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Controlled fermentation strategies to enhance the aroma profile of fruit spirits

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Abbreviations

a.a.	Absolute Alcohol
ABV	Alcohol By Volume
AMP	Antimicrobial Peptides
ANOVA	Analysis of Variance
DAP	Diammonium Phosphate
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GC-FID	Gas Chromatography with Flame Ionization Detection
GC-MS	Gas Chromatography-Mass Spectrometry
H.	*Hanseniaspora*
HCA	Hierarchical Cluster Analysis
HPLC	High Performance Liquid Chromatography
HS SPME	Headspace Solid Phase Microextraction
K.	*Kluyveromyces*
L.	*Lachancea*
LAB	Lactic Acid Bacteria
Lb.	*Lactiplantibacillus*
M.	*Metschnikowia*
MANOVA	Multivariate Analysis of Variance
O.	*Oenococcus*
OD	Optical Density
QDA	Quantitative Descriptive Analysis
S.	*Saccharomyces*
T.	*Torulaspora*
YAN	Yeast Assimilable Nitrogen

1. INTRODUCTION

Fruit spirits are popular alcoholic beverages that have been traditionally produced for ages. They are often perceived as a symbol of a country's culture and identity (e.g., Pálinka, Slivovitz, Kirschwasser). The production process of fruit spirits has evolved significantly over time, driven by advancements in technology, shifting consumer preferences, and a deeper understanding of the craft. Fruit spirits are the product of a complex biochemical process that starts with the fruit and continues with the mashing process, alcoholic fermentation, distillation, and maturation. The primary quality characteristic of fruit spirits is their aroma. The aroma profile of any fruit spirit is the product of a multitude of volatile compounds, which make significant contributions, although present in low concentrations. Volatile compounds originate from various sources, and the dynamic balance created among them is responsible for the unique aroma and sensory impression of fruit spirits (Śliwińska et al., 2015; Spaho, 2017). While some of the volatiles are derived directly from the raw material, others are produced or transformed by the yeast's metabolism during fermentation. Yeast plays a crucial and indispensable role in alcoholic fermentations. Usually, fermentation is carried out by a monoculture of yeast (primarily *Saccharomyces cerevisiae*), which provides a relatively high yield of ethanol and consistency in the aroma profile (Januszek et al., 2020a; Moreno et al., 2023). However, the involvement of non-*Saccharomyces* yeasts or species from the *Saccharomyces* genus other than *S. cerevisiae* during alcoholic fermentation has revealed distinctive products with enhanced aroma complexity (Comitini et al., 2011). Although an increasing number of publications demonstrate the undeniable potential of non-*Saccharomyces* to improve the sensory profile of beer and wine, there has been limited research on their use in distillates other than tequila, mezcal, and cachaça (Varela, 2016; Gschaedler, 2017). Moreover, hybrid yeasts have emerged as a promising and innovative alternative for fermentation, demonstrating their capacity and offering distinct advantages in the alcoholic beverage industry. Currently, there are only a limited number of studies describing fermentation trials, mainly in beer and cider (Magalhães et al., 2017a; Magalhães et al., 2017b; Bendixsen et al., 2021; Winans, 2022).

Another challenge in this area of research is addressing the metabolic requirements of different yeast strains. While the aroma production and fermentation performance of yeasts are genetically determined, these traits are also influenced by external factors, such as the composition of the fruit mash (including nutritional factors) and fermentation conditions. Yeast requires specific conditions to thrive and carry out fermentation efficiently. Maintaining optimal conditions helps ensure a healthy

yeast population and desirable fermentation outcomes. The chosen mash acidification method is essential in preventing the growth of spoilage microorganisms and ensuring the dominance of beneficial fermentation yeast strains. Moreover, many nutritional factors, in particular nitrogen, are critical for yeast survival and also affect fermentation performance and aroma compound production. Certainly, the production of high-quality distillates relies on the successful completion of alcoholic fermentation and the production of desirable aroma compounds by yeast strains. Therefore, the main focus of this work was to explore the complex interactions between a number of contributing factors related to the fermentation process.

Despite the importance of a properly conducted fermentation process, attention should be paid to the subsequent steps, such as distillation and maturation. The fermented material experiences additional modifications during distillation, as the heat enables the separation of undesirable volatiles and the concentration of desired ones (Heller & Einfalt, 2022). However, the obtained fresh distillates are not suitable for consumption due to their unpleasant and harsh taste and odor. They need a maturation period to soften the harsh notes, refine their sensory attributes, and improve their overall quality (Pecić et al., 2012).

2. OBJECTIVES

Producers are constantly seeking strategies to enhance the flavor diversity of their alcoholic beverages, aiming to improve style and differentiate their products. The aroma compounds that ultimately define the overall quality of these beverages originate from every stage of the manufacturing process, with particular emphasis on the pivotal role played by alcoholic fermentation. The main objective of this research was to study how changing and optimizing individual steps in the production process affects the quality parameters of the resulting fruit spirit.

Specific objectives:

- Study the efficiency of different chemical and biological acidification techniques in the process of fermenting fruit mash. Particularly, the goals were:
    - to determine the optimal ratio of phosphoric and lactic acid that provides adequate acid protection for the mash.
    - to implement novel acidification methods using microorganisms known for their rapid growth and increased organic acid (primarily lactic acid) production. Such characteristics may provide a bioregulatory effect, and additionally, may contribute to the formation of lactate esters, imparting a silky and soft character to the spirit.
- Assess the impact of different commercially available nutrient supplements on the fermentation kinetics and the production of aroma compounds by *Saccharomyces cerevisiae*.
- The aroma compounds synthesized by the yeast play a decisive role in shaping the sensory properties of fruit spirits. Therefore, increased research is devoted to the study of new, alternative yeasts that offer enhanced aroma compound production. The goals in this regard were:
    - to examine and compare the fermentation capacity of different hybrid yeasts in fruit mash and evaluate their potential use in the production of fruit spirits.
    - to investigate the fermentation performance of non-*Saccharomyces* strains alone or in sequential inoculation with *Saccharomyces cerevisiae* and determine the effect of their metabolism on the aroma profile of fruit spirits.
- Investigate the cumulative effect of all factors involved (nutrient treatment, acidification technique, and yeast strain) on the evolution of the aroma profile and overall sensory quality of fruit spirits. By exploring the changing patterns of aroma compounds from the raw material

through fermentation to the final step of distillation, a comprehensive understanding of the effect of the optimized conditions and parameters can be ascertained.

- Following distillation, the distillate still needs to mature in order to gain its full enjoyment value. Thus, it's crucial to examine the effect of specific parameters during maturation. The aim was to evaluate the influence of alcohol content and temperature on the changes in the volatile compounds of apple distillates during a 24-week maturation period.

3. LITERATURE REVIEW

3.1. The significance of alcoholic beverages in human history

Throughout history, the production of alcoholic beverages has been a glorious human tradition. The earliest archaeological evidence of brewing and winemaking dates back to 3000 BC (Vidrih & Hribar, 2016). On the other hand, brandy was first produced around 1000-1500 AD by alchemists in their search for the 'essence' of wine. Drinking spirits began just after the 16th century, and it became common in the 18th century when distillation costs decreased and alcohol consumption increased in both Europe and North America. The Dutch, who had a robust sea trade, played a key role in spreading alcoholic beverage consumption. To make their wine cargoes smaller, they promoted the production of brandy through the process of distillation (Comer, 2000; Wolf et al., 2008).

Nowadays, fruit spirits represent an important segment of the alcoholic beverage industry, largely as a result of the flavor quality and reputation these products have acquired on the market over the years (Wang et al., 2023). They are consumed all over the world and have become an indispensable part of numerous gastronomic cultures. Hungarians have their famous Pálinka (Harcsa, 2017); France is well-known for the Calvados (Ledauphin et al., 2003); Eastern European and Balkan countries are proud of their plum spirit (Slivovitz) (Satora & Tuszyński, 2008; Balcerek et al., 2017a); Germans and Swiss have their Kirschwasser (Nikicevic et al., 2011); while Mouro is produced in Greece (Soufleros et al., 2004).

According to European regulations, fruit spirit is a spirit drink produced exclusively by the alcoholic fermentation and distillation, with or without stones, of fleshy fruit or fruit must. The distillation of fruit spirits must occur at less than 86% ABV so that the spirit retains some character from the fruit produced. The minimum ethanol content of fruit spirits must be 37.5%, while the volatile substances content should be equal to or exceeding 200 g/hL of 100% vol. alcohol. In most cases, the maximum allowed methanol content in fruit spirits is 1000 g/hL of 100% vol. alcohol. However, for spirits produced from fruits such as apple, apricot, plum, mirabelle, peach, pear, blackberry, and raspberry, the maximum methanol content is set at 1200 g/hL of 100% vol. alcohol. On the other hand, for spirits produced from fruits like quince, juniper berry, Williams pear, blackcurrant, redcurrant, rosehip, elderberry, rowanberry, sorb apple, and wild service tree, the maximum allowed methanol content is 1350 g/hL of 100% vol. alcohol. Fruit spirits derived from stone fruits must not exceed a hydrocyanic acid content of 7 g/hL of 100% vol. alcohol. While fruit spirits may be sweetened to enhance their final taste, the final product cannot contain more than 18 grams of sweetening products

per liter, expressed as invert sugar. Additionally, these beverages are not permitted to be artificially flavored or colored (EC Regulation 2019/787).

3.2. The origin of aroma compounds in fruit spirits

Aroma is considered the key quality attribute of distilled beverages. Aroma is created by a complex combination of volatile organic compounds that arise from each successive stage of the manufacturing process (Flouros et al., 2003; Śliwińska et al., 2015). Primary aroma compounds derive from the raw material or type of fruit used in the production of the spirit; secondary aroma components are generated during alcoholic fermentation; tertiary aroma compounds arise during the distillation process; and quaternary aroma compounds are formed during the maturation process (Tesevic et al., 2005).

3.2.1. Raw material

The process of producing fruit spirits is complex and involves various influencing factors. However, the main physico-chemical and sensorial differences among spirits are due to the particular composition of their corresponding raw materials and the fermentation process (Santos et al., 2013). The choice of raw materials, such as specific fruits or their varieties, directly affects the flavor and aroma profile of the spirit. The chemical composition of fruits is influenced by many factors, including their geographical origin, method of cultivation, storage, and time of harvest (Śliwińska et al., 2015). Certain chemical components present in the fruit remain unchanged throughout the fermentation process, whereas others serve as precursors for newly formed compounds (Joshi et al., 2017). In order to achieve high-quality distillates, it is essential that the fruits possess suitable attributes; they should be healthy and harvested at proper maturity, have the proper sugar-acid balance, and possess the typical aromas (Joshi et al., 1999; Jagtap & Bapat, 2015; Joshi et al., 2017).

Fruit spirits are produced all over the world using various fruits, according to their availability in different countries and seasons (Santos et al., 2013). The use of specific fruits or traditional varieties in fruit spirit production often reflects regional heritage and cultural significance. In general, three categories of spirits can be distinguished: distillates obtained with pome fruits, with apples and pears being the most prevalent; those obtained with stone fruits, such as sweet cherries, sour cherries, plums, apricots, and peaches; and lastly, distillates obtained from berries (Christoph & Bauer-Christoph, 2007; López et al., 2017). Different fruits impart unique and distinct characteristics, contributing to the overall sensory perception of the spirit. The distinctive flavor of apple and pear distillates is characterized by the presence of typical aromas, produced through the enzymatic breakdown of fatty

acids into C6 fragments, including hexanol and trans-2-hexenol, as well as ethyl esters and acetates of hexanoic acid (Postel & Adam, 1989). The flavor of stone fruit spirits is primarily influenced by benzaldehyde, a compound characterized by a bitter-almond aroma (Spaho, 2017).

In addition, the impact of cultivars on the composition of volatile organic compounds in alcoholic beverages could be an issue for critical study. For instance, research has shown that the plum cultivars used play a fundamental role in the volatile profile of the obtained spirits (Vyviurska et al., 2017). Another study showed that the levels of ethyl octanoate, hexyl 2-methylbutanoate, 1-hexanol, benzaldehyde, and furfural in distillates are associated with the specific apple variety used (Versini et al., 2009). Additionally, the content of propanol, 2-methyl-1-propanol, and 2-phenylethanol (which imparts a pleasant rose flavor) varied among wines produced from three distinct apple varieties (Satora et al., 2008). So, the quality of the raw material will shape the quality of the final product.

The first step in fruit spirit production is fruit mashing. The primary objective of fruit mashing is to maximize the extraction of desirable compounds from the fruits. By breaking down the cellular structure and releasing juices, mashing facilitates the liberation of sugars, organic acids, and aroma compounds. The fruit is crushed to ensure that yeasts reach the sugar content and nutrients present in the mash. Different methods can be employed for fruit mashing, including traditional hand pressing, mechanical crushing, or the use of modern fruit processing equipment. The chosen technique should strike a balance between efficient extraction and avoiding excessive mechanical stress that could introduce unwanted bitterness or astringency from seeds or peels. Additionally, removing any undesirable components, such as stems or seeds, contributes to a cleaner final product. Modern practices call for special yeasts and pectin-decomposing enzymes to prevent the unwanted components from forming and to ensure the optimal formation of aromas and alcohol yield (László et al., 2016).

3.2.1.1. Enzymatic treatment of the mash prior to fermentation

The treatment of the mash with enzyme preparations is a commonly applied step in fruit processing. Enzymes are responsible for hydrolyzing polysaccharides like pectins in the fruit, which makes it difficult to extract juice from the mash or to clarify it. This process increases juice yield and reduces processing time. Various enzyme preparations are commonly employed as supplements because the natural enzymes found in fruits, yeasts, and other microorganisms present in the mash are often neither efficient nor sufficient at catalyzing the diverse biotransformation reactions required under winemaking conditions (Van Rensburg & Pretorius, 2000). The selection of enzymes is based on the activities required for a particular purpose. Commercial pectolytic enzymes are typically

derived from fungal sources, primarily *Aspergillus niger*, and are a mixture of various enzymes. Among these enzymes, the most significant ones include polygalacturonases and pectinlyases, which cleave long-chain polymers in a random manner, and pectinmethylesterases, which hydrolyze the methoxy groups, resulting in the production of methanol. Nevertheless, other accompanying enzymes such as cellulases, hemicellulases, proteases, oxidases, cinnamoylesterases, and β-glycosidases may also be present in commercial enzyme preparations (Lao et al., 1996).

The activity of pectolytic enzymes during fruit processing is not only associated with the breakdown of pectin, which leads to the liquefaction of the mash and an increase in ethanol yield, but also leads to a significant increase in the efficiency of extraction of the colored and aromatic fruit substances (Miljić et al., 2016). However, the presence of the pectin-methyl-esterase enzyme poses a primary challenge when using commercial preparations because it leads to the release of methanol, an alcohol toxic to humans (Revilla & González-SanJosé, 1998).

Methanol itself exhibits relatively low toxicity; however, products obtained from its metabolic transformations (formaldehyde and, in particular, formic acid) can pose a significant health risk to humans when ingested or inhaled. Methanol is initially oxidized to formaldehyde and subsequently to formic acid, which can accumulate in the bloodstream. The production of formic acid is responsible for the development of acidosis in cases of methanol poisoning. Humans have limited capacity to metabolize and detoxify formic acid, and symptoms of methanol poisoning can include headaches, severe abdominal pain, difficulty breathing, a weakening pulse, decreased body temperature, loss of vision, and, in severe cases, even death (Miljić et al., 2016).

3.2.2. Alcoholic fermentation

Alcoholic fermentation is a complex biochemical process performed by yeasts that utilize sugars and other constituents as substrates for their metabolism, converting these to ethanol, carbon dioxide, and other metabolic byproducts that contribute to the chemical composition and quality of the beverage (Buratti & Benedetti, 2016). Therefore, the use of quality yeast is one of the cornerstones of the production of high-quality spirit, and the success of the alcoholic fermentation depends on maintaining the viable yeast population at sufficient levels until all the fermentable sugars have been fully consumed (Zamora, 2009). At the metabolic level, yeasts are characterized by their ability to ferment a wide range of sugars, among which glucose, fructose, sucrose, maltose, and maltotriose predominate (Maicas, 2020). Fundamentally, yeasts metabolize the sugars and produce alcohol in order to obtain energy and multiply (Walker & Walker, 2018).

Yeasts use glycolysis as the main pathway for sugar catabolism (Figure 1). The glycolysis process begins with the breakdown of sugars to form pyruvate molecules. The glycolysis of a glucose molecule generates two molecules of pyruvate, four of ATP, and one of NADH. Pyruvate produced by glycolysis can be used by yeasts for several metabolic pathways. However, yeasts must regenerate NAD+ from NADH to re-establish the redox potential of the cell, which can be achieved either by fermentation or respiration. In most eukaryotes, this is dependent on the presence of oxygen. In aerobic conditions, pyruvate will be converted to acetyl-coA by the actions of a pyruvate dehydrogenase and head towards the citric acid cycle. Under anaerobic conditions, pyruvate is diverted towards fermentation. During fermentation, the two molecules of pyruvic acid are reduced to two molecules of ethanol and $2CO_2$, while the exchange of electrons that occurs in the process helps to build ATP (Huang et al., 2015; Malakar et al., 2020).

(A) (B)

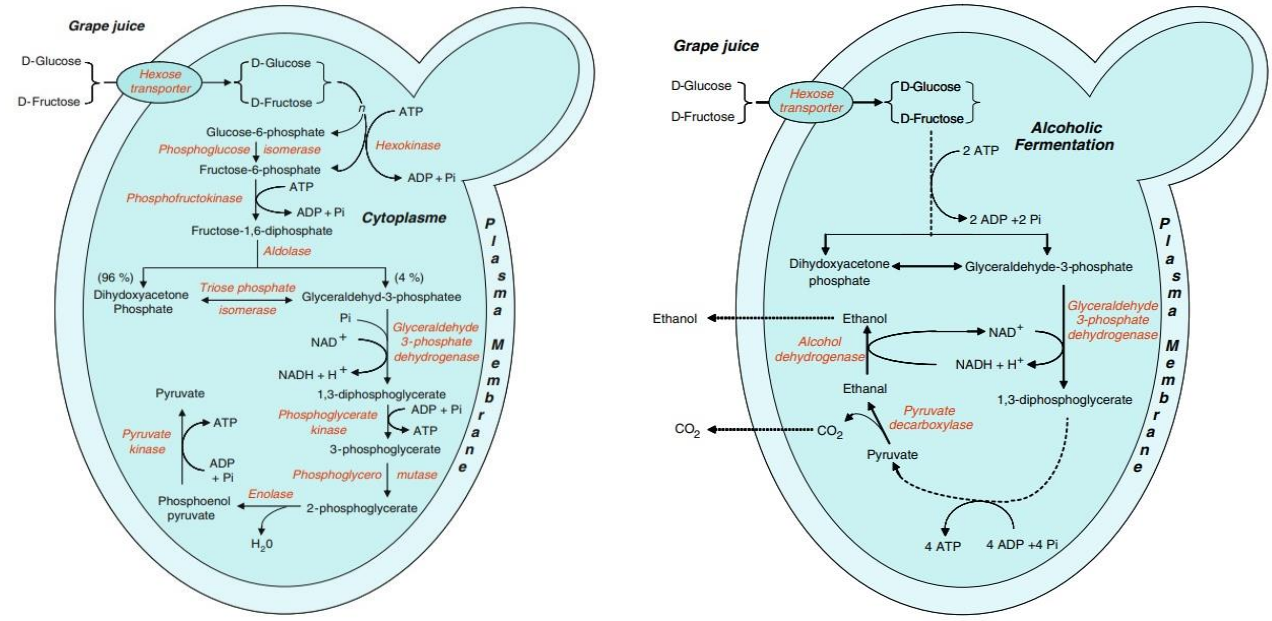


Figure 1. Biochemical mechanism of glycolysis (A); Alcoholic fermentation (B) (Zamora, 2009).

Twelve enzymes are involved in the conversion of glucose to ethanol and carbon dioxide. Ten of these enzymes break down glucose to pyruvate and generate ATP for yeast growth, while two others, namely pyruvate decarboxylase and alcohol dehydrogenase, convert pyruvate to the final fermentation products as yeast maintains its redox balance. Those enzymes have metal ion cofactor requirements, such as magnesium and zinc. As a result, the bioavailability of metal ions in fermentation media can affect the efficiency of sugar conversion to ethanol and the overall progress

of fermentation (Walker, 2004). During sugar fermentation by yeast, ethanol and carbon dioxide generation, energy (ATP) production, redox balancing, and yeast growth are all coupled, and the rate of fermentation is directly linked to the rate of yeast cellular reproduction (Walker & Walker, 2018).

3.2.2.1. Yeast secondary metabolism: The production of aroma compounds

The metabolism of fermenting yeasts comprises two stages: primary metabolism (glycolysis) and secondary metabolism. Primary metabolism is essential for growth, cell division, survival, and the production of metabolites such as ethanol, glycerol, acetaldehyde, and acetic acid. On the other hand, secondary metabolism is nonessential for growth and instead focuses on generating a wide range of small molecules known as aroma compounds, including higher alcohols, esters, volatile fatty acids, carbonyl compounds, and sulphur compounds (Figure 2) (Styger et al., 2011; Hirst & Richter, 2016). The secondary metabolism is influenced by various factors, like the initial substrate (fruit or grain), the availability of carbon and nitrogen sources, the specific yeast strain employed, and the fermentation conditions (Henschke & Jiranek, 1993; Verstrepen et al., 2004; Richter & Pugh, 2012; Hirst & Richter, 2016).

Higher alcohols

Higher alcohols, also known as fusel alcohols or fusel oils, are aliphatic and aromatic alcohols containing more than two carbon atoms. They are quantitatively the largest group of aroma compounds found in alcoholic beverages (Nykänen & Nykänen, 1977; Nykänen, 1986; Hazelwood et al., 2008; Mendes-Ferreira et al., 2011). The most abundant fusel alcohols detected in spirits are 1-propanol, 2-methyl 1-propanol, 2-methyl 1-butanol (active amyl alcohol), 3-methyl 1-butanol (isoamyl alcohol), and 2-phenylethanol (Swiegers & Pretorius, 2005; Mendes-Ferreira et al., 2011). Additionally, 1-hexanol was detected in Bartlett pear brandies (Willner et al., 2013), 1-butanol had a very high concentration in melon spirits (Zhang et al., 2022), and 2-butanol was observed in apple spirits known as Calvados (Guiné et al., 2021).

Higher alcohols are formed through two possible pathways: the catabolism of amino acids present in the fermentation substrate via the Ehrlich pathway (Dickinson et al., 2003) or the de novo synthesis of amino acids, also known as the anabolic pathway (Nisbet et al., 2014). As a result, the production of higher alcohols is linked to both nitrogen metabolism (the Ehrlich pathway) and carbon metabolism (the anabolic pathway) (Cordente et al., 2019). These compounds are primarily generated

during the active growth phase of yeast. Thus, factors that promote yeast growth simultaneously enhance the synthesis of higher alcohols (Dekoninck, 2012).

