 57.61
Ethyl dodecanoate	n.d.	n.d.	n.d.	n.d.	n.d.

According to the ANOVA, statistically significant differences ($p < 0.05$) were obtained for most of the sensory attributes assessed with the exception of overall odour intensity ($p = 0.61$), 'apple-like' in odour ($p = 0.08$), 'apple-like' in taste ($p = 0.10$), sourness in odour ($p = 0.19$), and astringency ($p = 0.98$). The results of sensory analysis with the exception of statistically insignificant parameters were then subjected to principal component analysis (PCA). The obtained PCA biplot is presented in Fig. 4. The Principal Component 1 accounted for 54.54% of the differences between the samples; the Principal Component 2 – for 17.18%. Based on the biplot, ciders produced with the apple must M2 possessed strong correlation with fruitiness, sweetness, and 'cooked apple' characteristic. However, the samples made with the apple must M2 contained more residual sugar at the end of fermentation than the samples made with M1 (Table 3). This might have partially contributed to an enhanced perception of sweetness and fruitiness. The ciders made with the must M1 strongly correlated with sourness, which corresponds well with its higher titratable acidity and higher malic acid content in finished ferments. Correlation with other parameters in these samples is negative, which means the intensities were considerably lower in comparison with the samples made with M2.

In the course of sensory analysis, the ciders also received additional commentary by the panel members. According to this, all samples made with the must M1 had an off-flavour described as 'animalic' and 'sulfur'. The occurrence of the off-flavour of significant intensity in the ciders made with M1 could also mask the fruitiness of these ciders. Based on the description, the most likely source of this off-flavour was proposed to be hydrogen sulphide. Indeed, the overproduction of hydrogen sulphide is regarded as one of the main challenges in cider production (Boudreau et al., 2017). The accumulation of hydrogen sulphide during fermentation can be related to multiple different factors such as susceptibility of yeast strain to produce it as well as nutritional composition of the environment (e.g., YAN and vitamins content) (Boudreau et al., 2017). The difference in initial YAN content was not the case in this study as it was

brought to the same level with Fermaid nutritional supplement prior to the start of fermentation. However, the difference in intrinsic amino acid and/or vitamin composition in the apple must could still be a key factor in off-flavour production. For example, Bohlscheid et al. (2011) have noted that biotin and pantothenic acid deficiency in the fermentation environment could result in excessive production of hydrogen sulphide by yeasts.

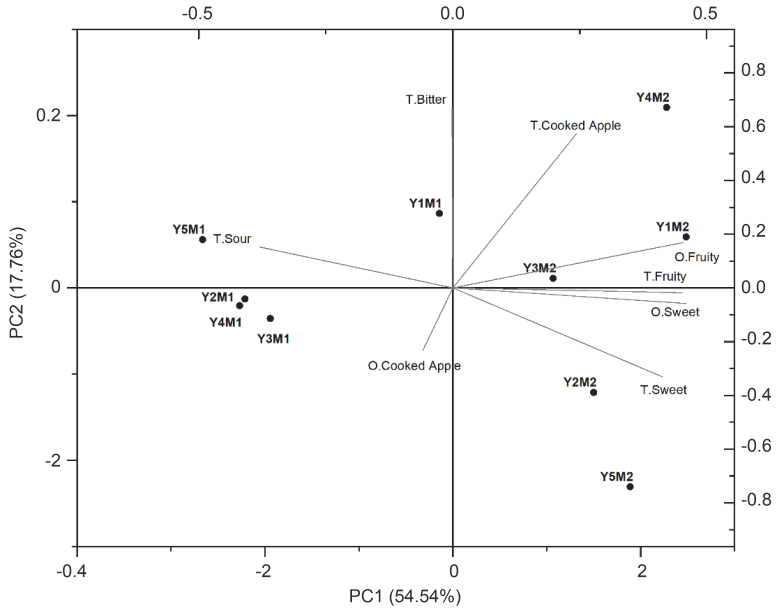

Figure 4. Grouping of the samples on the PCA biplot according to statistically significant ($p < 0.05$) sensory properties perceived at the end of fermentation with each combination of yeast (Y1-5) and fermentation matrix (M1 and M2).

CONCLUSIONS

Yeast performance in the fermentation of cider from the apple musts with similar nitrogen content was studied in this work. The differences in yeast fermentation activity, consumption of nutrients (sugars, malic acid, nitrogen), production of selected volatile esters and development of sensory properties were characterised. Based on the results the fermentative activity of yeasts and consumption of certain nutrients like fructose and nitrogen did not depend on the must. Malic acid consumption was found to depend on the initial malic acid content in the environment. The main differences between the two apple musts used for the fermentations were related to the content of volatile esters and sensory properties of cider. Despite the similar level of initial YAN content in the musts, the yeast performance in terms of the development of sensory properties was not the same. Lower volatile ester formation and synthesis the off-flavour was noted with one of the musts used for the experiments. Thus, initial nitrogen content adjustment did not

guarantee a good quality of the finished product. Since the sensory properties are the driving force behind consumer behaviour, a proper approach to off-flavour management should be implemented. Further research is required to establish the factors/combination of factors that could allow for simultaneous reduction of off-flavour production risks and an increase in the production of volatile esters in cider.