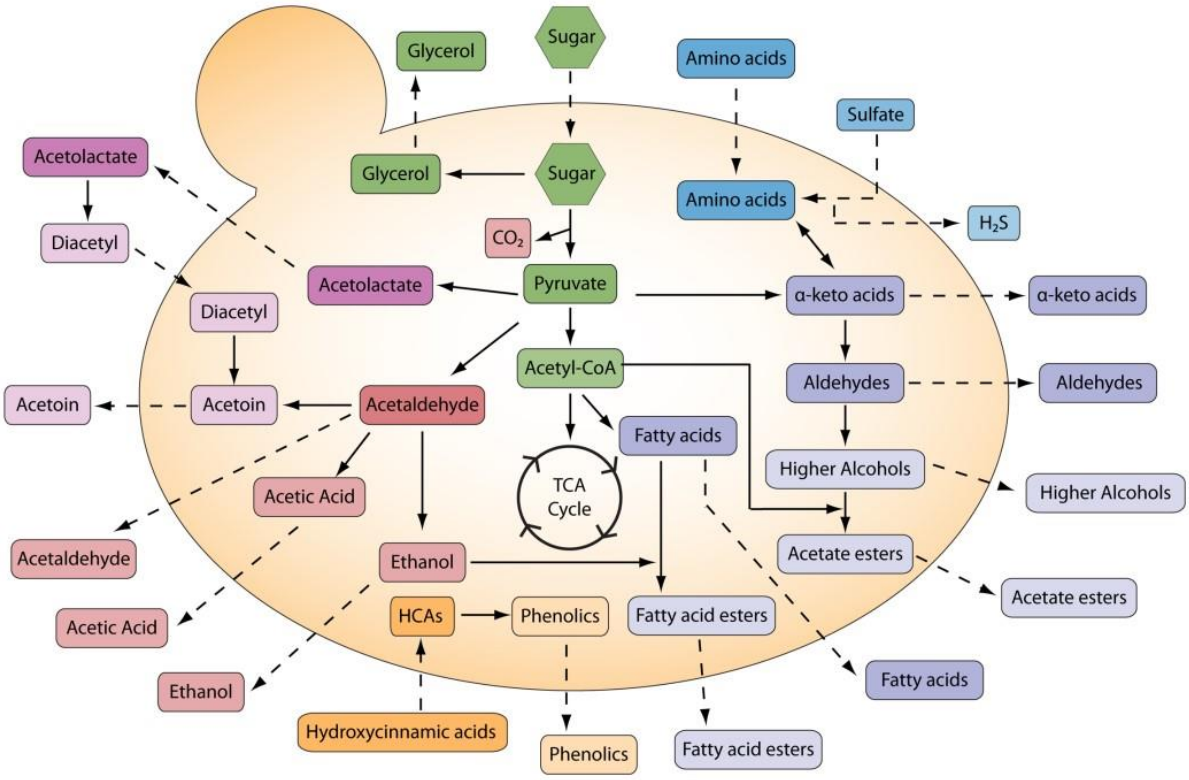


Figure 2. Overview of aroma compound production. The primary fermentation of pyruvate (green/red) leads to several carbon-based compounds, including ethanol and carbon dioxide. Pyruvate also feeds into the anabolism of amino acids, leading to the production of vicinal diketones (pink). The metabolism of amino acids is responsible for numerous aroma compounds, including higher alcohols and esters (purple) as well as sulfur-containing compounds (blue). Additionally, the phenolic compounds are derived from molecules found in the media (orange). Dotted lines indicate the import/export of compounds; solid lines represent biochemical reactions (not indicative of the number of reactions) (Dzialo et al., 2017).

Ehrlich (1907) was the first to establish the connection between amino acid metabolism and higher alcohol formation based on their structural similarity. He demonstrated that a surplus of external amino acids leads to an increase in the production of these alcohols. Conversely, when there is a shortage of amino acids, the pathways will inevitably favor anabolic routes (He et al., 2014). The first step in the Ehrlich pathway involves the transamination of the amino acid to form the corresponding α-keto acid analogue. Subsequently, multiple pyruvate decarboxylases catalyze the

conversion of the α-keto acid to an aldehyde (Sentheshanmuganathan, 1960). Last, an alcohol dehydrogenase catalyzes the NADH-dependent final step that reduces the aldehyde to fusel alcohol (Hazelwood et al., 2008). Yeast takes up the amino acids (valine, leucine, isoleucine, methionine, and phenylalanine) that undergo the Ehrlich pathway in a sequential order (Jones & Pierce, 1964).

Fusel alcohols can have positive or negative sensory impacts, depending on their concentration. Concentrations exceeding 400 mg/L can impart a strong, pungent smell and taste, whereas optimal levels below 300 mg/L confer fruity characters (Lambrechts & Pretorius, 2000; Swiegers & Pretorius, 2005). Fusel alcohols, such as propanol, butanol, and isobutanol, are known for their alcoholic scent; active amyl alcohol and isoamyl alcohol are associated with a marzipan-like or banana aroma; and 2-phenylethanol imparts floral aromas (Lambrechts & Pretorius, 2000). Higher alcohols are important precursors for ester production (Cordente et al. 2012). In the reaction between higher alcohols and acetyl-CoA, which is facilitated by alcohol acetyltransferases, acetate esters are formed (Mason & Dufour, 2000).

Esters

Esters represent the main class of flavor-active metabolites in alcoholic beverages (Procopio et al., 2011). Esters have a very low detection threshold and an intense smell (Wiśniewska et al., 2016). Their aromas are essential for the floral and fruity character of high-quality alcoholic beverages (Saerens et al., 2010).

During fermentation, two main types of esters are generated: acetate esters and fatty acid ethyl esters. Their formation is mediated intracellularly by fermenting yeasts. Acetate esters result from the esterification of acetyl-CoA and an alcohol (Saerens et al. 2010). Their synthesis is carried out by alcohol-O-acetyl (or acyl) transferases (AATases) (Dzialo et al., 2017). These acetates include ethyl acetate (solvent-like aroma), isoamyl acetate (banana aroma), and phenylethyl acetate (roses and honey aroma) (Saerens et al., 2007). The most frequently occurring ester is ethyl acetate. While low concentrations of ethyl acetate help smooth out the harsh odor of some alcoholic beverages, its presence at high concentrations can contribute to a so-called ‘vinegar flavor’ in products (Wiśniewska et al., 2016). The second group, the ethyl esters, is composed of ethanol and a medium-chain fatty acid (hexanoic acid, octanoic acid, decanoic acid, and dodecanoic acid). In the late exponential growth phase of yeast, medium-chain fatty acid intermediates are prematurely released from the cytoplasmic fatty acid synthase complex, leading to the initiation of ester synthesis (Taylor & Kirsop, 1977). These medium-chain fatty acids are activated by coenzyme A and then esterified in the presence of ATP,

ethanol, and enzymes (Saerens et al., 2010). Ethyl esters include ethyl butanoate, ethyl hexanoate (anise seed, apple aroma), ethyl octanoate (sour apple aroma), and ethyl decanoate (floral aroma) (Saerens et al., 2007).

Ethyl esters derived from long-chain fatty acids, including ethyl dodecanoate, ethyl tetradecanoate, ethyl hexadecanoate, and ethyl octadecanoate, contribute to a favorable aromatic profile when present in small amounts. At high concentrations, they are responsible for candle wax tones (Stanzer et al., 2023).

Carbonyl compounds

Carbonyl compounds, including aldehydes and ketones, are important flavoring agents in spirits (Stanzer et al., 2023). The identification of carbonyl compounds is highly significant, as it not only helps to determine the flavor attributes of spirits but also serves to identify anomalies that may indicate inconsistent manufacturing (Balcerek, 2010). Some of these compounds may be responsible for off-flavors, while others exhibit a range of fruity or floral aromas reminiscent of apples, lemons, or nuts (Kłosowski et al., 2017; Moreira et al., 2019).

Aldehydes are formed as intermediates during the production of fusel alcohols, arising from the decarboxylation of α-keto acids (Suomalainen & Lehtonen, 1979). Acetaldehyde accounts for 90% of the total carbonyl content in an alcoholic beverage (Januszek et al., 2020b). Acetaldehyde is a pyruvate intermediate that serves as a precursor for acetate, acetoin, and ethanol (Stanzer et al., 2023). In low concentrations, it contributes to a pleasant fruity aroma. However, if its concentration exceeds 125 mg/L, it can result in unpleasant rotting odors and a pungent and irritating aroma (Balcerek et al., 2017b; Portugal et al., 2017).

The content of the other aldehydes is generally very low. The most characteristic aromatic aldehyde of stone fruit spirits is benzaldehyde, which has an almond-like aroma. Certain yeasts can convert benzaldehyde into benzyl alcohol and benzoic acid (Lambrechts & Pretorius, 2000; Stanzer et al., 2023). Benzaldehyde and hydrogen cyanide are formed by the enzymatic degradation of amygdaline, a compound present in fruit seeds and stones. Hydrogen cyanide serves as the precursor to the genotoxic compound known as ethyl carbamate (Christoph & Bauer-Christoph, 2007). Furfural, a compound produced through the dehydration of pentoses, is also found in fruit distillates. Some authors attribute higher furfural concentrations to prolonged distillation periods (Balcerek, 2010). In plum spirits, various carbonyl compounds have been detected, including hexanal, octanal, heptanal, benzaldehyde, 2-undecanone, and damascenone (Velíšek et al., 1982). Ledauphin et al. (2006) showed

that 3-methylbutanal and hexanal are potential key aroma compounds in freshly distilled Calvados and Cognac.

Acetals are important aroma constituents in alcoholic beverages. They are formed through the condensation of aliphatic aldehydes and alcohols. Acetals contribute a delicate taste and aroma to alcoholic beverages. Moreover, they help to soften the sharp characteristics of Cognac flavor that are imparted by aldehydes (Balcerek, 2010).

3.2.2.2. Yeasts: the agents of the fermentation process

Long before Pasteur's discovery that yeasts are the agents that cause alcoholic fermentation, they had been in practical use for the production of beer, wine, and spirits (Barnett, 1997; Schehl et al., 2004). Traditionally, the fermentation was carried out spontaneously with a mixture of native microorganisms, including a large number of non-conventional yeasts that were naturally present on the fruit surfaces. These autochthonous yeasts are often referred to as 'wild', 'native', or 'indigenous' yeasts to distinguish them from exogenously added yeast starter cultures (Varela et al., 2009; Varela, 2016; Gschaedler, 2017).

Spontaneous fermentation is a complex process carried out by the sequential action of different yeast genera and species (Heard & Fleet, 1985; Romano, 2003). Studies have shown that non-*Saccharomyces* are the most prevalent yeast genera in the first stages of spontaneous fermentation (mainly belonging to *Hanseniaspora/Kloeckera, Candida, Pichia, Zygosaccharomyces, Schizosaccharomyces, Torulaspora, Kluyveromyces*, and *Metschnikowia* genera), while *Saccharomyces cerevisiae* strains are dominant during the latter stages (Pretorius, 2000; Fleet, 2008; Ciani et al., 2009; Comitini et al., 2011; Jolly et al., 2013; Gschaedler, 2017). This phenomenon and the causes underlying yeast interactions during fermentation are not fully understood. The ability of *S. cerevisiae* to outcompete other microbial species during alcoholic fermentation has traditionally been attributed to its high fermentative power and capacity to withstand harsh environmental conditions, i.e., low pH values, high levels of ethanol and organic acids, scarce oxygen availability, and depletion of certain nutrients (Albergaria & Arneborg, 2016).

According to Nissen et al. (2003), the early growth arrest of non-*Saccharomyces* yeasts (*Lachancea thermotolerans* and *Torulaspora delbrueckii*) is triggered by a cell-cell contact mechanism dependent on the presence of viable *S. cerevisiae* cells at high concentrations. Renault et al. (2013) supported the aforementioned assumption that the death of *T. delbrueckii* is mediated by a cell-to-cell contact mechanism. Some contradictory results were reported by Pérez-Nevado et al. (2006). They investigated the factors underlying the early death of *Hanseniaspora uvarum* and

Hanseniaspora guilliermondii by comparing their growth and fermentation profiles under single- and mixed-culture conditions with *S. cerevisiae* and concluded that the early death of the non-*Saccharomyces* yeasts was induced by unknown toxins produced by *S. cerevisiae*. Taillandier et al. (2014) also ruled out substrate competition and cell-to-cell contact as the main causes of the early death of *T. delbrueckii* in mixed-culture fermentations with *S. cerevisiae*, suggesting that death was induced by unknown metabolites produced by *S. cerevisiae*. Later, Albergaria et al. (2010) discovered that those metabolites or toxins correspond to antimicrobial peptides (AMPs) secreted by *S. cerevisiae* that inhibit the growth of several non-*Saccharomyces* yeasts, namely *K. marxianus*, *L. thermotolerans*, *T. delbrueckii,* and *H. guilliermondii*. The AMPs were then identified by Branco et al. (2014), who found that they derived from the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Probably, the secretion of GAPDH-derived AMPs corresponds to a defensive strategy used by *S. cerevisiae* to combat other microorganisms in complex microbial environments. Ultimately, in the investigation of Kemsawasd et al. (2015a), it was found that the two phenomena, i.e., cell-to-cell contact and secretion of AMPs, play a combined role in the early death of *L. thermotolerans* during mixed-culture fermentation with *S. cerevisiae*.

3.2.2.3. Fermentative potential of non-*Saccharomyces* yeasts

The traditional fermentation occurs spontaneously; as a result, the operational conditions are not fully controlled, and the quality of the final product is generally non-standardized (Cuvas-Limon et al., 2020). To end this chaos, the concept of controlled fermentation was introduced in the 1970s, which refers to the inoculation of the must with a specific, selected yeast strain (Gschaedler, 2017). The use of a selected yeast has several benefits, including reliable rapid fermentation, low risk of contamination, improved uniformity in fermentation rates, low competition for nutrients, higher beverage yield, low residual sugar concentrations, desirable flavor compounds, and preservation of the beverage's sensory qualities.

Saccharomyces cerevisiae is employed as a model microorganism in this context. *S. cerevisiae* predominates in the fermentation processes of most fruits due to its ability to tolerate fermentation conditions, including high initial sugar concentration, high acidity, variations in temperature, availability of nutrients, and, especially, increasing ethanol content (Lopes et al., 2019; Parapouli et al., 2020). However, simplifying the microflora by eliminating non-*Saccharomyces* yeasts also simplifies the analytical composition of the beverage. The yeast communities have a great potential to shape and enhance the aroma and flavor of alcoholic beverages (Varela, 2016).

Earlier studies considered non-*Saccharomyces* yeasts as 'wild' or 'spoilage' yeasts because they were often isolated from stuck or sluggish fermentations, or wines with anomalous analytical and sensorial profiles. This perception changed over the years, gaining relevance to the action of non-*Saccharomyces* yeasts in spontaneous fermentation and their positive contribution to the aroma complexity of the final product (Ciani et al., 2009; Maicas, 2020). The use of non-*Saccharomyces* yeasts alone has some drawbacks, as they are unable to complete the fermentation, leaving behind considerable residual sugar levels (Ciani & Picciotti, 1995; Ciani & Maccarelli, 1998; Jolly et al., 2003). Furthermore, they are likely to produce spoilage metabolites like acetic acid, acetaldehyde, acetoin, and ethyl acetate, along with off-odors like vinyl and ethyl phenols that are linked to the development of *Brettanomyces/Dekkera* spp. (Chatonnet et al., 1995). However, their positive traits have also been revealed and appreciated. Maturano et al. (2012) reported that non-*Saccharomyces* yeasts are able to produce extracellular enzymes that convert inactive compounds present in the must into their active aromatic forms, thereby enhancing the sensory quality of wines.

In this context, it has been proposed that non-*Saccharomyces* yeast strains should be included in mixed and multi-starter cultures alongside *Saccharomyces* strains to improve the chemical composition and sensory properties of alcoholic beverages while avoiding the undesirable compounds that these species might produce (Ciani et al., 2009; Andorrà et al., 2012). The positive impact of multi-starter fermentation on the complex flavor and quality of wine (Ciani et al., 2006; Azzolini et al., 2012; Gobbi et al., 2013), tequila (Arrizon et al., 2006; Lopez-Alvarez et al., 2012), and sugar cane spirit has been reported (Duarte et al., 2013; Amorim et al., 2016).

According to preliminary studies, when yeasts develop together under certain fermentation conditions, they do not passively coexist but rather interact and produce unpredictable compounds and/or varying levels of fermentation products, which can alter the chemical and aromatic composition of the beverage (Howell et al., 2006; Anfang et al., 2009). For instance, some negative enological characteristics of non-*Saccharomyces* yeasts may not be expressed or may be modulated by *S. cerevisiae* cultures. The fermentation of wine with a mixed culture of *T. delbrueckii* and *S. cerevisiae* resulted in a considerable reduction of acetaldehyde and acetic acid contents by 60% and 53%, respectively (Bely et al., 2008). Only 3 days of fermentation with *Wickerhamomyces anomalus* in sequential mixtures with *S. cerevisiae* were enough to improve the aroma quality of the cider (Ye et al., 2014a). According to Garde-Cerdán and Ancín-Azpilicueta (2006), there are beneficial interactions between non-*Saccharomyces* yeasts and *S. cerevisiae* in wine fermentation, as they demonstrated an increase in ester concentrations compared to pure fermentations.

3.2.2.4. Fermentative potential of hybrid yeasts

Another yeast alternative has emerged during the last decade, primarily in the brewing industry as well as in winemaking. An alternative to co-fermentation that avoids growth competition between species is the use of hybrid strains, where the genomes of different species are contained within a single cell (Bellon et al., 2011). Hybrids are thought to combine the metabolic properties of the two originating species and therefore may exhibit unique patterns of metabolism and end-product profiles (Borneman et al., 2011).

Natural hybrid yeasts exist in nature. The exchange of genetic material between species seems prevalent in all species of *Saccharomyces*. It has been proven that the lager beer yeast *Saccharomyces pastorianus* is a hybrid composed of the sub-genomes of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* (Nakao et al., 2009; Libkind et al., 2011; Pérez-Travéz et al., 2014; Gangl et al., 2017). This natural hybrid yeast is in charge of producing lager beer on a global scale and is a prominent industrial microorganism. Its success in the brewing environment is attributed to a combination of traits that are not commonly found in pure yeast species, particularly low-temperature tolerance and maltotriose utilization (Hebly et al., 2015; Krogerus et al., 2015; Krogerus et al., 2016). Moreover, natural hybrids between *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii* have been isolated from Belgian Trappist beers (González et al., 2008), whereas natural hybrids between *Saccharomyces cerevisiae* and *Saccharomyces uvarum* are often employed in winemaking (Le Jeune et al., 2007).

A number of techniques, such as spore-to-spore mating, mass mating, rare mating, and protoplast fusion, can be used in the lab to develop hybrid *Saccharomyces* strains (Figure 3). Hybridization enables the combination and enhancement of a range of phenotypic features from different and diverse parent strains. This method has been utilized to produce yeast hybrids with faster fermentation potential, higher rates of sugar utilisation, greater stress tolerance, and a broader spectrum of aroma compounds (Johnston, 1965; Spencer & Spencer, 1977; Legmann & Margalith, 1986; Gamero et al., 2011; Sanchez et al., 2012, Krogerus et al., 2016). Early research by Mukai and coworkers (2001) demonstrated that utilising an ale × sake intraspecific hybrid could boost the concentrations of 2-methylpropyl acetate (fruit aroma) and ethyl hexanoate (apple/aniseed aroma) in beer. Steensels et al. (2014) claimed that a 45% increase in 3-methylbutyl acetate (banana aroma) formation could be achieved by intraspecific hybrids. In winemaking, the use of de novo *S. cerevisiae* interspecific hybrids with *S. kudriavzevii, S. mikatae, S. paradoxus,* and *S. uvarum* proved their

potential to improve fermentation performance and aromatic diversity (Bellon et al., 2011; Bellon et al., 2013; Bellon et al., 2015; Lopandic et al., 2016).

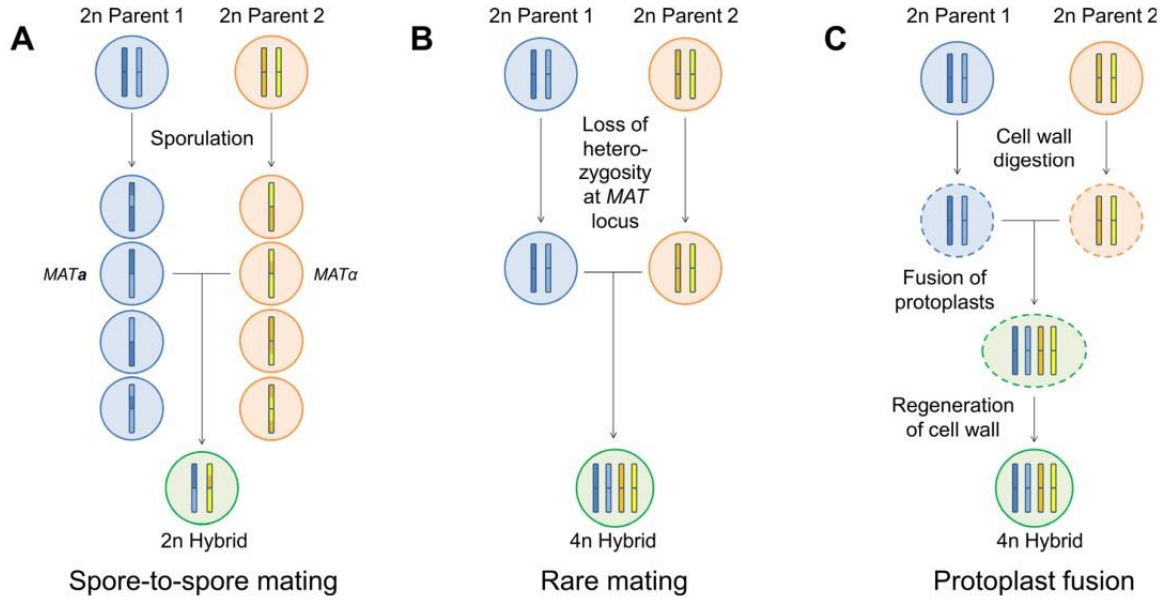


Figure 3. An overview of different hybridization methods (Krogerus et al., 2016)

A. During spore-to-spore mating, the diploid (2n) parent strains are first sporulated, after which haploid spores of the opposite mating type derived from the two parent strains are brought together and allowed to mate. A diploid (2n) hybrid is formed.
B. During rare matings, the diploid (2n) parent strains are brought together without any prior sporulation. The cells are not able to directly mate, but a rare spontaneous loss of heterozygosity at the mating-type locus can occur in a fraction of the population. As a result, diploid cells with a single mating type, which are able to mate, are formed. A tetraploid (4n) hybrid is formed.
C. During protoplast fusion, the cell walls of the diploid (2n) parent strains are first digested, after which the protoplasts are brought together and undergo fusion, followed by the regeneration of the cell wall. A tetraploid (4n) hybrid is formed.