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Appendix 3

Publication III

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The Effect of Apple Juice Concentration on Cider Fermentation and Properties of the Final Product

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Abstract: European legislation overall agrees that apple juice concentrate is allowed to be used to some extent in cider production. However, no comprehensive research is available to date on the differences in suitability for fermentation between fresh apple juice and that of reconstituted apple juice concentrate. This study aimed to apply freshly pressed juice and juice concentrate made from the same apple cultivar as a substrate for cider fermentation. Differences in yeast performance in terms of fermentation kinetics and consumption of nutrients have been assessed. Fermented ciders were compared according to volatile ester composition and off-flavor formation related to hydrogen sulfide. Based on the results, in the samples fermented with the concentrate, the yeasts consumed less fructose. The formation of long-chain fatty acid esters increased with the use of reconstituted juice concentrate while the differences in off-flavor formation could not be determined. Overall, the use of the concentrate can be considered efficient enough for the purpose of cider fermentation. However, some nutritional supplementation might be required to support the vitality of yeast.

Keywords: cider; juice concentrate; fermentation; gas chromatography; volatile esters; hydrogen sulfide

1. Introduction

Cider is a beverage produced by fermenting apple juice. The juice for the fermentation can be obtained in two different ways—either by having the juice freshly pressed and clarified or by reconstituting a juice concentrate to desired properties. The use of juice concentrates in the cider industry possesses several economic advantages, especially where mass production is concerned. Concentrates are available in large quantities, easily transportable (stable), and more affordable when purchased in bulk. However, freshly pressed apple juice allows more options due to blending juices from different apple varieties whereas the choice between different available apple cultivar concentrates is limited.

The specific definition for cider is country dependent and also legislation on cider differs—some countries have strict legislation and cider is well defined, whereas other countries are still developing specific legislation for cider. In Estonia, cider can be prepared from apple juice obtained from freshly pressed apples or apple juice concentrate with no particular limit on the percentage of apple components [1]. On the other hand, for example, French cider production policy declares that the product named "cider" must derive from the fermentation of fresh apple juice or a mixture of several juices. In this instance, apple juice may be partially obtained from the concentrate only if the latter

does not exceed 50% by volume [2]. In the UK, the pre-fermentation mixture should contain at least 35% apple juice [3].

The number of studies on cider properties and their development during fermentation has recently increased to a considerable extent. Most of these studies, however, have been largely focused on fermentation management aspects—the use of different apple varieties, ripening stage, yeast strains, and nitrogen supplementation [4–19]. There are no studies available that would directly compare freshly pressed apple juice and the concentrate made from the same juice pressing batch. This kind of research could potentially help to evaluate the effect of the juice concentration process on the fermentation and properties of the final product. Despite the economic advantages of the use of commercial apple juice concentrates in the cider industry, the definitive research on the differences in the performance is yet to be properly explored.

The purpose of this research was to provide a comparison of fresh apple juice and concentrate as a substrate for cider fermentation based on yeast performance and properties of fermented cider in terms of fermentation kinetics, volatile ester production, and off-flavor development potential.

2. Materials and Methods

2.1. Apple Juice Clarification and Concentration

This study used apple juice (Brix 9.80%; pH 3.38; titratable acidity 3.93 g L^{-1} in malic acid equivalent) industrially pressed from ripe Antei apples (150 L). Half of the juice (75 L) was clarified and concentrated. Clarification was based on the method described by Grampp et al. (1978) [20]. The juice was warmed to 40 °C from the storage temperature; 5 g hL^{-1} of commercial pectinase (Rapidase; Oenobrands SAS, Montferrier-sur-Lez, France) and 5 g hL^{-1} of glucoamylase (AMG 300L; Novozymes, Copenhagen, Denmark) were added. After 30 min at 40 °C, 100 mL hL^{-1} of silica sol (Baykisol® 30; E. Begerow GmbH & Co., Langenlonsheim, Germany) and 10 g hL^{-1} of dissolved gelatin (SIHA Clarifying Gelatine; E. Begerow GmbH & Co., Langenlonsheim, Germany) were added as flocculants. Flocculation occurred within 1.5 h at 40 °C. Clarified juice was filtered and concentrated using a vacuum concentration system (Didacta Italy; Fruit juice and syrup line, code 640,022). Due to volume restrains, the process was performed in three batches of 25 L. Each batch was concentrated separately from 9.8% to 30% Brix. The batches were then pooled and concentrated to the final Brix value of 67.8%. For fermentation, the concentrate was diluted with distilled water to 10% Brix (pH 3.45; titratable acidity 3.97 g L^{-1} in malic acid equivalents).

2.2. Free Amino Acid Content

Free amino acids were measured in the juice and reconstituted concentrate by UPLC (Acquity UPLC; Waters Corp., Milford, MA, USA) equipped with AccQ·Tag Ultra column and a UV detector according to the method applied by Lahtvee et al. (2014) [21]. Before analysis, the samples were diluted 1:2 with MilliQ, filtered (Whatman Spartan 13; Dassel, Germany), and derivatized using AccQ·Tag reagent. AccQ·Tag Ultra eluent B (linear gradient from 0% to 100%) was used as mobile phase at a flow rate of 0.3 mL min^{-1}. Three analytical replicates were measured for each sample.

2.3. Cider Fermentation

Apple juice and reconstituted apple juice concentrate (400 mL) were distributed into sterile 500 mL Duran bottles (DWK Life Sciences; Mainz, Germany). Each bottle was inoculated (5×10^6 CFU mL^{-1}) with a chosen yeast starter culture (Lallemand, Inc.; Quebec, QC, Canada). The yeast starter cultures used in the study were as follows: Y1 (*S. bayanus*; red and white wine yeast for demanding conditions), Y2 (*S. cerevisiae*; white wine yeast), Y3 (*S. bayanus*; sparkling wine yeast), Y4 (*S. cerevisiae*; red, rosé, and white wine yeast, selected through evolutionary adaptation), Y5 (*S. cerevisiae* with killer factor), Y6 (*S. cerevisiae*; white wine yeast selected through directed breeding). The fermentation vessels were sealed using screw caps with septums pierced with a syringe needle (20 G × 1″, 0.9 × 25 mm; Terumo

Medical Corporation, Somerset, NJ, USA) coupled with a microfilter (Millex PTFE Vent filters; Merck, Darmstadt, Germany) to vent carbon dioxide. In the headspace of each vessel, a piece of lead acetate paper strip (Whatman; GE Healthcare, Chicago, IL, USA) was placed to monitor hydrogen sulfide formation. The changes in color were assessed visually and grouped in comparison to blank paper strip and relatively to each other on a scale from "no color change" to "high-intensity color formation". It needs to be emphasized that the use of lead acetate paper strips has certain limitations. The change in color intensity of the strip as a function of hydrogen sulfide content in the headspace is not linear which makes even relative quantification impossible—the strips become saturated after which no further change in color can be observed despite hydrogen sulfide still potentially being produced by the yeast. Three nutritional strategies were applied: no additional nutritional supplementation (control); 9 g hL^{-1} of diammonium phosphate (DAP; Sigma Aldrich, St. Louis, MO, USA) at the start of fermentation as inorganic nitrogen supplement; 40 g hL^{-1} of organic supplement Fermaid O (Lallemand Inc, Montreal, QC, Canada). Fermentations were carried out at 18 ± 1 °C and 30 ± 1 °C by following carbon dioxide dissipation (mass loss) every 24 h. Fermentations were completed when the weight of fermentation vessels remained constant for three consecutive days. Experiments were conducted in duplicates. The total number of fermentations was thus 144 (2 fermentation matrices × 6 yeasts × 2 temperatures × 3 nutritional strategies × 2 biological replicates).

2.4. Fructose and Malic acid Content

Fructose and malic acid content before and after fermentation were analyzed using HPLC (Alliance HPLC; Waters Corp., Milford, MA, USA), BioRad HPX87H column, RI and UV detectors. Prior to analysis, the samples were diluted 1:10 with MilliQ and filtered (Whatman Spartan 13; Dassel, Germany). 5.0 mM H_2SO_4 solution was used as mobile phase with a flow rate of 0.6 mL min^{-1}. Standard solutions of fructose and malic acid were used for calibration curves. Two analytical replicates were measured for each sample.

2.5. Analysis of Volatile Esters

The presence of ethyl esters and acetate esters with a primary role in the formation and perception of fruity notes was monitored across the samples. The esters included in the study were as follows: ethyl acetate, isoamyl acetate, hexyl acetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and ethyl dodecanoate [15,22]. Quantification of volatile esters was performed according to the method previously described by Rosend et al. (2019) [15].