Besides interspecific hybrids, the construction of intergeneric hybrid strains is also possible through protoplast fusion. In intergeneric hybrid strains, the genomes of *Saccharomyces* and non-*Saccharomyces* yeasts are contained within one cell (Lucca et al., 2002; Ye et al., 2013). According to Wang et al. (2020), recombinant fusant yeast resulting from the fusion of *Saccharomyces cerevisiae* and *Candida ethanolica* parental strains shows great potential for producing high-quality, low-alcohol

cider. *Torulaspora delbrueckii* is another promising candidate that can be utilized in breeding programs to develop novel yeast strains for the production of aromatic wines with lower volatile acidity (Santos et al., 2008; Hart et al., 2016).

3.2.2.5. Nutritional requirements of yeast

Yeasts are eukaryotic microorganisms with a very diverse physiology, inhabiting a wide variety of ecological niches. Perhaps the most relevant habitat at this point is fruit surfaces. In this natural environment, yeasts can efficiently carry out their metabolism and fermentation activities as long as they have access to the necessary nutrients and substrates (Walker & Stewart, 2016). Although fermentation performance and flavor production of yeasts are genetically determined, these characteristics are also highly dependent on the fruit mash composition and environmental conditions. During alcoholic fermentation, yeast cells must withstand various environmental stresses, including osmotic stress, low pH, and temperature fluctuations. Additionally, their growth is conditioned by the availability of essential compounds such as fermentable sugars, nitrogen and phosphorus sources, vitamins, minerals, lipids, and oxygen. Variations in the availability and nature of these nutrients force the yeast cells to adapt accordingly. Yeast cells sense the amount and quality of external nutrients through multiple interconnected signalling networks, which allow them to adjust their metabolism, transcriptional profile, and developmental program to adapt readily and appropriately to changing nutritional states (Zaman et al., 2008).

In the past, it was believed that grape musts contained sufficient nutrients other than nitrogen to support yeast growth and fermentation (Ough et al., 1989). However, now it's clear that all nutritional components of grape must, apart from nitrogen, can influence the growth and metabolism of yeast cells and, consequently, the composition of the final wine and its sensory properties. When nutrients are present in insufficient or excess amounts, major fermentation problems such as sluggish fermentation, fermentation arrest, or the production of metabolites perceived as off-flavor compounds can arise (Bisson, 1999; Mendes-Ferreira et al., 2011). The presence of these nutrients in the medium influences the metabolic pathways linked to the production of aroma compounds (Bisson, 1999; Tesnière et al., 2015; Maicas, 2020). Thus, understanding and modelling the relationship between nutrient availability and the production of desirable aroma compounds by different strains must be one of the main objectives in the selection of industrial yeasts for the beverage industry (Carrau et al., 2008).

Nutrient limitations can modulate the yeast diversity of alcoholic fermentations, as one yeast species or strain may produce compounds or utilize a nutrient relevant to another species or strain (Albergaria & Arneborg, 2016). In spontaneous fermentations, where the initial microflora is primarily composed of non-*Saccharomyces* species, the consumption of amino acids and vitamins during the first days of fermentation can drastically restrict the subsequent growth of *S. cerevisiae* strains (Fleet, 2003). Taillandier et al. (2014) reported that in a sequential fermentation conducted in a medium containing 176 mg/L of initial assimilable nitrogen, *S. cerevisiae* was not able to develop due to nitrogen exhaustion by *T. delbrueckii* growth during the first 48 hours, leading to sluggish fermentation. There is evidence indicating that *Kloeckera apiculata* can deplete thiamine and other micronutrients in grape juice, which can impair *S. cerevisiae*'s ability to grow (Bisson, 1999).

Nitrogen Content

Nitrogen is a crucial nutrient for yeast growth and metabolism. In fruits, nitrogent exists in both inorganic (ammonium salt) and organic (proteins, peptides, and amino acids) forms. Yeast assimilable nitrogen (YAN) primarily consists of ammonium and amino acids (Bell & Henschke, 2005; Stanzer et al., 2023). Under enological conditions, yeasts require a minimum of 140-150 mg/L of YAN to complete fermentation within a reasonable period of time and prevent stuck fermentations (Bisson, 1999; Beltrán et al., 2005; Kemsawasd et al., 2015b; Gobert et al., 2019). However, this level is dependent on sugar concentration and winemaking practices (Bell & Henschke, 2005). In addition, it strongly depends on the genetic makeup of yeast species and clones that develop during the fermentation process (Hu et al., 2019; Prior et al., 2019; Seguinot et al., 2020; Stanzer et al., 2023).

In most instances, the majority of the alcoholic fermentation is carried out in nitrogen-depleted conditions, as all assimilable nitrogen sources are consumed during the exponential phase, which usually corresponds to the first 2 days of fermentation. Subsequently, the cells enter the stationary phase, triggered by the depletion of assimilable nitrogen (Tesnière et al., 2015). Hence, nitrogen supplementation, in particular in the form of diammonium phosphate (DAP), is a common practice to avoid problems such as stuck fermentation and the production of H_2S (Bell & Henschke, 2005; Mendes-Ferreira et al., 2010). Even so, excessive levels of ammonium can lead to problems such as the formation of ethyl carbamate, a compound with carcinogenic activity (González-Marco et al., 2010).

The concentration of the ammonium cation, NH_4^+, which is one of the preferred nitrogen sources of *S. cerevisiae*, was initially believed to impact the initiation and fermentability of the grape must (Ribéreau-Gayon et al., 2006a). However, recent data suggest that this nitrogen source is taken up

later than many amino acids (Crépin et al., 2012; Crépin et al., 2014). Yeasts take advantage of the uptake of these amino acids and use their alpha-amine group (except for proline) via deamination or transamination. The uptake and catabolism of these amino acids are tightly regulated and play a role not only in yeast growth, maintenance, and functioning but also in oenology, as certain fermentative aroma compounds originate from these amino acids (Mendes-Ferreira et al., 2011).

Aroma compounds directly associated with nitrogen metabolism, such as higher alcohols and their corresponding fatty acids and esters, are influenced by the total nitrogen concentration, the nature of nitrogen (inorganic and/or organic), and the timing of nitrogen addition (Beltran et al., 2005; Hernández-Orte et al., 2005; Barbosa et al., 2009). A study by Kemsawasd et al. (2015b) found that different nitrogen sources (i.e., nineteen amino acids, ammonium sulphate, and two complex nitrogen sources) had quite different impacts on the growth and fermentation performance of *S. cerevisiae, L. thermotolerans, M. pulcherrima, H. uvarum,* and *T. delbrueckii* during alcoholic fermentation. Santamaría et al. (2020) reported an improvement in the aromatic composition of red wines with the addition of inorganic nitrogen, although its organoleptic evaluation was not favored. Literature data show that the use of ammonium salts (inorganic form) as the sole nitrogen source, leads to an increase in the production of several compounds, including isoamyl acetate, linalool, 1-octanol, butyric acid, diethyl succinate, hexanoic acid, and octanoic acid. Whereas, the use of amino acids (organic form) as nitrogen sources results in higher levels of higher alcohols, indicating their direct catabolic formation via the Ehrlich pathway, as well as increased amounts of esters such as acetate esters, 2-phenethyl acetate, and ethyl esters (Barbosa et al., 2012).

Moreover, Seguinot et al. (2018) found that the timing of nitrogen addition had a more significant impact on aroma production than the nitrogen composition itself. Thus, when nitrogen is added to the initial fermentation medium, the amount of higher alcohols is lower compared to when nitrogen is added later in the fermentation process (Hernández-Orte et al., 2005). Nitrogen-limiting conditions lead to increased synthesis of higher alcohols via anabolic pathways. Basically, there are limited accessible amino acids for transamination, and most higher alcohols are generated from keto acids derived from sugars (Oshita et al., 1995). Nevertheless, in sufficient nitrogen supply, amino acids undergo transamination, resulting in an increase in the catabolic production of higher alcohols, while the anabolic production is diminished. Hence, the addition of nitrogen will lead to a reduction in the levels of higher alcohols, even when the necessary precursor amino acids are provided (Äyräpää, 1971). Several investigations have indicated that an increase in initial nitrogen content is associated with higher ester production, especially acetates of higher alcohols and ethyl esters (Hernández-Orte

et al., 2006a; Ugliano et al., 2010; Rollero et al., 2014). In their study, Barbosa et al. (2009) demonstrated that *S. cerevisiae* strains with nitrogen addition during the stationary phase experienced significant reductions in ethanol and acetic acid formation, while significantly increasing the production of the following compounds: 2-phenylethanol, ethyl isobutyrate, 2-phenylethyl acetate, ethyl 2-methylbutyrate, and ethyl propionate. Nevertheless, in some cases, the addition of nitrogen can impair the production of esters, depending on the yeast strain and chemical composition of the must (Beltran et al., 2005; Jiménez-Martí et al., 2007).

The abundance of research conducted in this field corresponds to wine. However, the study of Januszek et al. (2020b) demonstrated that the distillate produced by the apple variety with the highest nitrogen content exhibited the most diverse profile of volatile compounds. The initial nitrogen content in apples (ranges from 27 to 574 mg/L) is directly linked to the amino acid content, especially aspartic acid, asparagine, glutamic acid, and serine. Collectively, these amino acids account for 86 to 95% of the content and are easily assimilated by the yeast. Many of these amino acids serve as intermediates or precursors for the synthesis of higher alcohols (Valles et al., 2005; Ye et al., 2014b). According to Santos et al. (2015), ciders manufactured with low nitrogen content showed sluggish fermentation and around 50% less volatile compounds. High levels of amino acids like asparagine, aspartic acid, glutamic acid, and alanine in dessert apple musts are essential for the production of fusel alcohols and most esters by yeasts during cider fermentation. A year later, Santos et al. (2016) investigated the effect of amino acid supplementation in apple must for cider production. They found that adding aspartate and glutamate to apple musts increased the ester concentration in the cider by fourfold.

Recently, the use of yeast autolysates as a nutrient for yeasts has become a common practice during fermentation. These additives serve as a complex nutritive source, providing not only nitrogen compounds but also fatty acids and yeast cell walls. Fatty acids are essential for the formation of yeast plasma membranes, which play a crucial role in the active tranport system of different nutrients, including nitrogen compounds (Kunkee & Bisson, 1993). On the other hand, yeast cell walls can bind toxic substances in alcoholic fermentation such as medium-chain fatty acids (Lonvaud-Funel et al., 1985) and pesticide residues. In the study conducted by González-Marco et al. (2010), it was observed that the nutrient (complex yeast autolysate) enrichment of a nonlimited-in-nitrogen grape must did not favor the formation of either esters or alcohols in the wine obtained. The results demonstrate that when juice is sufficient in nitrogen, the addition of amino acids does not improve the volatile composition of the wine.

Minerals

Besides sugars and nitrogen, yeasts also need various minerals to be present in the medium for optimal growth and fermentation. The specific requirements vary depending on the yeast strain used, the fermentation media, and interactions with other components, particularly interactions involving trace metals (Jacques et al., 2003). Minerals, especially key metal ions, can impact yeast growth and metabolic processes during fermentation by influencing several critical parameters. These parameters include the rate of sugar conversion to ethanol, the final ethanol yield, cell viability and vitality, and stress tolerance (Walker, 2004). An imbalance in inorganic nutrition can result in complex and subtle changes in metabolic patterns and growth characteristics. The role played by many of these ions is both enzymatic and structural (Jacques et al., 2003). Potassium, magnesium, calcium, and zinc are cationic nutrients that play vital structural and functional roles in yeast cells and are particularly significant in fermentation processes.

Potassium, the most abundant cellular cation in yeast, serves as a major cofactor for enzymes involved in oxidative phosphorylation, protein biosynthesis, and carbohydrate catabolism (Walker, 2004).

Magnesium, the most abundant intracellular divalent cation in yeast, primarily functions as an enzyme cofactor. It provides protection to yeast cultures under stress conditions, including temperature and osmotic pressure, and plays a role in alcohol tolerance. Additionally, magnesium stimulates fermentation and the synthesis of essential fatty acids.

Calcium stimulates yeast growth but it is not a growth requirement. It is involved in membrane structure and function. The significance of Ca^{2+} uptake in yeast lies in the multifunctional role of this cation as a modulator of growth and metabolic responses. A proper ratio between calcium and magnesium positively influences fermentation rates.

Copper and iron ions serve as cofactors in numerous enzymes, including the redox pigments of the respiratory chain. Copper is an essential micronutrient at low concentrations but becomes toxic at higher levels. Yeast strains vary in their sensitivity to copper and negative effects on fermentation can be seen starting at concentrations of >10 ppm.

Trace levels of zinc are essential for yeast growth and other metal ions cannot fill this requirement. Zinc serves as an essential cofactor in a number of important metabolic enzymes, e.g. alcohol dehydrogenase (Walker & Stewart, 2016). Zinc deficiency results in low yields of yeast (can inhibit budding) and slow fermentations (Jacques et al., 2003).

Vitamins

Vitamins are among the essential nutrients that have a notable impact on yeast metabolism. Vitamins are important regulators and cofactors of numerous metabolic processes. Their principal function is enzymatic, and they generally act either as co-enzymes or enzyme constituents. Essential vitamin requirements for maximum fermentation rates are strain-dependent. Yeast strains exhibit significant diversity in their vitamin requirements. Furthermore, in a given strain, those requirements may also vary between active respiration and growth on the one hand, and alcoholic fermentation on the other. With the exception of mesoinositol, nearly all vitamins are essential for yeast as they operate as integral components of coenzymes, playing a catalytic role in yeast metabolism. Biotin is indispensable for most strains, while pantothenate is a necessity for many (Jacques et al., 2003). Thiamine and its biologically active phosphorylated forms are essential cofactors for several metabolic enzymes involved in central carbon metabolism pathways like glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle. Under oenological conditions, vitamin deficiencies can lead to impaired growth, cell death, and subsequently fermentation arrests (Labuschagne & Divol, 2021).

Oxygen and lipid availability

Saccharomyces cerevisiae is unquestionably the major ethanol-producing microorganism, owing to its unique physiological behaviour of exclusively growing in anaerobic conditions, which is remarkably unusual among yeast species (Visser et al., 1990). Moreover, *S. cerevisiae* is capable of producing ethanol even in the presence of excess oxygen when high sugar concentrations are present, such as in fruit mashes or musts, a phenomenon known as the Crabtree effect (Fiechter et al., 1981). Under such growth conditions, *S. cerevisiae*, a typical Crabtree-positive yeast, produces high ethanol yields and low biomass yields, while other yeasts like *T. delbrueckii* display a less pronounced Crabtree effect, resulting in lower ethanol yields and higher biomass yields (Merico et al., 2007). On the other hand, under similar growth conditions, Crabtree-negative yeasts like *Kluyveromyces marxianus* solely utilize the respiratory pathway for glucose breakdown (van Dijken et al., 1986). Consequently, under all growth conditions, Crabtree-positive yeasts are more likely to engage in alcoholic fermentation (van Dijken et al., 1993). In winemaking, it is a common practice to aerate grape musts before fermentation to promote the initial development of yeast and hasten ethanol production. This procedure benefits those yeast species that can ferment sugars in the presence of

oxygen (Crabtree-positive), enabling them to dominate the fermentation process (Boulton et al., 1996).

Under aerobic conditions, yeasts synthesize their own major sterols (mainly ergosterol) (Parks & Adams, 1978); however, this mechanism is limited under anaerobic conditions, such as during alcoholic fermentation. Yeast growth under anaerobiosis normally requires lipid supplementation (anaerobic growth factors) or oxygen addition in order to favour the synthesis of lipids (sterols and unsaturated fatty acids), which are crucial for maintaining the integrity of the plasma membrane (Andreasen & Stier, 1953; Andreasen & Stier, 1954). Insufficient availability of these nutrients can hinder both sugar uptake and yeast growth, resulting in an incomplete fermentation process. Oxygen or lipid supplementation not only restores yeast's fermentative activity but also affects the formation of volatile metabolites (Varela et al., 2012; Tesnière, 2019).

The addition of lipids to grape must has been found to increase the levels of esters, higher alcohols, and volatile acids. According to Varela et al. (2012), the combined effect of oxygen and lipid supplementation mostly affects higher alcohol concentrations. Another study (Rollero et al., 2014) was conducted to assess the combined impact of assimilable nitrogen and phytosterol content on the production of fermentative volatile compounds. The authors observed a correlation between the quantity of phytosterol and the generation of acetic acid, with higher concentrations of phytosterol leading to reduced levels of acetic acid. This observation could be attributed to potential differences in the need for acetyl-CoA during lipid synthesis, which is expected to be minimal when exogenous lipids like phytosterols are present. Moreover, variations were observed in the regulation of higher alcohol acetates and ethyl esters synthesis, suggesting that the availability of fatty acids primarily influences the production of ethyl esters, while the activity of alcohol acetyltransferases plays a key role in the generation of acetates.

3.2.2.6. Acidification techniques of fruit mash

The production of alcoholic beverages, especially alcoholic fermentation, is accompanied by a number of concerns, such as spoilage and undesirable changes in flavor associated with the metabolic activity of undesirable microorganisms (Jeon et al., 2015). It is well known that lower acidity and higher pH generally support the growth of microorganisms, including several unwanted or spoilage species. Therefore, it is essential to monitor the pH and acidity of the medium throughout the fermentation. The most common practise of acid management worldwide involves the addition of organic acids at the beginning of alcoholic fermentation to prevent the proliferation of spoilage microorganisms (LAB and other bacteria, moulds, and foreign yeasts).

In brewing and winemaking, mash acidification is achieved by adding tartaric, malic, or lactic acids (De Roos & De Vuyst, 2018; Comuzzo & Battistutta, 2019). Until 2004, the use of L-(+)-tartaric acid as a wine acidulant was the most common practise and the only one authorised by the International Organisation of Vine and Wine. However, the effectiveness of tartaric acid is often limited in warm regions due to the precipitation of potassium salts. In addition, when used in high amounts (3-4 g/L), tartaric acid can impart a noticeable and unnatural sour taste, impacting the sensory profile of the wine (Frost et al., 2017; Morata et al., 2019).

Distilleries have adopted different solutions to address this critical issue; yet, pH adjustment or acidification remains the most widely used technique. Typically, reducing the pH value of the mash to approximately 3.0 is considered a safe practice (Da Porto, 2002; Bovo et al., 2012). For pH correction of apple and pear mashes, Spaho et al. (2021) used a diluted solution of sulfuric acid. Bovo et al. (2012) demonstrated the effectiveness of sulfuric acid as an acidification tool for grape marc. The pH reduction during fermentation led to significant changes in the yeast-bacteria population ratio and yeast species turnover. These microbiological changes resulted in an improvement of the aromatic profile of the distillate (Grappa), mainly due to the reduction of the volatile compounds associated with potential off-flavors. In 2022, Blumenthal et al. attempted to produce an innovative spirit from coffee cherries. They adjusted the pH of the prepared mash with lactic and malic acids in order to prevent the uncontrolled growth of undesirable microorganisms.

Nowadays, as both science and industry are open to innovation, new alternatives that offer acid protection for the fermentation medium are being researched. Attention is being paid to specific microorganisms that can offer a natural acidification and rapid pH drop in the mash by producing mostly lactic acid and other organic acids as part of their metabolism (Vilela, 2018; Morata et al., 2018; Vicente et al., 2021).

An early study by Ribéreau-Gayon et al. (1975) revealed some intriguing characteristics of *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans),* including its high production of L-lactic acid, low production of volatile acidity, moderate alcohol productivity, and the absence of off-flavor production. Nowadays, the ability of *L. thermotolerans* to act as an acidifying agent and its potential use in wine and beer production are of increasing interest (Kapsopoulou et al., 2007; Hill, 2015; Domizio et al., 2016; Postigo et al., 2023).

L. thermotolerans possesses moderate fermentative power, this limitation obliges combining it with another, more fermentative yeast genera, such as *Saccharomyces,* to ensure complete fermentation of the sugars from the media. Additionally, employing yeasts with acidification

properties in mixed cultures with *S. cerevisiae* during alcoholic fermentation represents a convenient biological alternative. In the study conducted by Comitini et al. (2011), mixed fermentations of *L. thermotolerans* and *S. cerevisiae* showed some promising results, like reductions in pH and volatile acidity, as well as enhancements in total acidity, glycerol, and 2-phenylethanol production. The sequential inoculation of *L. thermotolerans* with *S. cerevisiae* has been demonstrated to be a powerful tool to modulate the acidity and ethanol of red wines in warm regions. The strain L3.1 of *L. thermotolerans* is capable of producing lactic acid by utilizing sugars as precursors, thereby decreasing the final alcohol content of the wine (Morata et al., 2019). The conversion of glucose to lactic acid by yeast involves the participation of the enzyme lactate dehydrogenase, which catalyzes the transformation of pyruvate into lactic acid (Sauer et al., 2010). According to scientific literature, the acidification capacity of *L. thermotolerans* species has been reported to range from 1 to 9 g/L in terms of lactic acid content and from 1 to 6 g/L in terms of total acidity (Benito, 2018). The achieved pH reductions can vary between 0.1 and 0.5 units, depending on the amount of L-lactic acid produced during alcoholic fermentation. These variations primarily depend on factors such as the selected strain, fermentation conditions, and inoculation method (Sgouros et al., 2020; Vicente et al., 2021). Among the different inoculation methods, sequential inoculation has shown the most favourable outcomes, as it allows *L. thermotolerans* to ferment alone for a longer period without competition from other microorganisms. Other non-*Saccharomyces* yeasts, such as *Starmerella bacillaris* or *Candida stellata,* also have acidifying potential because of the production of pyruvic and succinic acid (Vicente et al., 2022).

In addition to yeasts, different bacterial strains have been studied for their ability to induce biological acidification. *Oenococcus oeni* is well-known in winemaking for its ability to metabolize malic acid into lactic acid and CO_2 (Urbina et al., 2021). Dierings et al. (2013) suggest that *O. oeni* should be inoculated in the apple must after a decline of the yeast *S. cerevisiae* to achieve optimal viability and the best outcomes in cider production. Furthermore, *Lactobacillus* strains are good candidates for use as acidifying starters (Brizuela et al., 2018). They can synthesize only lactic acid from sugars and have no danger of acetic acid synthesis (Ribéreau-Gayon et al., 2006b; Onetto & Bordeu, 2015; Pardo & Ferrer, 2019). Lucio et al. (2016) claim that lactic acid produced by *Lactobacillus* strains may be a precursor of aromatic compounds such as ethyl lactate, thus enhancing the aromatic profile of the beverage. Additionally, these bacteria ensure microbiological stability through the antimicrobial effects of lactic acid and low pH. The results of Lowe et al. (2005) demonstrated that the biologically acidified beer with *Lactobacillus amylovorus* showed improved

characteristics, including a lighter colour, improved foam stability, and a more well-rounded taste with increased shelf life in comparison with the chemically and nonacidified beers. According to Vicente et al. (2022), the acidification effect increases if *Lactiplantibacillus plantarum* (former *Lactobacillus plantarum)* is inoculated before *S. cerevisiae* in mixed fermentations, as they do not tolerate high ethanol concentrations. Recently, Chen et al. (2023) showed that ciders produced by mixed inoculation with *Lb. plantarum* exhibited improved flavor because of their higher levels of volatiles such as esters and higher alcohols, as well as higher contents of non-volatile compounds like organic acids and polyphenols in comparison with the single culture of *S. cerevisiae*.

3.2.3. Distillation process

The distillation process is the subsequent step that follows fermentation. It involves the separation and concentration of alcohol from the fermented mash, resulting in the production of distilled spirits. Water and ethanol are the primary constituents of these beverages, making up around 99% of the overall content of spirits. Nevertheless, a vast array of compounds known as congeners have been identified in the water-ethanol mixture. Although present in very low concentrations, these congeners are crucial for the quality of the beverages (Spaho, 2017). The aroma profile of the spirits is defined by the raw material and fermentation process. Furthermore, during distillation, the heat applied in the boiler or pot can trigger chemical reactions among the existing congeners, leading to the formation of other compounds that enhance the complexity of the final distillate (López et al., 2017). Due to variations in boiling point, solubility in ethanol and water, and ethanol content in the vapour during distillation, the congeners will distil differently (Léauté, 1990).

The product obtained during distillation is separated into three fractions known as the head, heart, and tail. Each fraction consists of varying quantities and varieties of congeners. The heads contain a higher concentration of volatile compounds with low boiling points (acetaldehyde, acetone, and ethyl acetate) and other undesirable compounds. These compounds would give the distillates an unpleasant and sharp flavor, so they must be eliminated. The middle fraction, referred to as the "heart", contains reduced concentrations of undesirable compounds compared to other fractions. It is rich in ethanol, carries pleasant and fruity aromas, and ultimately transforms into the drinkable product. On the other hand, the final "tail" fraction possesses an unpleasant aroma attributed to the elevated concentration of fusel alcohols and other compounds with boiling points higher than ethanol (Bernot et al., 1990; Spaho, 2017).

Two distinct types of distillation equipment, namely the copper Charentais alembic (French style) and batch distillation columns (German style), are utilized in the manufacturing of fruit spirits.

Copper alembics have been generally utilized in small commercial and artisanal distilleries and medium-sized distilleries. Essentially, these systems consist of a copper boiler and a condenser (Silva et al., 2000). The internal reflux in alembics occurs as a result of condensation taking place in the head and the swan neck, which is primarily influenced by the external temperature. Modifying this reflux is possible by regulating the heating power in the boiler, making it a restricted system for controlling and adjusting the distillation process (Léauté, 1990; Rodríguez-Bencomo et al., 2016).

The traditional Hungarian method is considered to be the double distillation method, known as 'kisüsti', where 'kis' means small and 'üsti' refers to a traditional Hungarian pot or cauldron with a maximum size of 1000 liters. The 'kisüsti' distillation process involves two steps. The first step begins with the distillation of the mash, which has a relatively low alcohol content (2-10%), and results in the production of the first distillate (brute alcohol) with an alcoholic strength of 16-28%, containing all the volatile compounds because there is no separation of fractions. In the second step, the raw spirit undergoes re-distillation, increasing the alcohol content to 60-70% while also separating the spirit fractions (Harcsa et al., 2014; Korzenszky et al., 2020).

Batch distillation columns are composed of a copper pot still equipped with column plates and a dephlegmator. The dephlegmator within the column serves the purpose of partially condensing the distillate vapour, allowing a portion of it to return as counter-current distillate for further distillation. The plates in the column are usually copper sieve trays, which enable the distillate vapours to pass through. The counter-current distillate drains back down and accumulates on the subsequent lower plate, ready to undergo re-distillation. This creates the so-called reflux in the process known as rectification, resulting in an increase in the separating efficiency of different components (Claus & Berglund, 2005). Consequently, employing additional trays in the distillation process leads to a higher alcohol concentration and a lower congener concentration. Some of these congeners are pleasant aroma compounds, and it is not desired to excessively eliminate them during the alcohol purification process (Léauté, 1990; Spaho, 2017). Depending on the moment of distillation, changing the reflux rate can increase or decrease the concentration of specific compounds in the distillate (Osorio et al., 2004; Rodríguez-Bencomo et al., 2016). Lower reflux rates promote the transition of less volatile congeners into the condenser, resulting in a more complex product, albeit potentially contributing to an unrefined taste. Conversely, higher reflux rates are associated with improved cleanliness but may also result in a lack of aroma intensity (Goessinger & Lehner, 2007). So, reflux can be employed to customize the composition of the spirit according to consumer preferences (Rodríguez-Bencomo et al., 2016). Liebminger et al. (2021) showed that, in addition to the reflux rate, the heating ramp of the

pot is equally important as it easily impacts the relative volatility of tail congeners, allowing them to enter the condenser at the beginning of the distillation. The presence of tail congeners in the heart fraction can have detrimental effects on the quality of the final product. These findings indicate that it is important to control and monitor both process parameters in fruit spirit distillation processes. The study of Heller and Einfalt (2022) indicated that the thermal energy input and internal reflux rates are two crucial parameters that define the physical heat and mass transfer rates during fruit spirit distillation processes.

Both distillation methods are based on the same theoretical principles, i.e., mass and energy balances, heat and mass transfer, and vapour-liquid equilibrium, which play a crucial role in the selective separation of desirable and undesirable aroma compounds (García-Llobodanin et al., 2011; Spaho et al., 2013; Heller & Einfalt, 2022). The choice of distillation equipment leads to variations in the quantity of flavor compounds present in the final spirits.

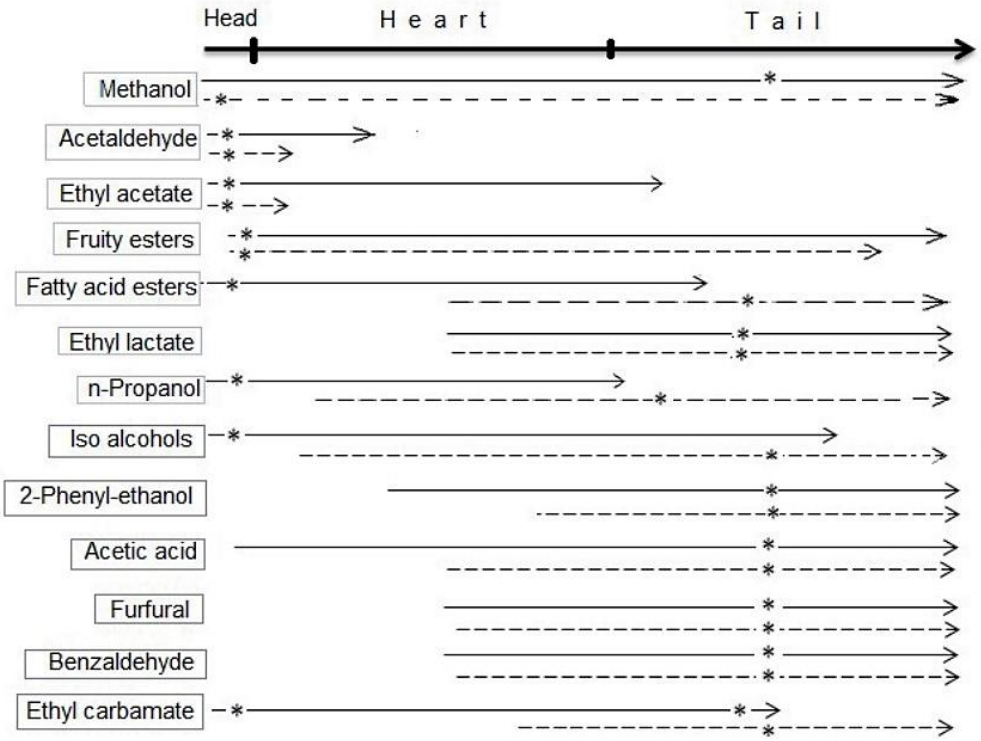


Figure 4. Distribution of main volatile compounds by using different distillation equipment: full line, alembic distillation; dashed line, column distillation and *, shows the cut where higher component is accumulated (Spaho, 2017).

As depicted in Figure 4, the main variations are seen in the distributions of methanol, propanol, higher alcohols, and fatty acid esters. Alembic stills yield fruit distillates with better aroma

characteristics, albeit at a slower pace and requiring more labour. On the other hand, column stills are known for purifying the distillate, resulting in a decent aroma and higher alcohol concentration (Spaho, 2017). In a study conducted by García-Llobodanin et al. (2011), the aromatic composition of pear spirits obtained through traditional alembic re-distillation was compared to that of spirits obtained through a single distillation in a packed column. The distillates from the column exhibited lower levels of toxic compounds (such as acetaldehyde and methanol), higher alcohol content, and elevated levels of higher alcohols and esters compared to the alembic re-distillates.

3.2.4. Maturation of fruit distillates

Maturation plays a crucial role in creating unique aromas in distilled beverages. Normally, fresh distillates are not suitable for consumption due to their inharmonious and pungent taste and odor. Consequently, a period of maturation is required in order to refine the organoleptic characteristics of the product (Pecić et al., 2012). The undesirable characteristics, such as "hotness," "rawness," and "greenness," often present in fresh distillates, diminish with aging. During storage, various physical and chemical reactions alter the concentration of compounds in the distilled beverage, resulting in a well-balanced and harmonious flavor.

The texture and characteristics of containers used during the aging process are key factors in the development of maturation flavors in distilled beverages (Wang et al., 2023). Most of the scientific literature focuses on wood aging (Coldea et al., 2020; López-Ramírez et al., 2013), which causes an aroma contribution from maceration and oxidative reactions (Matias-Guiu et al., 2020). Wood is a complex natural material primarily composed of lignin, cellulose, and hemicellulose. When wood comes into direct contact with the distilled beverage, numerous physicochemical interactions occur. In particular, semivolatile and non-volatile compounds of wood change the colour of the distillate and contribute to an enhanced flavor. For instance, compounds extracted from wood, such as lignin derivatives (vanillin), lactones (octalactone), and furan derivatives (furfural), may react with chemicals present in the distilled beverage, forming new molecules. These newly formed compounds are primarily esters (ethyl acetate) and ethers (vanillin ether), resulting from the reaction of ethanol with wood-extracted compounds (Wang et al., 2023). Permeable wooden barrels allow air to pass in and cause ethanol to evaporate; thus, the ethanol content decreases, and the aroma becomes more intense, complex, and concentrated (Christoph & Bauer-Christoph, 2007).

The primary factors influencing the chemical and sensory changes in distilled beverages are the type of wood and the heat treatment applied. Additionally, the toasting level of the barrels plays a significant role in the aroma profile. Brandies aged in strong toasted barrels exhibit the highest

intensities of woody, vanilla, caramel, smoky, spicy, and burned characteristics, which are mainly attributable to the high contents of furfural, coniferaldehyde, and vanillic acid (Wang et al., 2023).

Nevertheless, researchers have also been investigating the effects of aging various distillates in alternative inert containers like glass, plastic (Flouros et al., 2003), ceramic (Qiao & Sun, 2015), and stainless steel (Rodríguez Madrera et al., 2011). Moreover, several studies examined the effects of storage conditions (temperature, light exposure, etc.) on the evolution patterns of volatile compounds (Diéguez et al., 2002; Matias-Guiu et al., 2020). In this respect, Matias-Guiu et al. (2020) stated that when fruit spirits are stored at a temperature of 45 ºC, most compound levels are drastically reduced during the first 7 months of storage, and these differences remain consistent after 1 year. On the other hand, 5 ºC appeared to slightly favour the preservation of the volatile composition of the spirit. Regarding the composition of the distillate itself, Spaho et al. (2019) highlighted that the initial concentration of alcohol is critical for its aging. According to Różański et al. (2020), the decreases in the concentration of fusel alcohols and the increases in the concentrations of esters were positively correlated to the alcohol concentrations of rye and plum distillates.

Different components of the fresh distillate may react with each other during the maturation period, which is favored by its high ethanol content and storage conditions. Thus, the concentrations of ethyl esters of fatty acids increase during ageing, but the concentrations of esters of other alcohols, such as 3-methylbutyl acetate, decrease due to transesterification. Further reactions during ageing are the evaporation of aldehydes or their reaction to form acetals (Rodríguez Madrera et al., 2003; Christoph & Bauer-Christoph, 2007).

4. MATERIAL AND METHODS

4.1. Materials

4.1.1. Raw material

Apples (*Malus domestica* 'Jonathan', 'Golden Delicious', 'Jonagold', 'Gala') and pear juice concentrate (70 w/w%) were used in the study. The apples were obtained from local producers in Hungary, while the concentrate was purchased from Berrymix Ltd.

4.1.2. Microorganisms

The yeast strains used in the study included:

- Uvaferm 228™ (*Saccharomyces cerevisiae*)

Uvaferm 228 offers several advantages, including a quick fermentation start, controlled and reliable fermentation, tolerance towards high ethanol levels and low temperatures (~10 °C), high ethanol production, high ß-glucosidase activity enhancing fermentative aromas, and minimal formation of undesirable fermentation byproducts.

- Level2 Biodiva™ (*Torulaspora delbrueckii*)

Biodiva was chosen based on its ability to enhance wine aroma and mouthfeel complexity. Specific features of this strain include its tolerance to osmotic shock, low volatile acid production, and its capacity to enhance varietal and fermentation esters while also contributing to the mouthfeel.

- Viniflora Concerto™ (*Lachancea thermotolerans*)

Concerto is not intended to achieve alcoholic fermentation but to improve aroma complexity. It reduces the production of alcohol and improves the acidity of the wine by means of lactic acid production. Furthermore, it is characterized by low levels of acetic acid and volatile acid production. In particular, it can produce ethyl isobutyrate, a molecule recognized as a key component of fresh strawberry aroma.

- Laktia™ (*Lachancea thermotolerans*)

Laktia's unique attribute is the production of high levels of lactic acid during fermentation, which naturally enhance the medium's total acidity. In addition, Laktia produces low levels of volatile acidity

and contributes aromatic complexity to the product. It has a low alcohol tolerance, while it exhibits a high tolerance for high temperatures.

- Melody™ (mixed culture of 20% *Torulaspora delbrueckii*, 20% *Lachancea thermotolerans*, and 40% *Saccharomyces cerevisiae*)

These yeast blends provide the positive attributes and complexities of a "wild-fermentation", but with greater control and consistency, making them particularly suitable for Gin, Brandy, and Fruit Brandy production. Melody is an ideal mixture of *Saccharomyces* and non-*Saccharomyces* yeast, specifically developed to enhance flavors, aromas, and mouthfeel constituents.

- Oenoferm® X-treme (Hybrid *Saccharomyces cerevisiae*)

X-treme is a GMO-free hybrid yeast created by using protoplast fusion to merge the favorable traits of two different *Saccharomyces cerevisiae* strains. It is extraordinarily strong and resistant to low temperatures. Moreover, it promotes aroma production with an emphasis on minerality, with well-integrated fruity and spicy components.

- Oenoferm® X-thiol (Hybrid *Saccharomyces cerevisiae*)

X-thiol is a GMO-free hybrid yeast developed through protoplast fusion to combine the positive characteristics of two different *Saccharomyces cerevisiae* strains. The most important features of this strain include great fermentation strength, high ethanol tolerance, and the production of complex fermentation aromas with a fresh and fruity bouquet (pink grapefruit and blackcurrant).

- SafŒno™ HD S135 (Hybrid *Saccharomyces cerevisiae* x *Saccharomyces bayanus*)

HD S135 results from the hybridization of two yeast strains, with the goal of combining their most favorable attributes for polyphenol binding and resistance under difficult fermentation conditions. This yeast strain is known for its rapid onset of alcoholic fermentation, fast kinetics, moderate volatile acidity generation, and notable production of higher alcohols and esters.

- SafŒno™ HD S62 (Hybrid *Saccharomyces cerevisiae* x *Saccharomyces bayanus*)

HD S62 is the outcome of hybridizing two yeast strains, with the intention of combining their most advantageous traits to enhance polyphenol extraction and stability while also providing resistance to difficult fermentation conditions. This yeast strain is recognized for its rapid kinetics, efficient

fructose assimilation, and moderate potential for producing higher alcohols and esters, particularly ethyl esters.

- SafŒno™ HD A54 (Hybrid *Saccharomyces cerevisiae* x *Saccharomyces bayanus*)

This yeast strain, specially developed for white and rosé wines, is designed to enhance the production of floral and fruity higher alcohols (2-phenylethanol and isoamyl alcohol) and their associated acetate esters while maintaining a clean fermentation profile. Additionally, it preserves high total acidity and produces moderate levels of volatile acidity.

Additionally, two LAB strains were used:

- Smartbrev Harvest LB-1 (*Lactiplantibacillus plantarum*)

LB-1 is designed for sour beer production, crafted to impart clean and crisp flavors and aromas, while ensuring rapid and safe acidification for optimal results.

- WildBrew Sour Pitch ™ (*Lactiplantibacillus plantarum*)

Sour Pitch exhibits a fast pH drop that can be completed within 1-2 days. Exhibits high lactic acid and low acetic production. In addition, it contributes to a citrusy and tangy aroma and flavor with subtle fruity undertones.

The yeast and bacterial strains were provided by Chr. Hansen A/S (Hoersholm, Denmark), Kertrade Ltd. (Dunavarsány, Hungary), and Kokoferm Ltd. (Gyöngyös, Hungary).

4.1.3. Yeast nutrients

The applied yeast nutrient supplements and their compositions are detailed in Table 1.

4.1.4. Chemicals

Standards (acetaldehyde, methanol, 1-propanol, 2-butanol, 1-butanol, 2-methyl-1-propanol, phenethyl alcohol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-hexanol, trans-3-hexen-1-ol, cis-2-hexen-1-ol, benzyl alcohol, ethyl acetate, ethyl lactate, ethyl butyrate, isoamyl acetate, ethyl octanoate, diethyl succinate, hexyl acetate, 2-phenethyl acetate, phenylacetic acid, ethyl phenylacetate, ethyl myristate, myrcene, limonene, linalool, glucose, fructose, sucrose, succinic acid, acetic acid, lactic acid) and all chemicals of analytical grade were supplied by Sigma-Aldrich (Steinheim, Germany).

Table 1. Yeast nutrient supplements

Nutrient	Producer	Composition
VitaDrive F3	Erbslöh	Inactivated yeasts, yeast cell walls (30%), diammonium hydrogen phosphate (1%)
Vitaferm Ultra	Erbslöh	Diammonium hydrogen phosphate (60%), inactivated yeasts (provides aminoacids, minerals, and vitamins), yeast cell walls (11%), thiamine hydrochloride (0,065%)
Vitamon Combi	Erbslöh	Diammonium hydrogen phosphate (99,67%), microcrystalline cellulose, thiamine hydrochloride (0,13%)
Vitamon A	Erbslöh	Diammonium hydrogen phosphate
OptiMUM White	Lallemand Oenology	Inactivated yeast organic nitrogen content < 9,5% of dry matter (N equivalent)
Uvavital	Lallemand Oenology	Mixture of vitamins, nitrogen, arginine, Mg, Zn, sterols and fatty acids
V Starter Premium	Enologica Vason	Yeast autolysates naturally rich in aminoacidic compounds and prestigious vegetal polysaccharides 99,85%, thiamine 0,15%
Booster Aktiv Premium	Enologica Vason	Yeast hulls 50%, thiamine 0,1%, the rest are filtration adjuvants of vegetal origin
FosfoActiv Premium	Enologica Vason	Fine micrometry cellulose 50%, dibasic ammonium phosphate 37.5%, yeast cell walls 12.5%
Genesis Fresh	Oenofrance	Inerted yeast (provides amino acids and polysaccharides): 85%, microcrystalline cellulose 15%

4.2. Methods

4.2.1. Mashing and fermentation conditions

Upon delivery at the laboratory, the apples were manually sorted (mechanically harmed, decayed, and rotten fruits were excluded) and cleaned gently with water to remove dust and debris, and then crushed with a centrifugal mill. Fermentations were performed either on a laboratory scale or on a pilot scale. When lab-scale fermentations were conducted, the resulting apple mash was placed in 5 L Erlenmeyer flasks, each comprising 4 kg of mash. When performing pilot-scale fermentations, the apple mash was placed in 50 L stainless steel fermentation tanks, each containing 35 kg of mash. Then, the enzyme Lallzyme^{TM} HC (Lallemand, Montréal, QC, Canada) was used at a dose of 3 g/100 kg to break down the pectin molecules and enhance the liquefaction of the mash.

4.2.1.1. Acidification of the mash

The pH of the mash is typically adjusted to 3.0 to inhibit the growth of undesirable microorganisms. Laboratory-scale fermentations were carried out to test the efficiency of different

chemical and biological acidification techniques during mash fermentation (Figure 5). The mash from Gala apples was distributed into Erlenmeyer flasks. Phosphoric and lactic acid solutions (25% v/v) were used in different ratios: 100:0, 90:10, 80:20, 70:30, and 60:40, respectively. The first flask served as the control, where no acidification of the mash occurred. Whereas, the others received the pre-prepared acid solutions. Following the addition of the acid solutions, the mash was thoroughly mixed. Afterwards, the mash was supplemented with 40 g/100 kg of Uvavital™ yeast nutrients. Finally, fermentation was initiated by inoculating the yeast *S. cerevisiae* (Uvaferm 228, 40 g/100 kg) in the mash. On the other hand, in the last flasks, the mash was inoculated with *Lachancea thermotolerans* (Laktia, 25 g/100 kg), *Lactiplantibacillus plantarum* (Sour Pitch, 35 g/100 kg), and *Lactiplantibacillus plantarum* (LB-1, 35 g/100 kg). Whereas, *S. cerevisiae* (Uvaferm 228) was added 24 hours later in the mash. The flasks were sealed with airlocks, and the fermentation runs were conducted in triplicate at a temperature of 16±1 ºC for 15 days.