The samples were diluted 1:19 with distilled water into a 20 mL vial; 2-chloro-6-methylphenol (100 μg L^{-1}) was added as an internal standard (IS) for quantification. Volatile compounds were extracted by using solid-phase microextraction (DVB/Car/PDMS 30/50 μm Stableflex, 2 cm; Supelco, Bellefonte, PA, USA) at 45 °C for 20 min. Quantification of volatile compounds was performed using a gas chromatograph system (6890N; Agilent Technologies, Santa Clara, CA, USA) equipped with a mass spectrometer (GCT Premier TOF; Waters, Milford, MA, USA) and a DB5-MS column (30 m × 0.25 mm × 1.0 μm; J&W Scientific, Folsom, CA, USA). Helium was used as a carrier gas with a flow rate of 1.0 mL min^{-1}. The oven was programmed to ramp up from 40 °C at a rate of 7.5 °C min^{-1} to a final temperature of 280 °C with an additional holding time of three minutes (total run time 35 min). Mass spectra were obtained at ionization energy of 70 eV and a scan speed of 10 scans s^{-1}, with a mass scan range of 35 to 350 Da. Three analytical replicates were used for each sample. Analytical standards were used for the accurate identification of selected compounds. The concentrations were expressed as μg L^{-1} in the internal standard equivalent.

2.6. Sensory Assessment of Hydrogen Sulfide Related Off-Flavor

In this study, a sensory panel consisting of 8 trained assessors with previous experience in cider assessment carried out the sensory analysis. Prior to the analysis, the panel was familiarized with hydrogen sulfide related off-flavor by using spiked reference samples of different intensities.

The vocabulary was established for the description of the off-flavor ("rotten egg," "cabbage," "sulfuric"). The assessment scale was established as a 4-point category scale: "no perceived off-flavor," "low-intensity off-flavor," "moderate-intensity off-flavor," and "high-intensity off-flavor."

All samples were encoded with a randomized three-digit number. The samples were served in sniffing glasses and presented in sequential monadic order. Two analytical replicates were used for each sample. Prior to sensory analysis, all cider samples were adjusted for sweetness to balance out the sourness since the secondary malolactic fermentation was not carried out. In the course of the analysis, the assessors were asked to assess the intensity of perceived off-flavor (cumulative off-flavor in odor and taste) on the established scale. Samples were assessed independently by each assessor. The results were taken as averages across all replicates and assessors.

2.7. Data Processing

The results of chemical analysis were averaged across biological and analytical replicates. The analysis of variance was performed using R version 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria) and $p < 0.05$ was considered statistically significant. The results of GC-MS analysis were evaluated by partial least squares discriminant analysis (PLS-DA) using the R package 'mixOmics' 6.11.33 and presented as biplots. The response variable was constructed from a combination of juice type and nutritional supplement. Prior to the application of PLS-DA, the quantitation results were autoscaled.

3. Results and Discussion

3.1. Effect of Juice Concentration on Free Amino Acid Composition

The concentration of individual free amino acids (FAA) was measured in the juice and reconstituted apple juice concentrate prior to the start of fermentation and free amino nitrogen content (FAN) content was calculated (Table 1). Knowing the amount of added nutritional supplementation and FAN/NH_4^+ contained therein, the approximate amount of yeast assimilable nitrogen (YAN) in supplemented samples at the start of fermentation was also determined.

The most abundant free amino acids in the apple juice used in the study were asparagine, aspartic acid, and glutamic acid. This correlates with what has been shown previously in other studies [23,24]. Overall statistical significance according to the analysis of variance showed that the juice and reconstituted concentrate can be considered as different ($p < 0.05$), the latter containing much more free amino acids. Thus, the concentration of serine, glycine, threonine, ornithine, lysine, tyrosine, isoleucine, and leucine was deemed statistically significantly higher ($p < 0.05$) and, hence, attributed to the process of juice concentration. The industrial concentration of juices is a high-pressure boiling process that has been reported to possess several adverse effects on the juice. One of them is thermal degradation and evaporation of volatile compounds which may increase the proportion of free amino acids [25–28]. The increase could also be attributed to partial peptidolysis/proteolysis that may have led to the release of individual amino acids. Finally, the increase of free amino acids concentration could also be attributed to a shift in proportions of the components of the juice matrix during reconstitution–the loss of some intrinsic constituents during clarification pre-treatment shifts the proportions in favor of amino acids when the water is added back according to % Brix.

Table 1. Free amino acid (FAA) composition, free amino nitrogen (FAN) content before and yeast assimilable nitrogen (YAN) content after supplementation. Standard deviation is shown (n = 3).