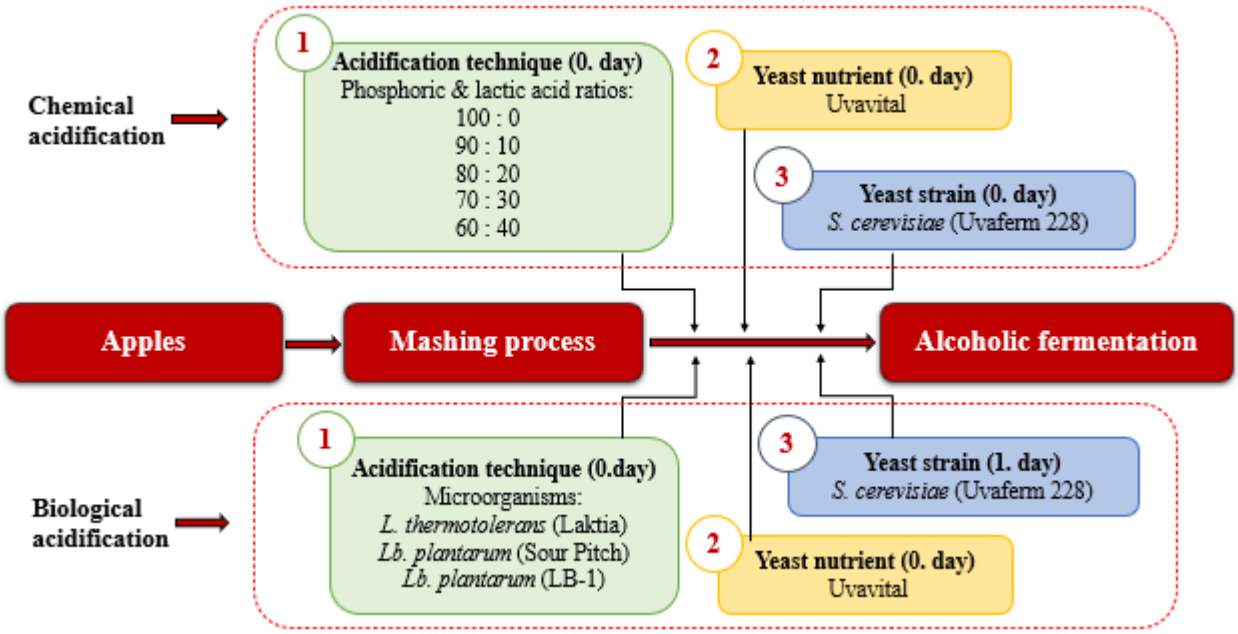


Figure 5. The schematic flowchart of the acidification process

4.2.1.2. Nutrient supplementations

Laboratory-scale fermentations were conducted to evaluate how various nutrient supplements affect the fermentation performance and the production of volatile compounds by *S. cerevisiae*. Details regarding the nutrient types, their compositions, and the experimental design can be found in Tables 1 and 2. Pear juice concentrate was used as the fermentation medium, which was diluted with

tap water to approximately 13 ºBrix and distributed among the Erlenmeyer flasks. No acid correction was performed in the juice. The juice was inoculated with 40 g/100 kg of *S. cerevisiae* (Uvaferm 228) yeast to initiate the fermentation process. The flasks were closed with airlocks, and the fermentation runs were performed in triplicate at a temperature of 16±1 ºC for 14 days.

Table 2. Experimental design of nutrient supplementation during fermentation process

	Day 0	Day 1	Day 2	Day 4	Day 7	Day 10
Control (no nutrients)						
N1	Vitamon A (30 g/hL)			Vitamon A (20 g/hL)		Vitamon A (20 g/hL)
N2	Vitamon A (55 g/hL)			Vitamon Combi (65 g/hL)		
N3	Vitaferm Ultra F3 (35 g/hL)			Vitaferm Ultra F3 (35 g/hL)		
N4	Vitadrive F3 (35 g/hL) + Vitaferm Ultra F3 (35 g/hL)			Vitaferm Ultra F3 (35 g/hL)		
N5	Vitadrive F3 (30 g/hL) + Vitamon A (30 g/hL)			Vitamon A (40 g/hL)		
N6	Uvavital (20 g/hL)			Uvavital (20 g/hL)		Uvavital (10 g/hL)
N7	Optimum White (30 g/hL)			Uvavital (10 g/hL)		
N8	V Starter Premium (20 g/hL)	Fosfoactiv (20 g/hL)			Booster Activ (10 g/hL)	
N9	Genesis Fresh (30 g/hL)		Vitamon Combi (30 g/hL)			

4.2.1.3. Hybrid yeasts

Laboratory-scale fermentations were performed to evaluate the fermentation potential of various hybrid yeasts in comparison to an industrial strain of *S. cerevisiae* (Figure 6). Apples of the Jonagold cultivar were mashed and then distributed into Erlenmeyer flasks. The pH of the mash was corrected to 3.0 using a diluted solution of phosphoric and lactic acid (25% v/v) in a ratio of 90:10. Subsequently, 40 g/100 kg of Uvavital yeast nutrient was added to the mash. Controlled alcoholic fermentations were initiated by adding rehydrated yeasts to each flask. The yeast strains X-treme, HD

S135, HD S62, and HD A54 were inoculated at a dose of 25 g/100 kg, while X-thiol was added at a rate of 35 g/100 kg, and *S. cerevisiae* (Uvaferm 228) was incorporated at a rate of 40 g/100 kg of mash. The flasks were sealed with airlocks, and the fermentation runs were conducted in triplicate at a temperature of 16±1 ºC for 14 days.

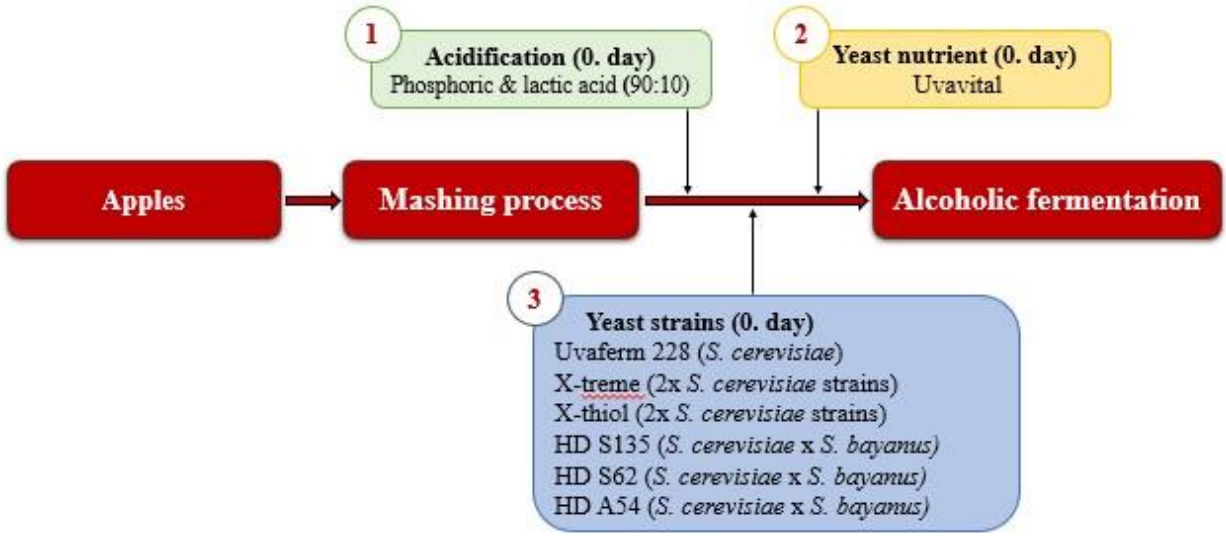


Figure 6. The schematic flowchart of the fermentation process

4.2.1.4. Non-*Saccharomyces* yeasts

Pilot-scale fermentations were carried out to assess the fermentation performance of non-*Saccharomyces* strains on Jonathan apple mash (Figure 7). The pH of the mash was adjusted to 3.0 using a mixture of phosphoric and lactic acid in a ratio of 90:10. Thereafter, 20 g/100 kg of Uvavital yeast nutrient was added to each tank. Fermentations were initiated by adding rehydrated yeast starters. *T. delbrueckii* (Biodiva) and *L. thermotolerans* (Concerto) were sequentially inoculated with *S. cerevisiae* (Uvaferm 228). Initially, the non-conventional yeast was inoculated at a concentration of 25 g/100 kg, followed by the addition of the *Saccharomyces* yeast at 30 g/100 kg three days later. In the case of Melody, a yeast mixture, the inoculation was performed in a single step at 30 g/100 kg. The tanks were sealed with air-tight covers fitted with airlocks, enabling the release of carbon dioxide. Fermentations were conducted in triplicate at 16±1 °C until no further changes were observed in the apparent extract.

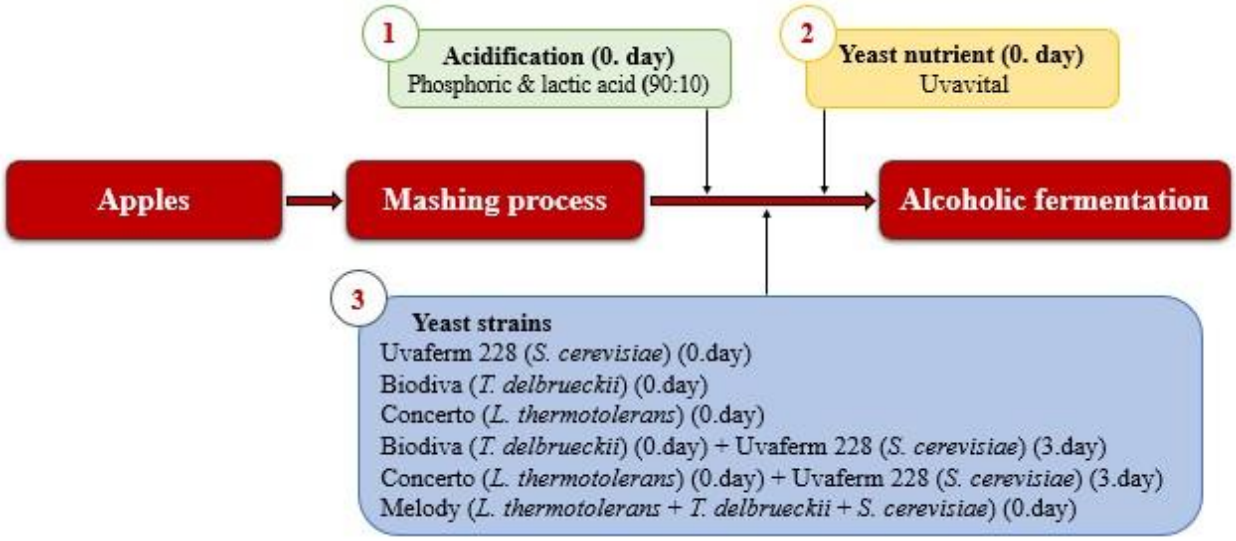


Figure 7. The schematic flowchart of the fermentation process

4.2.1.5. Combinatorial effect of yeast strains and mash treatments during fermentation

After individually testing various parameters (acidification method, nutrient supplements, and yeast strains), the most optimal alternatives were chosen and combined in a new experiment. The mash, prepared from Gala apples, was treated as outlined in Table 3. Briefly, the pH of the mash was adjusted to 3.0 using a mixture of phosphoric and lactic acid in a 70:30 ratio, or the mash was inoculated with *Lb. plantarum* (LB-1) at a dosage of 35 g/100 kg. Afterwards, the mash was supplemented with yeast nutrients. Nutrient treatment 1 consisted of Vitamon A (55 g/100 kg) and Vitamon Combi (65 g/100 kg), while nutrient treatment 2 included Genesis Fresh (30 g/100 kg) and Vitamon Combi (30 g/100 kg). The yeasts were rehydrated following the manufacturer's instructions and then inoculated into the mash at the following doses: *S. cerevisiae* (Uvaferm 228) at 40 g/100 kg, hybrid *S. cerevisiae* (X-treme) at 30 g/100 kg, and *L. thermotolerans* (Concerto) at 25 g/100 kg. Once the corresponding yeasts were inoculated, all the tanks were closed with airtight lids fitted with airlocks, enabling the release of carbon dioxide. The fermentation runs were carried out in triplicate at a temperature of 16±1 ºC until no further changes were observed in the soluble solids content (ºBrix).

Table 3. Experimental design of the fermentation process

Samples	Acidification method	Nutrient supplements	Yeast strain
Apple mash 1	70:30 (Lactic acid : Phosphoric acid)	Vitamon A (0.day) + Vitamon Combi (4.day)	Uvaferm 228 (*S. cerevisiae*) (0.day)
Apple mash 2	70:30 (Lactic acid : Phosphoric acid)	Vitamon A (0.day) + Vitamon Combi (4.day)	X-treme (Hybrid *S. cerevisiae*) (0.day)
Apple mash 3	70:30 (Lactic acid : Phosphoric acid)	Vitamon A (0.day) + Vitamon Combi (4.day)	Concerto (*L. thermotolerans*) (0.day) + Uvaferm 228 (3.day)
Apple mash 4	LB-1 (*Lb. plantarum*)	Vitamon A (0.day) + Vitamon Combi (4.day)	Uvaferm 228 (*S. cerevisiae*) (2.day)
Apple mash 5	LB-1 (*Lb. plantarum*)	Vitamon A (0.day) + Vitamon Combi (4.day)	X-treme (Hybrid *S. cerevisiae*) (2.day)
Apple mash 6	LB-1 (*Lb. plantarum*)	Vitamon A (0. day) + Vitamon Combi (4.day)	Concerto (*L. thermotolerans*) (2.day) + Uvaferm 228 (5.day)
Apple mash 7	70:30 (Lactic acid : Phosphoric acid)	Genesis Fresh (0.day) + Vitamon Combi (2.day)	Uvaferm 228 (*S. cerevisiae*) (0.day)
Apple mash 8	70:30 (Lactic acid : Phosphoric acid)	Genesis Fresh (0.day) + Vitamon Combi (2.day)	X-treme (Hybrid *S. cerevisiae*) (0.day)
Apple mash 9	70:30 (Lactic acid : Phosphoric acid)	Genesis Fresh (0.day) + Vitamon Combi (2.day)	Concerto (*L. thermotolerans*) (0.day) + Uvaferm 228 (3.day)
Apple mash 10	LB-1 (*Lb. plantarum*)	Genesis Fresh (0.day) + Vitamon Combi (2.day)	Uvaferm 228 (*S. cerevisiae*) (2.day)
Apple mash 11	LB-1 (*Lb. plantarum*)	Genesis Fresh (0.day) + Vitamon Combi (2.day)	X-treme (Hybrid *S. cerevisiae*) (2.day)
Apple mash 12	LB-1 (*Lb. plantarum*)	Genesis Fresh (0.day) + Vitamon Combi (2.day)	Concerto (*L. thermotolerans*) (2.day) + Uvaferm 228 (5.day)

The time of nutrient addition and yeast inoculation is given in brackets.

4.2.2. Distillation Process

When laboratory-scale fermentations were carried out, the distillation process was performed on glass distillation equipment with a capacity of 3 L (Figure 8A). The temperature of the heater was maintained at 100 °C. Cool water circulated continuously throughout the system at a fixed flow rate. Distillation was carried out slowly and continuously, allowing for the proper separation of head, heart, and tail fractions.

When pilot-scale fermentations were carried out, the distillation process was performed in a steam-heated still equipped with a rectifying column and dephlegmator (Hagyó Spirit Company, Miskolc, Hungary) (Figure 8B). The distillation unit was computer-controlled, and process parameters, including condenser temperature, reflux ratio, and heating program, were set through software. The rectifying column was equipped with three bubble cap trays: the lower tray held 70%, the middle tray held 45%, and the upper tray was bearing only 15% condensate, which flowed back as reflux from the dephlegmator (condenser). After the completion of fermentation, all batches were distilled using the same distillation settings. The collected distillates were separated into three fractions: heads, hearts, and tails. Heart fractions were subjected to further analysis.

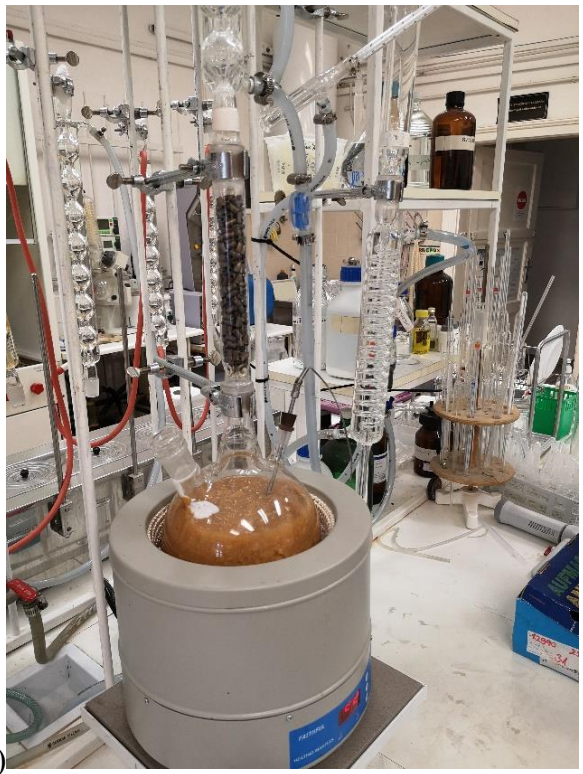
(A)  (B) 

Figure 8. (A) Glass distillation system and (B) steam-heated still equipped with a rectifying column and dephlegmator

4.2.3. Maturation Process

After evaluating the fermentation potential of non-*Saccharomyces* yeasts and recognizing their positive influence on the aroma profile of apple distillates, the two most promising strains were employed to produce apple spirits. These spirits were subsequently subjected to a maturation period. Golden Delicious apples served as the substrate for a pilot-scale fermentation, wherein *T. delbrueckii* (Biodiva) and *L. thermotolerans* (Concerto) were used in sequential inoculation with *S. cerevisiae* (Uvaferm 228). The mash was treated similarly, as described in Section 4.2.1.4.

The distillates produced from the fermentation of apple mash by *S. cerevisiae* (Uvaferm 228), *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228), and *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228), had an alcoholic strength of 80.6% v/v, 84.8% v/v, and 81.8% v/v, respectively. The fresh distillates were divided into two batches. The first batch of distillates was kept at their original alcohol content, whereas the second batch of each distillate was diluted with deionized water to an ABV of 60% v/v. Representative samples were taken from both batches of each distillate and placed in 100 mL glass containers with plastic lids for airtight sealing. The samples were stored at controlled temperatures of 10 °C and 25 °C for 24 weeks. For GC-FID analysis, samples were collected from each glass container at three consecutive time points: 0, 12, and 24 weeks of maturation. The samples were maintained at -20 °C until analysis. The fresh (unmatured) distillates were used as a control.

4.2.4. Chemical analysis (traditional analytical methods)

The fermentation processes were monitored continuously by measuring critical parameters including refraction (°Brix) (PAL-1 Refractometer, Atago, Tokyo, Japan), pH (FE20-Kit FiveEasy™ Benchtop pH Meter, Mettler Toledo, Greifensee, Switzerland), and reducing sugar content (Schoorl & Regenbogen, 1917).

The titratable acidity was determined by potentiometric titration with 0.2 N NaOH to pH 6.8. The volatile acidity of the fermented mashes was assessed by steam distillation (Büchi K-350 distillation equipment) and titration with 0.1 N NaOH and expressed as acetic acid equivalents.

The ethanol content of the distillates was quantified by a DMA 35N density meter (Anton Paar, Graz, Austria). To measure the alcohol content of fermented mashes, first the mash underwent distillation using a steam distillation unit (Büchi K-350, Switzerland). The resulting distillate was collected in a 100 mL volumetric flask and then filled to the mark with distilled water. The alcohol content was subsequently measured using the digital DMA 35N density meter.

Yeast assimilable nitrogen (YAN) was determined by Formol titration (Gump et al., 2001). Yeast growth was determined by the measurement of the optical density (OD) at 600 nm with a spectrophotometer (Helios Gamma, Unicam, UK). All measurements were performed in triplicate.

4.2.5. Analysis of sugars and organic acids (HPLC)

The quantities of sugars and organic acids in the mash were determined using HPLC, following the method described by Chinnici et al. (2005). Briefly, the sample aliquots were centrifuged at 14,000× g for 10 minutes. The resulting supernatants were filtered through a 0.45 µm membrane (Waters, Milford, MA, USA). Subsequently, the samples were analyzed in triplicate by the Thermo Scientific Surveyor Plus HPLC System (Thermo Fisher Scientific, Waltham, MA, USA), consisting of an autosampler, Refractor Index (RI) and Photodiode Array (PDA) detectors, and a thermostatically controlled column compartment set at 45 °C. An ion exclusion column, Aminex HPX-87H (BioRad, Hercules, CA, USA), was employed with 5 mM H_2SO_4 as the eluent, with a flow rate of 0.5 mL/min. Data acquisition and integration were performed using the ChromQuest 5.0 software package (Thermo Fisher Scientific, Waltham, MA, USA). Standards of sugars (glucose, fructose, and sucrose) and organic acids (lactic, acetic, and succinic) were used to identify and quantify the components in the samples. All measurements were performed in triplicate.

4.2.6. Analysis of volatile compounds (GC-FID)

Chromatographic analyses of selected volatile compounds were carried out using a GC-FID system (Perichrom PR2100, Alpha MOS, Toulouse, France), according to the method outlined by Rodríguez Madrera and Valles (2007). The compounds were separated on a CP-WAX 57CB (Agilent Technologies, Santa Clara, CA, USA) capillary column (50 m x 0.32 mm x 0.2 µm). The injector and detector temperatures were 260 °C and 275 °C, respectively. For major compounds, the oven temperature program was as follows: the initial isotherm at 60 °C for 10 min, raised to 70 °C at a rate of 8 °C/min, then raised to 220 °C at a rate of 15 °C/min, with a final isotherm of 220 °C for 15 min. For minor compounds, the oven temperature was set as follows: the initial isotherm at 35 °C for 5 min, raised to 60 °C at a rate of 5 °C/min, then raised to 90 °C at a rate of 10 °C/min, and finally raised to 220 °C at a rate of 8 °C/min, with a final isotherm at 220 °C for 10 min. Helium was used as the carrier gas at a flow rate of 3 mL/min. External standards were used to identify and quantify the components in the sample. All samples were measured in triplicate. The concentrations of volatile compounds are provided in mg/L alcohol 100% (v/v).

4.2.7. Analysis of volatile compounds (HS-SPME/GC-MS)

4.2.7.1. Extraction of volatile aroma compounds - HS SPME

Headspace solid-phase microextraction (HS-SPME) was employed to extract and concentrate the volatile compounds from the fruit, fermented mash, and spirit samples, following the procedures described by Ferreira et al. (2009) and Nešpor et al. (2019) with some modifications in order to suit all sample types. Briefly, 10 mL of spirit samples (10% v/v) were pipetted into 20 mL glass vials, along with 0.5 g sodium chloride, and mixed well. Due to the high ethanol content of the obtained spirits (82.5-86.5% v/v), they were diluted with distilled water to 10% v/v ethanol before solid-phase microextraction. Subsequently, samples of fruit and fermented mashes (3.75 g each) were blended and homogenized with 5 ml of distilled water and 0.5 g NaCl (0.5% w/v), then immediately transferred into 20 mL glass vials. The vials were tightly capped with a PTFE/silicone septum and placed in a 45 °C water bath for 30 minutes to reach an equilibrium state. Samples were extracted using a SPME device with a 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (Supelco, Bellefonte, PA, USA). Prior to headspace sampling, the SPME fiber was conditioned in the GC injector port for 30 min at 250 °C, as specified by the manufacturer. Then, the fiber was plugged into the headspace of the vial for 30 minutes at 45 °C using continuous magnetic stirring at 1000 rpm. After extraction, the fiber was thermally desorbed for 5 minutes into the GC injection port at 250 °C.

4.2.7.2. GC-MS analysis

Chromatographic analysis was performed using an Agilent 6890N Gas Chromatograph coupled with a 5975 C MSD Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA). The volatile compounds were separated on a non-polar HP-5MS (30 m x 0.25 mm i.d., 0.25 μm film thickness) capillary column (Agilent Technologies, Santa Clara, CA, USA). The analyses were performed following the method described by Rodríguez Madrera and Valles (2007), with slight modifications in the method for measuring major compounds. The oven temperature program was set as follows: the initial temperature of 50 °C was held for 10 min, raised to 70 °C at a rate of 8 °C/min, then to 160 °C at a rate of 10 °C/min, and finally raised to 220 °C at a rate of 15 °C/min, where it was held for 10 minutes. As a carrier gas, hydrogen was used with a constant flow rate of 1 mL/min. Injections were performed in splitless mode, and the inlet temperature was held constant at 250 °C. The temperatures of the ion source, transfer line, and quadrupole analyzer were 230, 220, and 150 °C, respectively. The MS was operated in an electron ionization mode (70 eV) with a scan range of 30-300 *m/z*. Agilent Enhanced MSD ChemStation software handled the GC and MS parameters. Agilent

MassHunter Qualitative Analysis 10.0 software was used for data processing. For the determination of Kovat's indeces (KIs), a C8–C20 n-alkane series was used. The volatile compounds were identified by matching mass spectra with spectra of reference compounds in the National Institute of Standards and Technology (NIST08) Mass Spectral Search Program. The Kovats indexes and the mass spectra were compared with those from the NIST library. All measurements were performed in triplicate.

4.2.8. Sensory Analysis

4.2.8.1. Sensory evaluation of the distillates produced by non-*Saccharomyces* yeasts

Organoleptic properties of the spirits produced with the involvement of non-*Saccharomyces* yeasts as described in Section 4.2.1.4. were evaluated using the 20-point scale test (MSZ ISO 11132, 2013). Four weeks before the evaluation, samples were diluted with distilled water to 43% (v/v) ethanol. The sensory evaluation was performed by a trained panel of seven female and eight male participants. The tasting procedure incorporated four criteria and a scale from 5 to 1. The criteria were as follows:

- Cleanliness (technological purity): presence/absence of head and tail fractions and other technological defects (e.g., moldy mash, pickling).
- Fruit character: the typical aroma of the distillate in terms of intensity and quality in the nose and on the palate.
- Mouthfeel: examination of the flavors that can be felt in the mouth, their permanence, pleasantness, and elegance.
- Harmony: evaluation of overall impressions of the product and testing of the harmony of taste and smell.

4.2.8.2. Sensory evaluation of apple spirits

The sensory evaluation of the spirits produced, as described in Section 4.2.1.5., was performed using QDA methodology. The sensory analysis was conducted by a panel of eight trained panelists aged between 28 and 50 years old. All the assessors were experts in the field and had prior experience in similar studies. Following a panel discussion until a consensus on the sensory descriptors was reached, the final evaluation sheet included eight descriptors for aroma (fruity, floral, citrus, pungent, vegetal/herbaceous, grassy, waxy, and phenolic), six descriptors for taste (sweet, spicy, tart, bitter, apple, and astringent), and one descriptor for appearance (clearness). Additionally, the overall performance (aroma and taste) was assessed. Two weeks before the evaluation, the samples were

diluted with water to an ethanol content of 40% v/v and stored at 4°C. A consistent sample volume of 30 mL was evaluated in spirit glasses at room temperature. The samples were coded and provided to panelists in a randomized order. The intensity of each sensory attribute was rated on a 5-point hedonic scale, defined as follows: 1= very weak; 2= weak; 3= moderate; 4= strong; 5= very strong. In overall impressions (total performance), the scale was defined as follows: 1= dislike extremely; 2= dislike; 3= neither like nor dislike; 4= like; 5= like extremely (Lobo et al., 2005).

4.2.9. Statistical Analysis

All experiments and measurements were conducted in triplicate. Data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test was employed to determine the difference between means using SPSS software (Version 20.0, SPSS Inc., Chicago, IL, USA). All the tests were done with a significance level of 5% ($\alpha = 0.05$).

4.2.9.1. Methods used to analyze the effect of maturation process

The volatile compounds of apple distillates were compared by three-way repeated measures of ANOVA model with between-subject factors 'temperature' (10 ºC and 25 ºC), 'alcohol content' (>80% v/v and 60% v/v), and 'yeast type' (*S. cerevisiae, L. thermotolerans* + *S. cerevisiae*, and *T. delbrueckii* + *S. cerevisiae*) as well as with within-subject factor 'time' (0, 12, and 24 weeks). After having transformed the raw values by the ln function, the normality of the model residuals was accepted by the absolute values of their skewness and kurtosis, as they were all below 2 and 4, respectively. The homogeneity of variances was tested by the ratio of maximum and minimum variances. The sphericity assumption was considered satisfied since Greenhouse-Geisser's epsilon values were all above 0.6. In case of a significant overall test, follow-up univariate analysis was performed using Bonferroni's Type I error correction. Finally, the results obtained with different yeast types were compared by Games-Howell's post hoc tests, while the estimated marginal means of the volatile compounds after different times elapsed were compared by Bonferroni's adjustment. Moreover, principal component analysis (PCA) was performed for the 25 compounds. Before the dimension reduction process, the data were scaled and tested for normality the same way as in the previous analysis. The results are visualized with biplots. The statistical analyses were performed using R software (version 4.2.2, R Core Team, Vienna, Austria).

4.2.9.2. Methods used to analyze the effect of combined treatments

To compare the fresh apple mash with 12 different fermented apple mash samples, volatiles analysis was performed by one way MANOVA with dependent variables: reducing sugars, total sugars, titratable acidity, pH, volatile acidity, ethanol, YAN, and sugars' consumption.

The 12 apple spirits and control samples were compared according to their compounds by one-way MANOVA model with dependent variables: acetaldehyde, methanol, 1-propanol, ethyl acetate, 2-methyl-1-propanol, 1-butanol, ethyl propionate, propyl acetate, 3-methyl-1-butanol, 2-methyl-1-butanol, isobutyl acetate, ethyl butyrate, 1-hexanol, cis-2-hexen-1-ol, isoamyl acetate, myrcene, ethyl hexanoate, phenethyl alcohol, ethyl octanoate, and ethyl decanoate. Isobutyl acetate, 1-hexanol, ethyl hexanoate, and phenethyl alcohol were ln transformed while myrcene was sqrt transformed to ensure the normality requirement.

The 12 samples and the control (GC-MS data) compounds at the three phases of fermentation (mid, end, and dist) were compared by repeated measures MANOVA. The MANOVA models were followed by one-way ANOVA models with Type I error correction. The normality of the residuals was accepted by the absolute values of their skewness and kurtosis s they were all below 2. Levene's test was performed to check the homogeneity of variances (p >0.05). In the end, Tukey's post hoc tests were run if homogeneity of variances was satisfied, and Games–Howell's method was used when this assumption was violated. In case of repeated measure MANOVA, sphericity violation was controlled by Greenhouse-Geisser's epsilon ($\varepsilon > 0.5$). Between subject (process) effect was tested by estimated marginal mean based on Sidak's correction. Hierarchical cluster analysis with Ward linkage was performed to find out the similarities among the apple spirit samples based on the GC-FID results. Heatmap analysis using normalized GC-MS data was used to illustrate the dynamic evolution of volatile compounds during the production process of apple spirit. Finally, after standardization, PCA was performed to explore the contribution of different volatile compounds to the aroma profile of apple spirits during different phases of the production process. The sensory data were visualized with spider plots after having standardized the data to make them comparable. The statistical analyses were performed using R software (version 4.3.1 'Beagle Scouts', R Core Team, Vienna, Austria) with the MVA (Everitt & Hothorn, 2022), ggplot2 (Wickham, 2016), and ggbiplot (Vu, 2011) packages.

5. RESULTS AND DISCUSSION

5.1. Chemical and biological acidification of fruit mash

Monitoring the acidity and pH of the mash during fermentation is essential in order to prevent the proliferation of spoilage microorganisms and the undesirable flavor changes associated with their metabolic activity. Therefore, this study aimed to assess the efficiency of different chemical and biological acidification techniques in the process of fermenting fruit mash and their impact on the quality of the final spirit.

5.1.1. Acidification kinetics

The pH of the fresh apple mash was 3.69, which was adjusted to 3.0 in the chemically acidified samples (Figure 9A). During fermentation, a slight variation in pH ranging between 2.94 and 3.10 was observed in these samples, indicating that the growth of possible harmful microorganisms remained inhibited throughout.

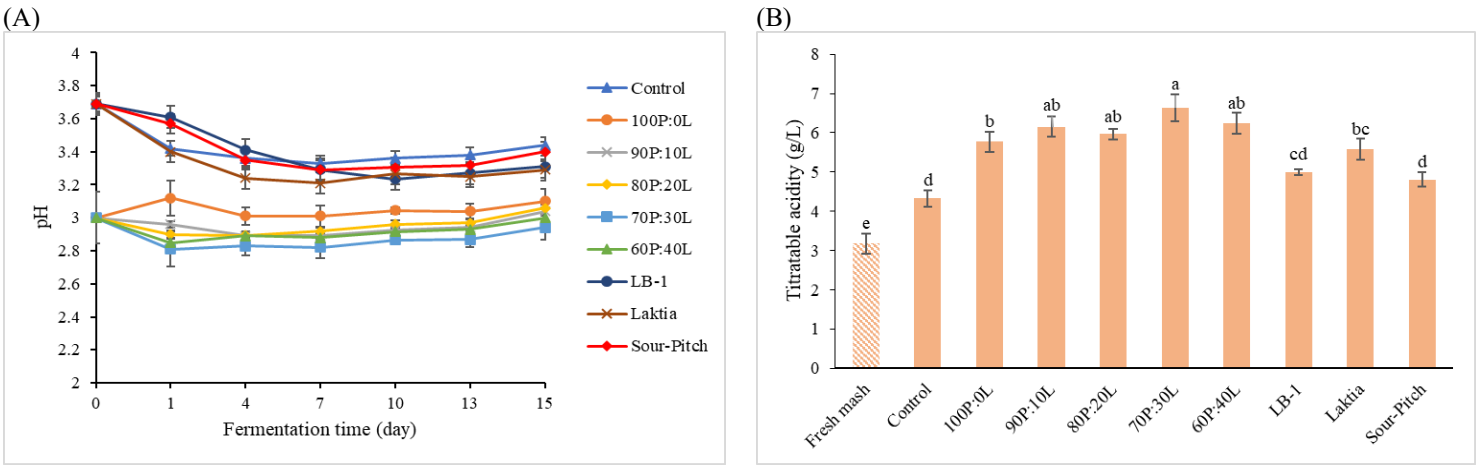


Figure 9. Changes of the pH during the fermentation process (A) and titratable acidity of fresh and fermented apple mashes (B)

Bioregulators were effective in reducing the pH of the fermentation medium to a satisfying level. The yeast *Lachancea thermotolerans* (Laktia) stood out in particular because it was able to reduce the pH of the medium by 0.4 units to 3.29. In the study of Morata et al. (2019), a specific strain of *L. thermotolerans* was highlighted for its ability to lower the pH by approximately 0.5 units, thus eliminating the need for chemical acidification. Among the two studied *Lactiplantibacillus plantarum* strains, LB-1 displayed better results by reducing the pH of the mash to 3.31. The results are consistent

with the findings of Lucio et al. (2016), who demonstrated that different *Lb. plantarum* strains were capable of reducing the medium's pH by more than 0.35 units.

The titratable acid contents of mashes that underwent both chemical and biological acidification displayed considerable variation, as depicted in Figure 9B. Notably, the chemically acidified sample 70P:30L had the highest measured titratable acidity at 6.63 g/L. Regarding microorganisms, *L. thermotolerans* (Laktia) demonstrated an 88% increase in the total titratable acidity of the apple mash (5.58 g/L), surpassing *Lb. plantarum* (LB-1) (4.99 g/L) and *Lb. plantarum* (Sour-Pitch) (4.81 g/L). According to Kapsopoulou et al. (2007), the sequential inoculation of *L. thermotolerans* and *S. cerevisiae* in grape must fermentation led to a noteworthy 70% increase in titratable acidity. This aligns with the findings of Morata et al. (2019), who demonstrated a similar outcome, achieving a total acidity increase from 4 to over 9 g/L through sequential fermentation with *L. thermotolerans* and *S. cerevisiae*. Whereas, a two-fold increase in total acidity by *Lb. plantarum* was reported in cider production (Chen et al., 2023).

Table 4. Physicochemical parameters of fresh and fermented apple mashes

	Refraction (w/w%)	Reducing sugars (g/L)	Total sugars (g/L)	Volatile acidity (g/L)	Ethanol (vol%)	Sugars' consumption (%)
Fresh apple mash	11.90 ± 0.25	89.80 ± 2.97	110.38 ± 5.27	n.a.	n.a.	n.a.
Fermented apple mash						
Control	4.30 ± 0.10 a	7.20 ± 0.21 a	7.65 ± 0.25 ab	0.52 ± 0.06 a	6.10 ± 0.10 ab	92.53 ± 1.49 a
100P:0L	4.10 ± 0.17 a	6.96 ± 0.12 ab	7.42 ± 0.14 abc	0.61 ± 0.01 a	6.30 ± 0.10 a	92.73 ± 1.35 a
90P:10L	4.30 ± 0.20 a	7.16 ± 0.16 a	7.74 ± 0.19 a	0.46 ± 0.04 a	6.20 ± 0.17 a	92.45 ± 1.26 a
80P:20L	4.10 ± 0.17 a	6.58 ± 0.27 bcd	7.12 ± 0.26 bc	0.55 ± 0.09 a	5.90 ± 0.30 abc	92.99 ± 1.27 a
70P:30L	4.00 ± 0.10 a	6.10 ± 0.33 d	6.19 ± 0.23 e	0.46 ± 0.03 a	6.20 ± 0.26 a	93.80 ± 1.48 a
60P:40L	4.20 ± 0.10 a	6.84 ± 0.14 abc	7.31 ± 0.14 abc	0.46 ± 0.06 a	5.60 ± 0.10 bc	92.82 ± 1.22 a
LB-1	4.12 ± 0.10 a	6.35 ± 0.11 cd	6.87 ± 0.12 cd	0.57 ± 0.06 a	5.40 ± 0.17 c	93.21 ± 1.34 a
Laktia	4.00 ± 0.20 a	6.30 ± 0.19 cd	6.30 ± 0.19 e	0.52 ± 0.08 a	5.40 ± 0.18 c	93.71 ± 1.40 a
Sour Pitch	4.10 ± 0.20 a	6.40 ± 0.18 bcd	6.42 ± 0.16 de	0.49 ± 0.06 a	5.40 ± 0.10 c	93.60 ± 1.41 a

Data are expressed as mean ± standard deviation; n.a.: not analyzed. Values with different letters in the same column are significantly different according to Tukey's HSD test ($p < 0.05$).

The main physicochemical parameters of fresh and fermented apple mashes are summarized in Table 4. No significant differences were observed among samples concerning the dynamics of refraction changes and sugar consumption. Reducing sugars, with an initial concentration of 89.80 g/L, were satisfactorily utilized in both chemically and biologically acidified mashes within 15 days. Among all the tested samples, the highest alcohol content was produced in the sample acidified solely with phosphoric acid (6.30% v/v). On the other hand, the lowest alcohol content (5.40% v/v)

resulted from the mash inoculated with the bioregulators *L. thermotolerans* (Laktia) and *Lb. plantarum* (LB-1 and Sour Pitch). Decreased ethanol yields may be due to the diversion of carbohydrates for bacterial growth and the production of lactic acid (Narendranath et al., 1997). Morata et al. (2019) reported that when *L. thermotolerans* is used in sequential inoculation with *S. cerevisiae*, wines result in a 0.5% v/v decrease in ethanol content. The volatile acid content of the fermented mashes ranged from 0.46-0.61 g/L, with no significant variations.

5.1.2. The metabolism of sugars and organic acids during the fermentation process

HPLC measurements revealed no major differences in residual sugar concentrations among the samples at the end of the fermentation process (Figure 10A). In each fermentation trial, negligible saccharose (< 0.50 g/L), glucose (< 1.13 g/L), and fructose (< 3.63 g/L) contents were detected, confirming the completion of the process by the yeast.

Acidity significantly impacts the sensory perception and quality of the beverage. Certain organic acids originate from the raw material, whereas others are formed during alcoholic fermentation. The variations in total acidity and pH observed during fermentation could be attributed to the distinct patterns of lactic, succinic, and acetic acid production in each sample (Figure 10B). The initial mash contained 0.52 g/L of succinic acid. Among the chemically acidified samples, the highest amount of succinic acid was produced in the mash 100P:0L (0.73 g/L) and 90P:10L (0.72 g/L). On the other hand, mashes inoculated with bioregulators, such as *L. thermotolerans* (Laktia) (0.67 g/L), *Lb. plantarum* (LB-1) (0.70 g/L), and *Lb. plantarum* (Sour Pitch) (0.66 g/L), exhibited lower production of succinic acid. According to Li et al. (2021a), *Lactobacillus* has the ability to produce succinic acid by utilizing citric acid generated during the tricarboxylic acid cycle. A study by Binati et al. (2019a) found that the average concentration of succinic acid in grape musts inoculated with 13 strains of *L. thermotolerans* was 0.5 g/L.

Fermentations involving *L. thermotolerans* (Laktia) showed the highest final concentrations in lactic acid (1.26 g/L). Kapsopoulou et al. (2007) found even higher levels of lactic acid production (1.80 g/L) using the same inoculation method in grape must. Another study showed that the production of lactic acid by *L. thermotolerans* reached the amount of 4.8 and 5.5 g/L in single and sequential fermentations with *S. cerevisiae* (Morata et al., 2019). Moreover, Hranilovic et al. (2021) reported varying final lactic acid concentrations for *L. thermotolerans* in sequential fermentations with *S. cerevisiae* (ranging from 1.0 to 8.1 g/L), depending on the *L. thermotolerans* strain.

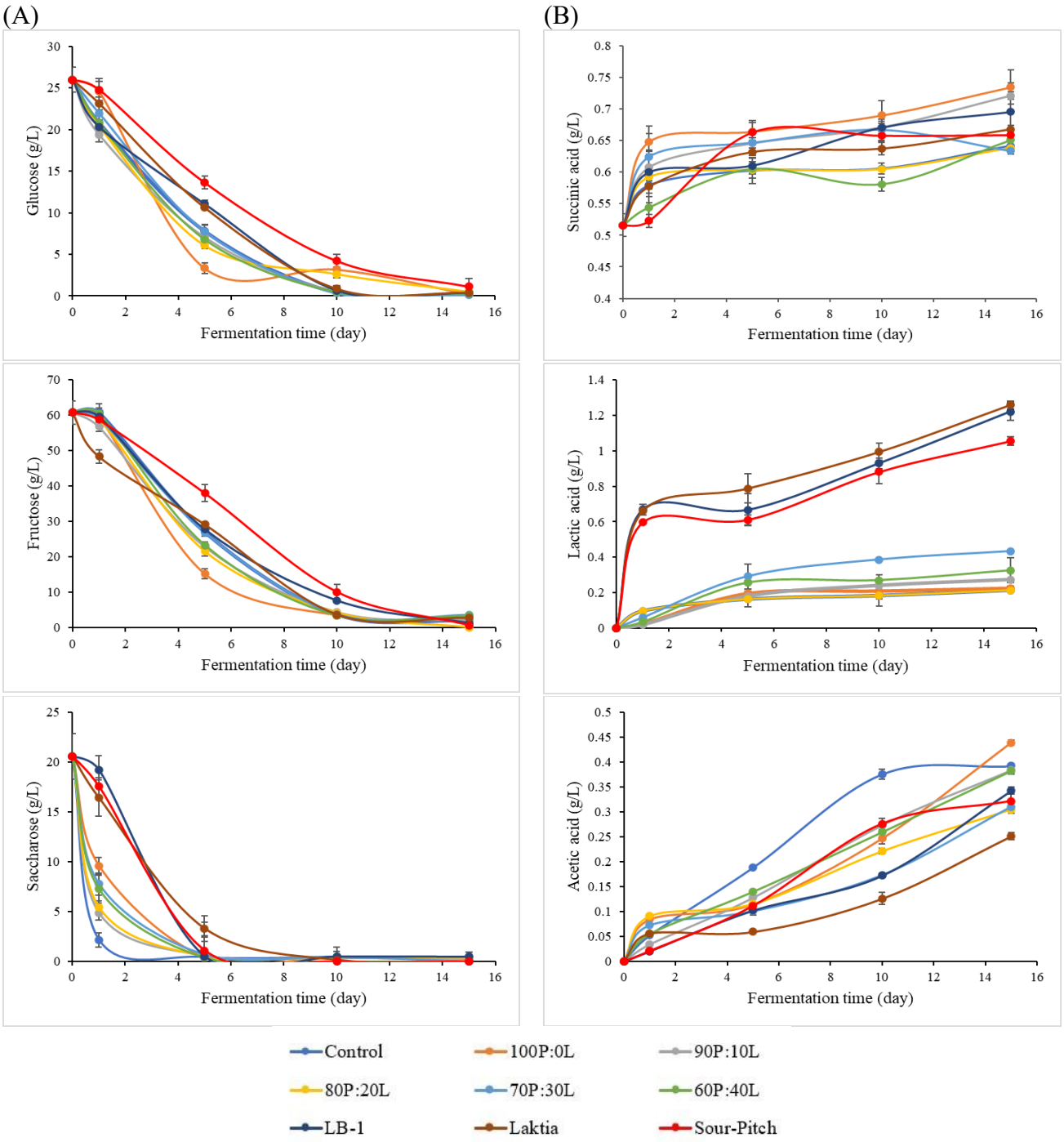


Figure 10. The concentrations of sugars (A) and organic acids (B) in apple mash during the fermentation process

Lb. plantarum strains, LB-1 and Sour Pitch, also showed high lactic acid production potential, 1.22 and 1.05 g/L, respectively. *Lb. plantarum* can produce lactic acid from malic acid degradation and from sugar metabolism (Pardo & Ferrer, 2018). During wine production, the combined fermentation of *S. cerevisiae* and *Lb. plantarum* was shown to produce 2.66 g/L of lactic acid and

0.40 g/L of acetic acid (Onetto & Bordeu, 2015). In our study, *Lb. plantarum* strains in fermentation with *S. cerevisiae* (Uvaferm 228) produced less acetic acid, 0.34 g/L (LB-1) and 0.32 g/L (Sour Pitch), respectively. The amount of lactic acid produced by *S. cerevisiae* (Uvaferm 228) in the samples acidified with chemicals was below 0.40 g/L.