FAA, mg L^{-1}	Juice, 9.8% Brix	Concentrate, 10% Brix
His	1.71 ± 0.32	2.27 ± 0.44
Asn	269.60 ± 24.48	315.03 ± 32.70
Ser	6.28 ± 0.93	9.21 ± 0.82
Gln	3.26 ± 0.60	2.87 ± 0.26
Arg	21.68 ± 1.10	19.36 ± 3.63
Gly	0.32 ± 0.04	1.19 ± 0.21
Asp	38.10 ± 5.26	44.42 ± 4.46
Glu	32.43 ± 3.54	38.15 ± 3.67
Thr	2.75 ± 0.45	3.62 ± 0.33
Ala	8.85 ± 1.56	10.91 ± 1.01
Pro	2.32 ± 0.37	3.22 ± 0.33
Orn	0.27 ± 0.02	0.51 ± 0.10
Cys-cys	0.30 ± 0.03	0.34 ± 0.03
Lys	0.82 ± 0.09	1.93 ± 0.08
Tyr	0.88 ± 0.06	1.04 ± 0.22
Met	1.06 ± 0.16	1.27 ± 0.12
Val	3.93 ± 0.73	4.41 ± 0.46
Ile	2.34 ± 0.45	2.90 ± 0.33
Leu	1.18 ± 0.23	3.21 ± 0.39
Phe	1.97 ± 0.16	1.98 ± 0.21
Trp	0.57 ± 0.10	0.41 ± 0.03
FAN, mg L^{-1}	76.13 ± 7.29	87.91 ± 9.62
+ DAP YAN, mg L^{-1}	95.03 ± 7.29 [1]	106.81 ± 9.62 [1]
+ Fermaid O YAN, mg L^{-1}	93.33 ± 7.29 [2]	105.11 ± 9.62 [2]

[1] Taking into consideration that DAP contains 21% on nitrogen by mass. [2] According to the manufacturer's instructions, 40 g hL^{-1} dose of Fermaid O is equivalent to 17.2 mg L^{-1} of YAN.

3.2. Fermentation Kinetics

The fermentation kinetics results of ciders prepared from either fresh juice or juice concentrate, fermented with different yeast at two different temperatures with or without a nutrient are provided in Figure 1. In all samples, fermentations were successfully completed with no signs of sluggish fermentation.

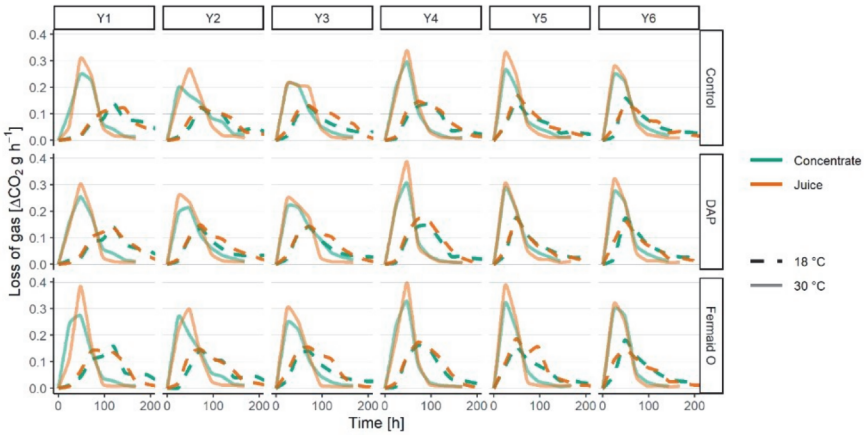

Figure 1. Fermentation kinetics of cider samples prepared from fresh juice or juice concentrate with different yeasts (Y1-Y6) at 18 or 30 °C. The samples done with the concentrate are represented by a green line and with the juice by an orange line. The samples fermented at 18 °C are represented by a dashed line of appropriate color; by a solid line at 30 °C.

Expectedly, some differences between strains could be observed while the temperature was the main factor affecting yeast fermentative activity. The yeast fermented at least two times faster at 30 °C. No particular difference in fermentative activity can be noted between the juice and reconstituted concentrate at 18 °C. However, at 30 °C, the fermentation activity on reconstituted concentrate was slightly inferior to the juice. A loss of certain nutrients during the concentration process might have affected the ability of yeast to cope with higher temperatures but the change is not drastic enough to significantly impact the effectiveness of the process. In this case, the supplementation fills the necessary nutritional gap enough to make the yeast perform better.

3.3. Assimilation of Fructose

Apple juice and its concentrate contained 8.08 ± 0.42% and 8.33 ± 0.26% of fructose before the start of fermentation, respectively. The amount of residual fructose in cider samples fermented under different conditions is shown in Figure 2. Based on the pattern of fructose consumption the yeasts used in the study can be divided into fructophobic and fructophilic ($p < 0.05$). The similar behavior in yeasts has been previously shown by Rosend et al. (2019) [15].