Previous studies have reported that *L. thermotolerans* produces low concentrations of acetic acid, below 0.24 g/L, but with a high strain variability of up to 50%, showing variations from 0.03 to 0.58 g/L (Hranilovic et al., 2018; Binati et al., 2019a; Hranilovic et al., 2021; Vicente et al., 2021). During apple mash fermentation, *L. thermotolerans* (Laktia) produced 0.25 g/L of acetic acid. Whereas in the chemically acidified samples, 100P:0L showed the highest acetic acid content (0.44 g/L), followed by 90P:10L and 60P:40L (0.38 g/L). The lowest amount of acetic acid was detected in the sample 70P:30L (0.31 g/L).

5.1.3. Aroma profile of the obtained distillates

The use of different mash acidifying techniques resulted in significant variations in the analytical profiles of the obtained distillates (Table 5). Concentrations of acetaldehyde, the main carbonyl compound in fruit distillates, were very diverse. The lowest amounts of acetaldehyde were measured among the chemically acidified samples, with the lowest value detected in 90P:10L (12.32 mg/L a.a.) and the highest in 80P:20L (14.76 mg/L a.a.). In comparison to the Control (11.50 mg/L a.a.), the biologically acidified samples, *Lb. plantarum* (LB-1), *Lb. plantarum* (Sour Pitch), and *L. thermotolerans* (Laktia), favored higher acetaldehyde production, resulting in concentration increases of 6.63, 6.98, and 7.44 mg/L a.a., respectively. Gobbi et al. (2013) found a concentration of 22.22 mg/L a.a. of acetaldehyde in wines produced by *L. thermotolerans* in sequential inoculation with *S. cerevisiae*, which they claimed to be higher than values measured in wines produced by pure cultures of *S. cerevisiae* (18.42 mg/L a.a.) and *L. thermotolerans* (17.12 mg/L a.a.). In contrast, Li et al. (2021a) reported a decrease in acetaldehyde levels after the sequential fermentation by *Lb. plantarum* of dealcoholized fruit wines. Benzaldehyde is responsible for imparting a marzipan-like aroma or a bitter almond aroma. Its production is typically associated with the enzymatic degradation of amygdalin found in apple seeds (Lambrechts & Pretorius, 2000; Stanzer et al., 2023). Benzaldehyde was detected in some of the investigated samples, with the highest content found in 100P:0L (1.83 mg/L a.a.) and *L. thermotolerans* (Laktia) (1.55 mg/L a.a.).

Table 5. Volatile aroma compounds identified in the apple distillates

Compounds (mg/L alcohol 100% v/v)	Control	100P:0L	90P:10L	80P:20L	70P:30L	60P:40L	LB-1	Laktia	Sour Pitch
Acetaldehyde	11.50 ±1.12 c	13.13 ±0.86 bc	12.32 ±1.04 bc	14.76 ±0.88 b	14.29 ±1.25 b	13.25 ±0.46 bc	18.13 ±0.84 a	18.94 ±1.09 a	18.48 ±0.84 a
Benzaldehyde	1.41 ±0.10 a	1.83 ±0.20 a	n.d.	1.72 ±0.27 a	0.72 ±0.18 b	n.d.	0.33 ±0.04 b	1.55 ±0.13 a	n.d.
Methanol	3642.80 ±126.63 b	3332.52 ±96.83 bc	3082.38 ±130.49 c	3355.55 ±80.11 bc	2570.55 ±85.54 de	2347.11 ±120.03 e	4423.12 ±102.61 a	3548.30 ±88.88 b	2703.23 ±151.93 d
1-Propanol	1742.32 ±96.23 ab	1472.27 ±123.16 bc	972.80 ±85.25 de	1554.65 ±116.17 b	1185.47 ±67.17 cd	837.98 ±113.28 e	1168.39 ±105.50 cd	1873.22 ±124.77 a	1129.82 ±123.30 de
1-Butanol	159.04 ±19.84 b	166.93 ±17.37 ab	74.60 ±7.95 d	165.67 ±21.61 ab	149.57 ±13.70 bc	77.39 ±8.40 d	75.02 ±5.50 d	210.82 ±30.54 a	104.19 ±13.38 cd
1-Hexanol	68.95 ±3.42 cd	82.94 ±4.99 bc	21.57 ±1.03 ef	90.00 ±2.32 b	63.21 ±3.91 d	19.24 ±1.19 ef	14.66 ±0.93 f	127.61 ±13.26 a	33.86 ±3.68 e
2-Methyl-1-propanol	1162.65 ±104.47 a	957.65 ±88.65 abc	677.95 ±149.82cde	973.05 ±78.18 ab	633.57 ±71.87 de	542.04 ±88.13 e	677.46 ±100.68cde	1068.34 ±128.22 ab	839.30 ±64.92 bcd
3-Methyl-1-butanol	1882.60 ±94.42 b	1898.97 ±115.58 b	830.98 ±119.51 e	1998.22 ±87.68 b	1513.90 ±111.21 c	772.97 ±65.55 e	675.05 ±51.47 e	2519.55 ±131.66 a	1129.96 ±109.67 d
2-Methyl-1-butanol	477.71 ±21.19 ab	440.77 ±23.97 b	207.04 ±12.83 d	425.56 ±25.48 b	295.11 ±30.14 c	188.98 ±29.08 d	190.49 ±25.60 d	534.85 ±48.65 a	302.04 ±20.82 c
Trans-3-hexen-1-ol	0.15 ±0.01 d	0.25 ±0.01 c	0.47 ±0.05 b	n.d.	0.68 ±0.04 a	n.d.	0.12 ±0.03 d	0.13 ±0.01 d	n.d.
Cis-2-hexen-1-ol	n.d.	0.18 ±0.01 b	0.37 ±0.03 a	n.d.	n.d.	0.09 ±0.01 d	0.08 ±0.00 d	0.11 ±0.00 cd	0.15 ±0.00 bc
Benzyl alcohol	n.d.	0.79 ±0.01 a	n.d.	n.d.	n.d.	n.d.	0.53 ±0.01 b	0.21 ±0.01 c	n.d.
Phenethyl alcohol	0.24 ±0.01 a	n.d.	n.d.	n.d.	0.04 ±0.00 d	n.d.	0.19 ±0.01 b	n.d.	0.10 ±0.00 c
Ethyl acetate	213.77 ±41.37 b	200.19 ±15.42 b	168.46 ±25.85 b	313.42 ±36.16 a	83.71 ±17.55 cd	151.28 ±28.49 bc	59.76 ±14.62 d	327.68 ±15.26 a	180.80 ±16.02 b
Ethyl propionate	0.18 ±0.01 b	0.16 ±0.01 b	0.20 ±0.01 b	0.19 ±0.02 b	0.32 ±0.01 a	0.08 ±0.00 c	0.17 ±0.01 b	0.09 ±0.01 c	0.19 ±0.02 b
Ethyl butyrate	0.57 ±0.02 c	0.25 ±0.01 ef	0.20 ±0.01 f	0.30 ±0.02 e	0.18 ±0.00 f	0.46 ±0.01 d	0.85 ±0.05 b	0.01 ±0.00 g	1.06 ±0.09 a
Ethyl lactate	0.24 ±0.01 e	0.16 ±0.01 f	0.39 ±0.01 d	0.54 ±0.01 bc	0.58 ±0.03 abc	0.40 ±0.01 d	0.65 ±0.02 a	0.61 ±0.06 ab	0.52 ±0.01 c
Ethyl benzoate	0.83 ±0.02 a	0.32 ±0.01 b	0.04 ±0.00 c	0.02 ±0.00 cd	0.03 ±0.01 cd	0.02 ±0.01 cd	0.02 ±0.00 cd	0.02 ±0.01 cd	0.01 ±0.00 d
Ethyl octanoate	2.66 ±0.10 e	4.86 ±0.13 c	3.55 ±0.23 d	6.01 ±0.36 b	1.40 ±0.09 g	1.23 ±0.02 g	1.93 ±0.07 f	5.32 ±0.16 c	6.85 ±0.13 a
Ethyl decanoate	0.95 ±0.08 c	1.43 ±0.05 b	0.18 ±0.01 e	0.49 ±0.01 d	3.84 ±0.18 a	0.30 ±0.01 de	1.03 ±0.08 c	0.98 ±0.04 c	0.94 ±0.08 c
Ethyl myristate	0.15 ±0.01 c	n.d.	0.20 ±0.00 b	n.d.	0.15 ±0.01 c	n.d.	0.37 ±0.00 a	0.03 ±0.00 d	n.d.
Ethyl formate	0.02 ±0.00 ab	0.02 ±0.00 bc	0. 01 ±0.00 bc	0.01 ±0.00 bc	0.01 ±0.01 bc	0.01 ±0.00 bc	0.002 ±0.00 c	0.01 ±0.01 bc	0.03 ±0.01 a
Ethyl hexanoate	1.75 ±0.22 a	1.13 ±0.19 c	1.29 ±0.04 bc	1.59 ±0.18 ab	0.56 ±0.17 d	0.50 ±0.07 d	1.39 ±0.02 abc	0.57 ±0.04 d	0.63 ±0.02 d
Ethyl phenylacetate	0.01 ±0.01 f	0.10 ±0.01 d	0.19 ±0.02 b	0.03 ±0.00 f	0.18 ±0.01 b	0.06 ±0.00 e	0.13 ±0.01 c	0.25 ±0.01 a	0.05 ±0.00 e
Diethyl succinate	0.01 ±0.00 e	0.14 ±0.00 c	0.02 ±0.00 e	0.04 ±0.01 d	0.03 ±0.00 de	0.02 ±0.00 de	0.32 ±0.02 a	0.26 ±0.00 b	0.03 ±0.00 de
Isoamyl acetate	3.16 ±0.04 c	2.83 ±0.14 c	4.71 ±0.23 b	5.52 ±0.20 a	1.47 ±0.07 e	2.87 ±0.19 c	2.06 ±0.12 d	1.06 ±0.12 e	2.17 ±0.24 d
Propyl acetate	0.10 ±0.01 a	0.05 ±0.01 bc	0.04 ±0.01 cd	0.05 ±0.00 bcd	0.04 ±0.01 bcd	0.03 ±0.00 d	0.03 ±0.01 d	0.06 ±0.01 b	0.05 ±0.00 bcd

Isobutyl acetate	n.d.	0.17 ±0.01 d	0.05 ±0.01 e	0.32 ±0.02 c	0.68 ±0.02 b	0.05 ±0.01 e	0.09 ±0.00 e	0.27 ±0.02 c	1.09 ±0.07 a
Butyl acetate	0.07 ±0.00 d	0.13 ±0.03 c	0.18 ±0.00 b	0.27 ±0.01 a	0.03 ±0.00 e	0.16 ±0.00 bc	0.02 ±0.01 e	0.03 ±0.00 e	0.02 ±0.01 e
Hexyl acetate	0.29 ±0.01 d	0.53 ±0.02 c	0.83 ±0.01 b	1.51 ±0.03 a	0.20 ±0.00 e	0.16 ±0.00 f	0.03 ±0.00 g	n.d.	n.d.
2-Phenethyl acetate	0.14 ±0.00 b	0.21 ±0.01 a	n.d.	0.04 ±0.00 c	0.21 ±0.02 a	n.d.	n.d.	0.02 ±0.00 c	n.d.
Phenylacetic acid	n.d.	0.15 ±0.01 c	n.d.	0.27 ±0.00 a	0.19 ±0.01 b	n.d.	n.d.	0.07 ±0.00 d	n.d.
Limonene	0.02 ±0.01 cd	0.05 ±0.01 b	0.07 ±0.00 a	0.03 ±0.01 c	0.01 ±0.00 d	0.02 ±0.00 cd	0.08 ±0.00 a	0.03 ±0.01 cd	0.03 ±0.01 cd
Myrcene	0.07 ±0.01 a	0.04 ±0.00 c	0.05 ±0.00 b	0.00 ±0.00 e	0.03 ±0.01 cd	0.04 ±0.00 cd	0.01 ±0.00 e	0.03 ±0.00 d	0.03 ±0.00 cd
Linalool	0.06 ±0.00 d	0.19 ±0.01 c	0.30 ±0.01 b	n.d.	0.40 ±0.01 a	n.d.	n.d.	0.08 ±0.01 d	n.d.

Data are expressed as mean ± standard deviation; n.d.: not detected. Values with different letters in the same row are significantly different according to Tukey's HSD test ($p < 0.05$).

Higher alcohols constitute one of the largest groups of aroma compounds in distillates. They are synthesized during fermentation either catabolically, through the degradation (transamination and carboxylation) of amino acids via the Ehrlich pathway, or anabolically, via the biosynthesis route from the carbon source (Satora et al., 2008). At low quantities, they impart fruity and floral notes to the distillate, but at higher levels, they are characterized by penetrating odors that mask the aromatic finesse (Tsakiris et al., 2013). As reported in Table 5, *L. thermotolerans* (Laktia) in mixed fermentation with *S. cerevisiae* (Uvaferm 228) appears to promote the formation of the majority of higher alcohols. The sample *L. thermotolerans* (Laktia) exhibited the highest contents of amyl alcohols (2519.55 mg/L a.a. and 534.85 mg/L a.a.), 1-propanol (1873.22 mg/L a.a.), 1-butanol (210.82 mg/L a.a.), 1-hexanol (127.61 mg/L a.a.), and 2-methyl-1-propanol (1068.34 mg/L a.a.). Several studies on wine fermentation have shown that *L. thermotolerans* produces fewer higher alcohols compared to *S. cerevisiae* (Gobbi et al., 2013; Balikci et al., 2016). In sequential fermentations, Benito et al. (2015) observed that *L. thermotolerans* resulted in approximately 13% lower final concentrations of higher alcohols compared to the *S. cerevisiae* control. However, some studies have reported the opposite effect, with mixed cultures of *L. thermotolerans* and *S. cerevisiae* leading to higher production of higher alcohols, with an approximate increase of 80-100 mg/L a.a. (Comitini et al. 2011). In a study by Chen et al. (2018), it was found that 1-propanol increased by approximately 20 mg/L a.a., while 3-methyl-1-butanol and 2-methyl-1-butanol decreased by 24 and 22 mg/L a.a., respectively. The presence of two *Lb. plantarum* strains, particularly LB-1, in the mash during alcoholic fermentation led to reduced production of all higher alcohols compared to the Control. It appears that the bacterial

strain has a significant impact on the production of higher alcohols during fermentation. According to Chen et al. (2023), the sequential inoculation of *Lb. plantarum* with *S. cerevisiae* in ciders led to an increase of 381.05 mg/L a.a. in 3-methyl-1-butanol and 19.27 mg/L a.a. in 2-methyl-1-propanol compared to the *S. cerevisiae* control. The sample 90P:10L was distinguished by higher concentrations of trans-3-hexen-1-ol and cis-2-hexen-1-ol, 0.47 mg/L a.a. and 0.37 mg/L a.a., respectively. Phenethyl alcohol is an aromatic alcohol that imparts a rose-like odor to the product (Lambrechts & Pretorius, 2000). Interestingly, low concentrations of phenethyl alcohol were produced in the samples co-inoculated with *Lb. plantarum* (LB-1) (0.19 mg/L a.a.) and *Lb. plantarum* (Sour Pitch) (0.10 mg/L a.a.). Moreover, a trace amount of this compound was detected in sample 70P:30L (0.04 mg/L a.a.). The highest amount of benzyl alcohol was detected in sample 100P:0L at 0.79 mg/L a.a.. This compound probably results from benzaldehyde reduction (Silva Ferreira et al., 2014), which was also found in the highest concentrations in the same sample.

Esters are formed during the fermentation process and are responsible for the pleasant fruity and floral aromas in alcoholic beverages. They have a greater influence on the overall profile of spirits than higher alcohols (Belda et al., 2017; Stanzer et al., 2023). Among the distillates, the most prevalent ester was ethyl acetate, with concentrations ranging from 59.76 mg/L a.a. (*Lb. plantarum* - LB-1) to 327.68 mg/L a.a. (*L. thermotolerans* - Laktia). Gobbi et al. (2013) reported an ethyl acetate level of 47.82 mg/L a.a. in wine after sequential inoculation with *L. thermotolerans*, whereas Dutraive et al. (2019) reported a significantly higher value of 127.97 mg/L a.a. for ethyl acetate. Ethyl acetate, when present in low concentrations (up to 200 mg/L a.a.), contributes to a desirable and fruity character in wine. However, at higher concentrations, it can result in a solvent/nail varnish-like aroma (Sumby et al., 2010; Satora et al., 2016). Based on this information, the acidification of the mash with *Lb. plantarum* (LB-1) (59.76 mg/L a.a.) or 70P:30L (83.71 mg/L a.a.) can modulate the production of ethyl acetate, ensuring that positive characteristics prevail in the final product. The metabolism of *S. cerevisiae* (Uvaferm 228) appears to be greatly influenced by the ratio of phosphoric and lactic acid used for mash acidification. Specifically, the ratio of 80P:20L had a positive impact on the production of ethyl acetate (313.42 mg/L a.a.), isoamyl acetate (5.52 mg/L a.a.), ethyl hexanoate (1.59 mg/L a.a.), hexyl acetate (1.51 mg/L a.a.), and butyl acetate (0.27 mg/L a.a.). Furthermore, the ratio of 70P:30L resulted in high production levels of ethyl decanoate (3.84 mg/L a.a.), ethyl propionate (0.32 mg/L a.a.), and 2-phenethyl acetate (0.21 mg/L a.a.). In sample *Lb. plantarum* (LB-1), the highest concentrations of ethyl myristate and diethyl succinate (with fruity, fermented, and floral aroma notes) were observed at 0.37 mg/L a.a. and 0.32 mg/L a.a., respectively. The sample of *Lb. plantarum* (Sour

Pitch) contained the highest levels of isobutyl acetate (1.09 mg/L a.a.), known for its apple-like aroma; ethyl octanoate, which imparts a sweet and fruity aroma (6.85 mg/L a.a.); and ethyl butyrate (1.06 mg/L a.a.) with floral, strawberry, and sweet aroma notes (Sumby et al., 2010).

In the biologically acidified samples, the highest levels of ethyl lactate were detected. Ethyl lactate production might be favored by the presence of lactic acid and its esterification reaction with ethanol (Pereira et al., 2011). Therefore, the involvement of lactic acid-producing strains in the mash favored the production of ethyl lactate. Among the samples, *Lb. plantarum* (LB-1) exhibited significantly higher concentrations of this compound at 0.65 mg/L a.a., followed by *L. thermotolerans* (Laktia) (0.61 mg/L a.a.) and *Lb. plantarum* (Sour Pitch) (0.52 mg/L a.a.). Numerous studies (Binati et al., 2019b; Dutraive et al., 2019; Morata et al., 2019; Hranilovic et al., 2021; Urbina et al., 2021) have reported the ability of *L. thermotolerans* to produce significant quantities of ethyl lactate. They also demonstrated that fermentations involving *L. thermotolerans* yield higher concentrations of total esters compared to *S. cerevisiae* controls. Our study also supports these findings, as we observed concentrations of ethyl octanoate, ethyl decanoate, ethyl phenylacetate, diethyl succinate, and isobutyl acetate that were 2.66, 0.03, 0.24, 0.25, and 0.27 mg/L a.a. higher in sample *L. thermotolerans* (Laktia) compared to the control. Significant differences in volatile compound production were observed among different *Lb. plantarum* strains. Particularly, the *Lb. plantarum* (Sour-Pitch) induced the production of higher amounts of ethyl acetate, ethyl propionate, ethyl butyrate, ethyl octanoate, isoamyl acetate, propyl acetate, and isobutyl acetate compared to *Lb. plantarum* (LB-1). As indicated by various studies (Mtshali et al., 2010; Lerm et al., 2016), *Lb. plantarum* strains possess the genetic potential to influence the aroma profile of wines.

Trace amounts of terpenes were detected in the samples. Terpenes generally originate from the raw materials used during fermentation. The sample *Lb. plantarum* (LB-1) exhibited the highest level of limonene. The content of myrcene in the investigated samples was lower compared to the control (0.07 mg/L a.a.). Linalool was not detected in the samples inoculated with *Lb. plantarum* strains, its concentration in the sample *L. thermotolerans* (Laktia) was 0.08 mg/L a.a., while the highest content of this compound was observed in 70P:30L.

Ultimately, the use of a 70:30 ratio of phosphoric and lactic acids has led to the most favorable enological and aromatic outcomes among the chemically acidified samples.

Effective mash acidification was achieved by introducing *L. thermotolerans* (Laktia) and *Lb. plantarum* strains (LB-1 and Sour Pitch). Among the microbial strains, *Lb. plantarum* (LB-1) stood out not just for its bioregulatory role (primarily through lactic acid production), but also for its capacity

to influence and modulate the volatiles formed during fermentation. Notably, *Lb. plantarum* (LB-1) lowered the levels of higher alcohols and ethyl acetate while enhancing the production of esters, in particular ethyl lactate.

5.2. The impact of nutrients on yeast metabolism and aroma compound production during fermentation

The nutritional composition of the fermentation medium can affect the growth and metabolism of yeast cells, subsequently influencing the volatile composition and sensory properties of the final spirit. Thus, the goal was to evaluate how various nutrient combinations impact the fermentation performance of *S. cerevisiae* (Uvaferm 228). Preliminary experiments were conducted to assess the impact of different nutrient supplements, either individually or in combination, on apple mash and various fruit juices. Finally, from the tested combinations (shown in Table A1), the alternatives resulting in the best outcomes were reevaluated in a new experiment.