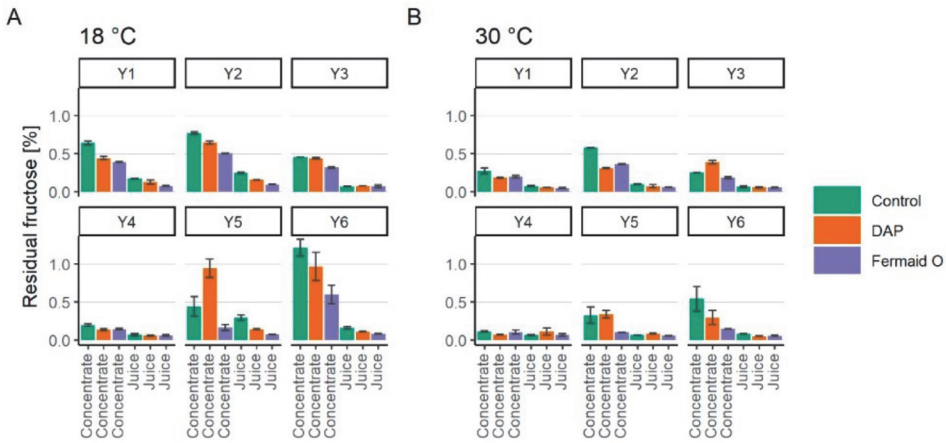

Figure 2. Residual fructose concentration at the end of fermentations prepared from fresh juice or juice concentrate with different yeasts (Y1-Y6) at 18 or 30 °C ($p < 0.05$). The color green represents control samples, orange—supplemented with DAP (diammonium phosphate), purple—supplemented with Fermaid O. (**A**) represents the samples fermented at 18 °C; (**B**)—at 30 °C. Red lines represent initial malic acid content either in juice or reconstituted concentrate.

The yeasts left behind larger amounts of residual fructose in reconstituted concentrate (up to 1.29% residual fructose) in comparison to the fermentations performed with apple juice (up to 0.32% residual fructose). This observation correlates with the differences in fermentation kinetics shown previously (Figure 1). Fructophilic yeast (Y3, Y4) consumed the same amount of fructose regardless of temperature or applied nutritional supplementation. On the other hand, fructose assimilation by fructophobic yeasts (Y1, Y2, Y5, Y6) was slightly improved with nutrient addition, with Fermaid O being more effective than DAP ($p < 0.05$). Higher fermentation temperature was also shown to have a stimulating effect on fructose assimilation by generally fructophobic yeast used in this study. However, it should be emphasized that this might not be the case for other yeast species. For example, low-temperature fermentations have been previously reported as preferred for non-*Saccharomyces* yeasts [29–31].

3.4. Assimilation of Malic Acid

At the start of fermentation, apple juice contained 32.07 ± 2.24 mM of malic acid while the reconstituted concentrate 56.68 ± 4.47 mM. The higher malic acid content in the concentrate was most likely caused by removal/loss in other constitutes contributing to the Brix value during clarification and evaporation.

The concentration of residual malic acid in cider samples fermented under different conditions in comparison to initial amounts are shown in Figure 3. In general, malic acid consumption by the yeast was rather low. Temperature and nutritional supplementation had no stimulating effect on malic acid assimilation. The main difference in malic acid consumption can be noticed only when comparing the juice and reconstituted concentrate ($p < 0.05$). Thus, malic acid consumption was higher in the environment with higher initial malic acid content. This can be tied to previous reports of a more efficient enzymatic conversion of malic acid to pyruvate at higher extracellular malate concentrations [15,32,33]. The K_m of Mae1p enzyme responsible for malic acid decarboxylation is reported at 50 mM which is close to the concentration of malic acid in juice concentrate [32].

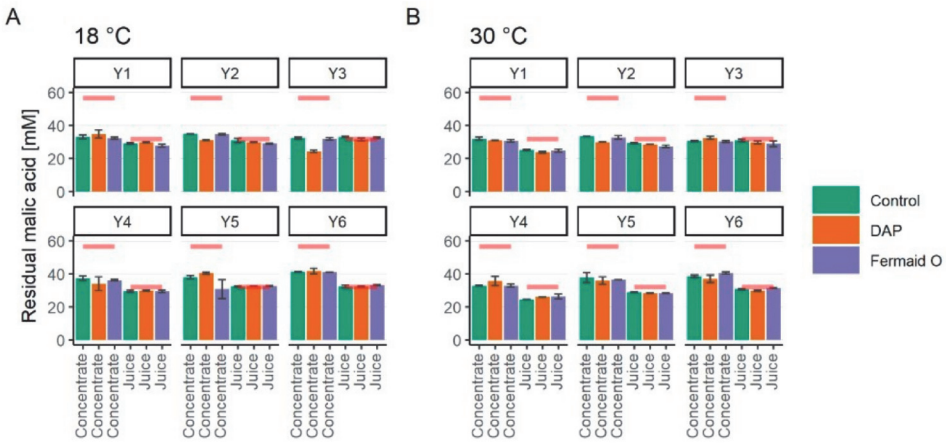

Figure 3. Residual malic acid concentration at the end of fermentation ($p < 0.05$). The color green represents control samples, orange—supplemented with DAP, purple—supplemented with Fermaid O. (**A**) represents the samples fermented at 18 °C; (**B**)—at 30 °C. Red lines represent initial malic acid content either in juice or reconstituted concentrate.

3.5. Production of Volatile Esters

The presence of 8 esters was monitored across the samples. PLS-DA was employed as a statistical approach to observe the differences in ester production (Figure 4). PLS-DA method identifies the differences between the sample groups, which are shown as ellipses on the biplots with overlap indicating similarity. The characteristic differences between the groups are evaluated on an axis that goes through the origin point at (0,0) towards the position of a volatile ester label. The best representation of the results was achieved when viewing the samples according to the yeast species—*S. bayanus* yeasts (Figure 4A) were compared to *S. cerevisiae* yeasts (Figure 4B). According to the biplots, all ciders fermented with *S. cerevisiae* yeasts clustered closely together showing more consistency across the samples in volatile ester composition than *S. bayanus* yeasts.

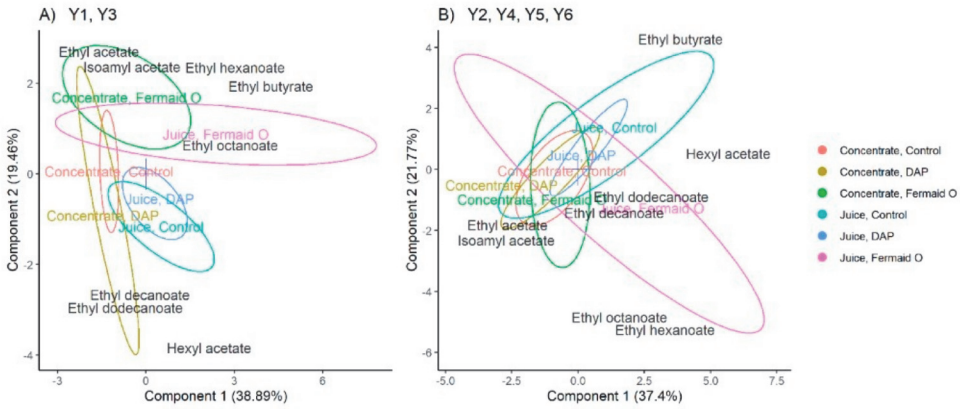

Figure 4. PLS-DA biplots of volatile ester production in cider samples. (**A**) *S. bayanus* yeasts; (**B**) *S. cerevisiae* yeasts. Colors correspond to the combinations of juice type and nutritional supplement. Ellipses indicate confidence region at 0.95 level. The number in parenthesis shows variance captured by the component.

The cider samples made with the concentrate correlated with the highest production of isoamyl acetate (up to 6700 μg L^{-1} in the concentrate; up to 5100 μg L^{-1} in the juice). Isoamyl acetate can be synthesized by yeast either from amino acids leucine and valine or from isoamyl alcohol [34,35]. As was noted previously, the diluted concentrate had elevated amounts of most of the identified amino acids, including leucine. Higher content of leucine might have been successfully utilized by the strains used in this study for isoamyl acetate production. *S. cerevisiae* yeasts used in the study accounted for the highest amount of isoamyl acetate produced (up to 6700 μg L^{-1}) as a result of additional leucine utilization than *S. bayanus* yeasts (up to 5300 μg L^{-1}).

According to the biplots, the use of juice concentrate in cider fermentation has also resulted in the production of selected long-chain fatty acid esters (ethyl decanoate, ethyl dodecanoate). Long-chain fatty acid esters possess a characteristic "fatty" or "soapy" odor description; their overproduction when using juice concentrate in fermentations might increase the risk of the off-flavor formation [36,37]. On the other hand, the same esters have also been reported to increase the perceived fruitiness in wine [38].

It should be noted that long-chain fatty acids esters have been previously tied to the onset of cell death [39–41]. Due to their low permeability through the cell membrane, the long-chain fatty acid esters are released along with other compounds (e.g., amino acids, fatty acids, lipids, glycoproteins, mannoproteins) into the environment during cell autolysis [39]. The correlation of long-chain fatty acids esters with the cider samples produced using concentrate could signify a higher cell death rate in comparison to the samples prepared with apple juice.

The nutritional supplementation approaches used in this study did not possess any significant influence on the production of volatile esters in *S. ceverisiae* strains. On the other hand, organic supplementation has increased the production of medium-chain fatty acid ethyl esters (ethyl butyrate, ethyl hexanoate, ethyl octanoate) in *S. bayanus* strains and reduced the formation of long-chain fatty acid esters. Supplementation of nitrogen with organic sources has proven to be efficient at stimulating ethyl ester production in multiple previous studies [7,38,42]. The supplementation requirements for the visible increase in ester production, however, have shown in this study to be strain-specific.

3.6. Hydrogen Sulfide Production and Off-Flavor Development

Hydrogen sulfide production in the cider samples was assessed by using indicative lead acetate paper strips in the headspace of fermentation vessels. Due to the limitations of the method, conclusive

quantitative decisions on the differences in hydrogen production cannot be made. Hence, the conclusions are provided as estimates only.

The H_2S production determined by the color intensities of the reaction on the lead acetate paper strip and the respective intensities of the sensorially perceived sulfuric off-flavor are provided in Tables 2 and 3, respectively. The pattern of hydrogen sulfide production was strain-specific, which has also been noticed in other studies [43,44]. Most of the strains used in this study produced hydrogen sulfide during fermentation but generally not enough to produce sulfuric off-flavor of high intensity. Y6 (*S. cerevisiae* strain) has been declared by a manufacturer as a yeast with a low capacity to produce hydrogen sulfide. According to the results of our experiments, it indeed showed the lowest production of hydrogen sulfide.

Table 2. Color reaction intensity of H_2S (hydrogen sulfide) formation on the selective lead acetate paper strip during fermentations prepared from fresh juice or juice concentrate with different yeasts (Y1-Y6) at 18 or 30 °C. − No color change; + Low intensity color formation; ++ Moderate intensity color formation; +++ High intensity color formation.

Nutrient	**Control**				**Fermaid O**				**DAP ***			
Matrix	**Juice**		**Concentrate**		**Juice**		**Concentrate**		**Juice**		**Concentrate**	
°C	**18**	**30**	**18**	**30**	**18**	**30**	**18**	**30**	**18**	**30**	**18**	**30**
Y1	++	−	++	++	+++	++	++	++	+++	++	++	++
Y2	++	−	++	−	++	−	++	++	++	−	++	+
Y3	++	++	++	++	++	+	++	++	++	+	++	+
Y4	+++	−	+++	+	+++	−	+++	++	+++	−	+++	+++
Y5	+	+	+	+	++	++	++	++	+++	+++	+++	+++
Y6	−	−	−	−	+	−	+	−	−	−	−	−

* Diammonium phosphate.

Table 3. Sulfur off-flavor occurrence and its intensity in cider samples. − No off-flavor perceived; + Low intensity off-flavor; ++ Moderate intensity off-flavor; +++ High intensity off-flavor.

Nutrient	**Control**				**Fermaid O**				**DAP**			
Matrix	**Juice**		**Concentrate**		**Juice**		**Concentrate**		**Juice**		**Concentrate**	
°C	**18**	**30**	**18**	**30**	**18**	**30**	**18**	**30**	**18**	**30**	**18**	**30**
Y1	+	−	−	−	++	−	−	−	++	−	−	−
Y2	−	−	−	−	−	−	−	−	−	−	−	−
Y3	−	−	−	−	−	−	−	−	−	−	−	−
Y4	+	−	++	−	+	−	++	−	++	−	++	++
Y5	−	−	−	−	−	−	−	−	++	++	++	++
Y6	−	−	−	−	−	−	−	−	−	−	−	−

In general, lower temperature fermentation (18 °C) resulted in higher production of hydrogen sulfide in the headspace by the end of the fermentation. This can be attributed to the better accumulation of the hydrogen sulfide due to low fermentation speed and prolonged viability of the yeast culture [45]. No notable differences in hydrogen sulfide production (reaction on the test strip) could be observed between apple juice and concentrate. The addition of nutrients prior to fermentation did not reduce hydrogen sulfide formation and, in some cases, even promoted it. For example, hydrogen sulfide production increased with the addition of a nutritional supplement in ciders fermented with Y5 yeast. The increase, however, was intense enough to produce a perceived off-flavor only in the case of DAP addition.

4. Conclusions

Industrially prepared apple juice concentrate was successfully applied in this study to observe the differences in cider fermentation in comparison to fresh apple juice. The process of clarification and concentration was shown to affect the concentration of initial amino acids and malic acid—both increased significantly after the treatment. In terms of fermentation kinetics, the concentrate was shown to be slightly inferior at higher temperatures to the fresh apple juice, most likely due to the partial loss of nutrients. Such was also evident from the fructose consumption patterns. As expected, the increased malic acid concentration in the concentrate increased also its consumption by yeast. The production of volatile esters was also affected by the use of the concentrate. When using the concentrate all yeast strains under study showed increased production of long-chain fatty acid esters (ethyl decanoate, ethyl dodecanoate) which might signify a higher cell death rate in the cider samples fermented with the concentrate. Increased synthesis of isoamyl acetate was also noted in the samples fermented with the concentrate which could be attributed to the higher concentration of leucine in it, which is a precursor for isoamyl acetate synthesis. This effect was, however, specific to the species of the yeast used as was noticed mainly with *S. cerevisiae* strains. No differences in the hydrogen sulfide related off-flavor formation was observed between the fresh apple juice and the concentrate. In conclusion, the use of the apple juice concentrate can result in rather similar cider fermentation kinetics and quality as in the case with fresh apple juice; however, the fermentation might require additional nutritional supplementation to compensate for the loss of some nutrients and support the viability of the yeast cell.

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Teadusartiklid

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