5.2.1. Fermentation kinetics

Nine nutrient treatments (Table 2), differing in their potential to support yeast growth and aroma compound formation, were studied in pear juice under fermentation conditions. Table 6 and Figures 11 and 12 summarize the evolution of the fermentation processes. The fermentation kinetics of *S. cerevisiae* (Uvaferm 228) in pear juice were determined by monitoring reducing sugar consumption rates during fermentation (Figure 11A). Nutrient addition to the fermentation medium had a positive impact on the efficiency of sugar utilization. In the first week of fermentation, the yeast had already consumed 84.56 to 91.75% of the available reducing sugars. The best pattern of sugar utilization was observed in the trial using Nutrient 9. Among all ten trials, the Control variant resulted in the highest residual concentration of reducing sugars (6.99 g/L), whereas the samples treated with Nutrient 2 (5.11 g/L) and Nutrient 9 (5.12 g/L) demonstrated the lowest concentrations.

Negligible pH variations were observed among the samples. The titratable acidity content increased in most samples in response to nutrient supplementation. The results are in accordance with previous studies (Vilanova et al., 2012). Nevertheless, the treatment of the pear juice with nutrients 1, 2, and 5 resulted in a lower titratable acidity compared to the Control. According to Torrea et al. (2011), the addition of nitrogen leads to a significant decrease in titratable acidity, with musts that received a combination of amino acids and ammonium nitrogen exhibiting the lowest values.

Table 6. Physicochemical parameters of fresh and fermented pear juices

	Refraction (w/w%)	Total sugars (g/L)	Sugars' consumption (%)	pH	Titratable acidity (g/L)	Volatile acidity (g/L)	Ethanol (vol%)
Fresh pear juice	12.80 ± 0.26	94.74 ± 4.21	n.a.	3.34	4.31 ± 0.13	n.a.	n.a.
Fermented pear juice							
Control	6.40 ± 0.17 a	7.37 ± 0.27 a	88.30 ± 4.03 a	3.41 ± 0.08 a	5.48 ± 0.11 abc	0.42 ± 0.05 a	4.7 ± 0.26 a
Nutrient 1	6.00 ± 0.10 abc	6.13 ± 0.20 cd	89.60 ± 3.77 a	3.43 ± 0.10 a	5.19 ± 0.07 c	0.42 ± 0.11 a	4.5 ± 0.17 a
Nutrient 2	5.60 ± 0.17 cd	5.54 ± 0.10 e	90.22 ± 3.90 a	3.49 ± 0.16 a	5.38 ± 0.12 bc	0.45 ± 0.06 a	4.6 ± 0.10 a
Nutrient 3	5.90 ± 0.17 bc	5.81 ± 0.27 de	89.94 ± 4.05 a	3.34 ± 0.09 a	5.67 ± 0.18 ab	0.45 ± 0.07 a	4.6 ± 0.10 a
Nutrient 4	6.10 ± 0.10 ab	6.63 ± 0.09 bc	89.07 ± 3.88 a	3.35 ± 0.14 a	5.48 ± 0.10 abc	0.42 ± 0.15 a	4.6 ± 0.20 a
Nutrient 5	6.20 ± 0.20 ab	6.44 ± 0.19 bc	89.28 ± 3.96 a	3.45 ± 0.13 a	5.38 ± 0.20 bc	0.45 ± 0.13 a	4.6 ± 0.10 a
Nutrient 6	6.10 ± 0.10 ab	6.65 ± 0.22 bc	89.06 ± 3.92 a	3.38 ± 0.12 a	5.67 ± 0.08 ab	0.45 ± 0.09 a	4.6 ± 0.10 a
Nutrient 7	5.90 ± 0.17 bc	6.11 ± 0.25 cde	89.63 ± 4.13 a	3.41 ± 0.19 a	5.77 ± 0.16 a	0.50 ± 0.14 a	4.6 ± 0.10 a
Nutrient 8	6.10 ± 0.20 ab	6.74 ± 0.17 b	88.96 ± 4.05 a	3.40 ± 0.09 a	5.67 ± 0.10 ab	0.50 ± 0.15 a	4.5 ± 0.17 a
Nutrient 9	5.40 ± 0.10 d	5.55 ± 0.17 e	90.22 ± 3.85 a	3.36 ± 0.07 a	5.58 ± 0.11 ab	0.50 ± 0.10 a	4.6 ± 0.20 a

Data are expressed as mean ± standard deviation; n.a.: not analyzed. Values with different letters in the same column are significantly different according to Tukey's HSD test ($p < 0.05$).

The supplementation of the juice with nutrients showed no significant effect on the volatile acid and ethanol yields of the samples (Table 6). The results align with the findings of González-Marco et al. (2010).

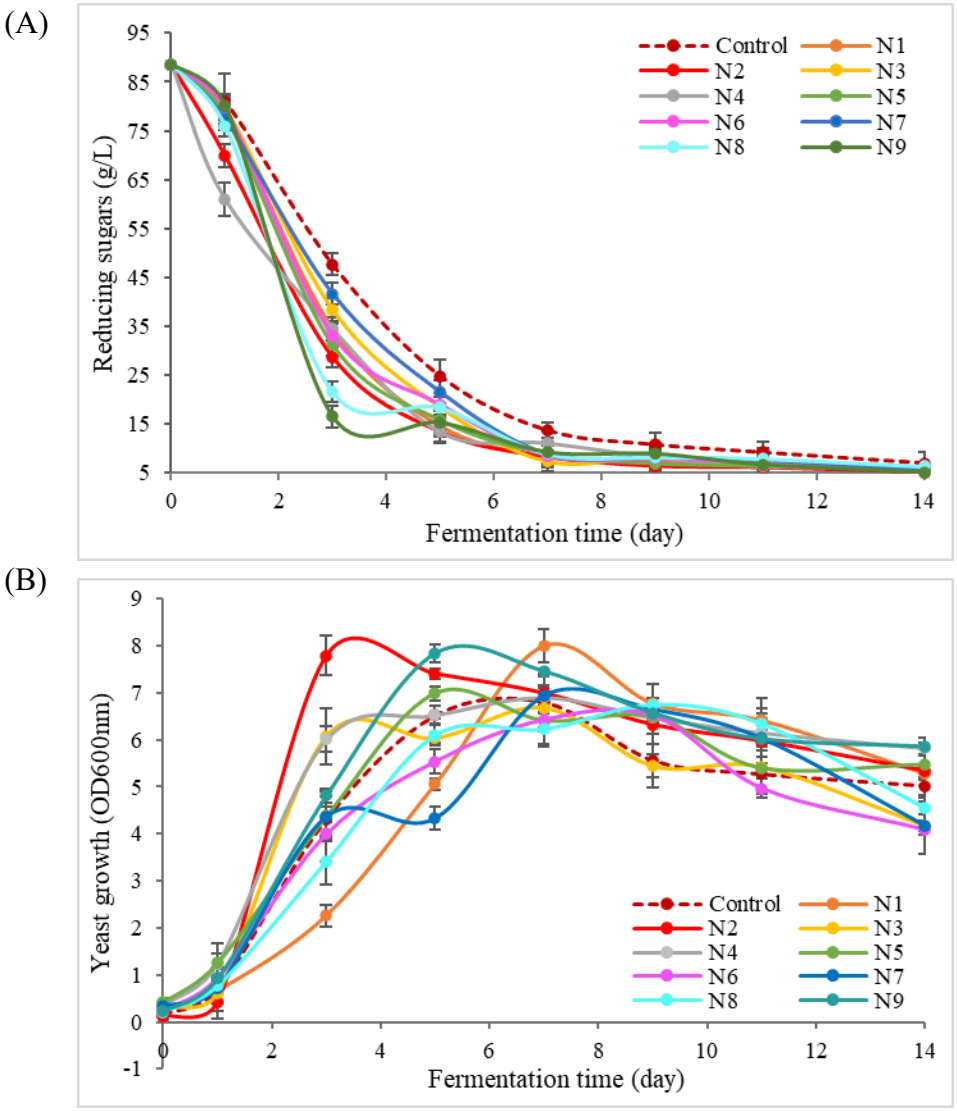


Figure 11. Characterization of refraction changes (A) and population growths (monitored at 600nm) during fermentation (B)

Figure 11B illustrates the impact of nutrient additions on OD (Optical Density) measurements throughout the fermentation process. Surprisingly, distinct growth patterns emerged across the various fermentation trials. While samples treated with nutrients displayed more substantial population growth, fluctuations were also evident. The peak population size was attained at different time points across the different samples. All the variants characterized the exponential phase differently. Moreover, they diverged from each other in the transition between the exponential and stationary

phases. We can emphasize Nutrient 2, which exhibited the most rapid population growth and reached its peak on the third day of the fermentation process. Similar increases in OD values were noted for Nutrient 9 on the fifth day. A longer exponential phase was observed in the case of Nutrient 1 compared to the other samples and the Control. These results reveal *S. cerevisiae*'s preferences toward the nutrients tested. YAN (Yeast Assimilable Nitrogen) composition affects yeast growth rate with complex mixtures favouring higher rates than single compounds (Bell & Henschke, 2005). The richness of media affected the stationary cell concentrations, maintaining the OD values at a high level for a substantial duration. On the 4th day of fermentation, the second addition of nutrients to Nutrient 3 and Nutrient 7 led to an increase in OD values. This correlation indicates that an additional nitrogen dose introduced in the medium during the first half of fermentation aids yeast in overcoming growth challenges (Beltran et al., 2005). Nevertheless, the measured OD values in Nutrient 3 and 6 were consistently lower than those in the Control throughout fermentation. The decline of nutrients (sugars and YAN) in the media notably influenced the OD values over time.

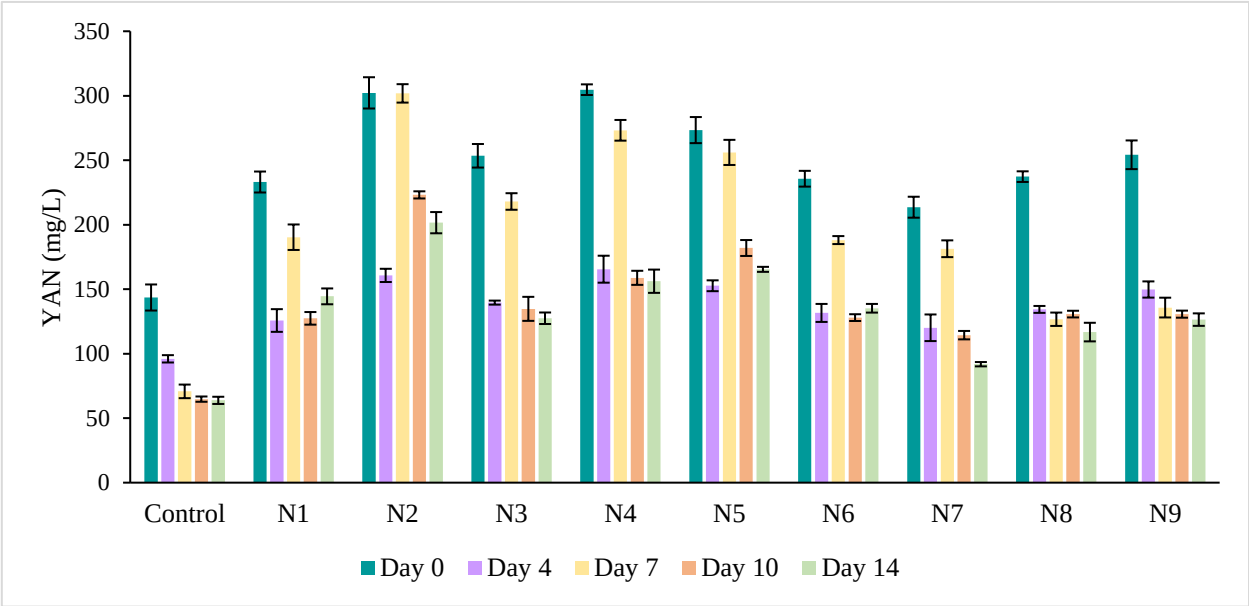


Figure 12. Changes of YAN concentration during fermentation

The diverse patterns of nitrogen uptake and utilization, as reported by numerous studies in wine, can be attributed to the variability in yeast strains and fermentation media employed (Bell & Henschke, 2005; Fairbairn et al., 2017). Therefore, it is essential to consider the potential for diverse responses to nutrient supplementation, stemming from variations in yeast genetic backgrounds and the heterogeneous chemical composition of fermentation media.

The YAN utilization patterns during fermentation by *S. cerevisiae* (Uvaferm 228) are presented in Figure 12. As outlined by many studies, the minimum assimilable nitrogen quantity required to complete alcoholic fermentation is 140 mg/L, although this requirement varies depending on the yeast strain carrying out the process and may be as high as 300-400 mg/L (Bely et al., 1990; Boudreau et al., 2017; Ma et al., 2018). The YAN content in the fresh pear juice was 143.56 mg/L. YAN matrix composition and quantity varied among samples that were inoculated with *S. cerevisiae* (Uvaferm 228). A more robust rate of YAN utilization was observed during the initial four days of fermentation, corresponding to the exponential growth phase of yeasts. Within this period, yeasts actively use YAN, which allows for faster yeast reproduction and therefore rapid fermentation. This utilization rate was higher in the samples treated with different nutrients (up to 50%) in comparison to the Control (33%). Consistent with studies in wine, YAN plays a crucial role in establishing a yeast population early in the fermentation process, while sugar sustains that population by facilitating the conversion of sugar into alcohol and carbon dioxide (Gutiérrez et al., 2012). On the 4th day of fermentation, the majority of samples received an additional nutrient supplementation, which could explain the sudden increase in YAN content in the middle of fermentation. The yeasts continued to utilize YAN in the second half of fermentation, albeit at a significantly lower rate. Regardless of the timing and quantity of nutrient addition, *S. cerevisiae* (Uvaferm 228) exhibited the highest YAN utilization in Nutrient 7. Nutrient 7 is composed of 99.85% yeast autolysates. González-Marco et al. (2010) proposed that the enhanced YAN utilization in samples supplemented with yeast autolysates (rich in fatty acids) might be attributed to a reduced deterioration of the yeast plasma membrane in the must. Consequently, the transport system of the yeast membrane would be less affected by the ethanol concentration at the end of fermentation.

5.2.2. Aroma profile of the obtained distillates

Nutrient treatments exhibited a significant effect on the fermentation performance of *S. cerevisiae* (Uvaferm 228) and the production of aroma compounds (Table 7). The literature presents contradictory data concerning the impact of nutrient supplementation in the fermentation medium on the volatile composition of alcoholic beverages. However, YAN sources have been recognized as potential precursors for volatile compounds in *S. cerevisiae* (Hazelwood et al., 2008).

Consistent with previous findings, acetaldehyde contents increased in response to nutrient treatments (Torrea et al., 2011). The methanol content of the samples treated with Nutrient 6 and 8 was lowered by 23.05% and 16.81%, respectively.

In general, the formation of higher alcohols is known to increase upon nitrogen limitation (Lambrechts & Pretorius, 2000). 1-Propanol production appears to be an exception, with higher concentrations formed with increasing available nitrogen and has been specifically correlated with large quantities of added ammonium (Giudici & Kunkee, 1994; Carrau et al., 2008). Similarly, in our study, an elevation in 1-propanol concentrations was noted in spirits that received nutrient supplementations compared to the Control, reaching the peak level in Nutrient 9 (409.70 mg/L a.a.), Nutrient 2 (405.14 mg/L a.a.), and Nutrient 1 (404.58 mg/L a.a.). This increasing trend was also evident in the case of 2-methyl-1-propanol and phenethyl alcohol across all nutrient-treated samples. The results are in accordance with the literature (Liu et al., 2017). The generation of increased concentrations of higher alcohols could be linked to the presence of particular amino acids in nutrient treatments or might be associated with the biosynthesis pathway from carbohydrate sources. Hernández-Orte et al. (2005) suggests that the anabolic pathway for higher alcohol formation is significant only when nitrogen levels are low, and beyond a certain threshold (likely between 250 and 300 mg/L), formation predominantly occurs through the catabolic pathway.

A less clear relationship was shown for other higher alcohols. Slight variations in 1-butanol concentrations were noted among samples. A decrease was observed by the addition of Nutrient 2, 4, 6, 7, 8. Conversely, an increase in the 1-butanol concentration was observed in spirits that received Nutrient 1, 3, 5, 9 supplementation, compared to the Control. Overall, treated samples exhibited a decline in 1-hexanol concentrations, with a notable decrease of around 50% observed in Nutrient 6 when compared to the Control. Hernández-Orte et al. (2005) previously reported reductions in 1-hexanol content in wines, irrespective of the type of nitrogen supplementation employed (ammonium or amino acids). The nature of the nitrogen supplement appears to induce distinct patterns of amyl alcohols production. Inorganic nitrogen (Nutrient 1, 2 and 6) led to a reduction in these compounds, while organic or mixed supplements yielded elevated levels compared to the Control. Likewise, Vilanova et al. (2012) documented a reverse relationship between amyl alcohol concentrations and DAP supplementation in Albarino wine. Whereas, it has been reported that the addition of organic nitrogen in the form of amino acids in the must, increased the production of isoamyl alcohol by 43% (Liu et al., 2017).

Although benzyl alcohol, trans-3-hexen-1-ol, and cis-2-hexen-1-ol were found in the Control variant, they were not detected in most samples treated with nutrients.

Table 7. Effect of the nutrient addition during fermentation on the concentration of volatile compounds in spirits

Compounds (mg/L alcohol 100% v/v)	Control	Nutrient 1	Nutrient 2	Nutrient 3	Nutrient 4	Nutrient 5	Nutrient 6	Nutrient 7	Nutrient 8	Nutrient 9
Acetaldehyde	2.80 ± 0.14 cd	3.23 ± 0.10 abc	3.11 ± 0.07 bc	3.10 ± 0.14 bc	3.42 ± 0.06 ab	2.56 ± 0.15 d	2.92 ± 0.17 bcd	3.67 ± 0.39 a	3.33 ± 0.23 ab	3.66 ± 0.12 a
Benzaldehyde	0.35 ± 0.02 d	0.51 ± 0.01 ab	0.45 ± 0.05 bc	0.40 ± 0.02 cd	0.53 ± 0.01 ab	0.42 ± 0.01 cd	0.17 ± 0.00 e	0.54 ± 0.05 a	0.39 ± 0.04 cd	0.55 ± 0.03 a
Methanol	580.46 ± 22.68 b	662.59 ± 17.37 a	582.94 ± 34.49 b	582.75 ± 33.46 b	647.02 ± 18.53 ab	604.46 ± 23.10 ab	446.62 ± 20.76 c	615.04 ± 22.89 ab	482.86 ± 32.15 c	588.13 ± 18.22 b
1-Propanol	264.26 ± 7.88 c	404.58 ± 8.26 a	405.14 ± 19.97 a	370.03 ± 27.77 a	360.33 ± 23.32 ab	383.80 ± 19.26 a	270.52 ± 19.08 c	313.63 ± 18.50 bc	310.13 ± 11.17 bc	409.70 ± 10.53 a
1-Butanol	36.49 ± 1.53 abc	38.72 ± 3.77 ab	33.64 ± 2.59 abc	38.56 ± 2.81 ab	36.30 ± 4.07 abc	39.28 ± 3.88 ab	28.20 ± 2.22 c	30.44 ± 2.21 bc	31.83 ± 5.23 bc	41.65 ± 3.04 a
1-Hexanol	6.26 ± 0.15 c	5.93 ± 0.23 cd	5.00 ± 0.40 e	6.39 ± 0.12 c	6.16 ± 0.36 c	9.39 ± 0.48 a	3.17 ± 0.03 f	5.65 ± 0.13 cde	5.37 ± 0.12 de	8.00 ± 0.15 b
2-methyl-1-propanol	836.47 ± 38.84 c	883.18 ± 41.19 c	893.85 ± 66.79 c	1153.82 ± 68.37 ab	1166.07 ± 54.89 ab	1320.52 ± 51.91 a	934.23 ± 56.58 c	1133.09 ± 82.45 b	1262.28 ± 78.38 ab	1265.82 ± 71.35 ab
3-methyl-1-butanol	2466.71 ± 98.55 de	2296.46 ± 108.41 ef	2129.52 ± 90.25 f	2945.74 ± 126.33 b	2809.11 ± 107.73 bc	3343.20 ± 162.81 a	2263.28 ± 97.36 ef	2493.82 ± 136.11 cde	2516.04 ± 112.49 cde	2701.83 ± 101.13 bcd
2-methyl-1-butanol	301.72 ± 6.57 efg	262.17 ± 12.90 g	251.14 ± 13.03 g	325.28 ± 14.22 def	345.61 ± 16.06 cde	371.02 ± 23.67 bcd	274.56 ± 15.33 fg	392.13 ± 26.74 abc	436.42 ± 32.16 a	418.37 ± 15.67 ab
Trans-3-hexen-1-ol	0.02 ± 0.00 c	0.04 ± 0.00 b	0.02 ± 0.00 c	0.08 ± 0.00 a	n.d.	n.d.	n.d.	n.d.	0.01 ± 0.01 c	n.d.
Cis-2-hexen-1-ol	0.04 ± 0.00 a	n.d.	n.d.	n.d.	n.d.	n.d.	0.04 ± 0.00 a	n.d.	n.d.	n.d.
Benzyl alcohol	0.11 ± 0.00 b	0.14 ± 0.00 a	n.d.	0.08 ± 0.00 d	n.d.	n.d.	0.09 ± 0.00 c	n.d.	n.d.	n.d.
Phenethyl alcohol	0.71 ± 0.00 f	0.83 ± 0.01 ef	0.91 ± 0.01 de	1.98 ± 0.05 a	1.14 ± 0.07 b	0.89 ± 0.03 de	0.83 ± 0.01 e	1.10 ± 0.03 bc	0.99 ± 0.08 cd	1.12 ± 0.04 b
Ethyl acetate	233.59 ± 8.67 bc	425.35 ± 10.72 a	114.56 ± 11.70 f	152.19 ± 7.11 e	210.28 ± 11.98 cd	155.70 ± 17.75 e	93.96 ± 8.06 f	179.04 ± 14.11 de	169.63 ± 10.91 e	264.96 ± 12.55 b
Ethyl propionate	1.08 ± 0.15 cd	1.22 ± 0.05 cd	1.33 ± 0.12 cd	1.31 ± 0.23 cd	1.07 ± 0.13 cd	0.99 ± 0.01 d	1.62 ± 0.37 abc	1.48 ± 0.17 bcd	2.19 ± 0.27 a	2.06 ± 0.28 ab
Ethyl butyrate	0.32 ± 0.01 bc	0.37 ± 0.01 b	0.36 ± 0.01 bc	0.31 ± 0.03 c	0.31 ± 0.01 c	0.23 ± 0.01 d	0.22 ± 0.00 d	0.34 ± 0.01 bc	0.22 ± 0.00 d	0.45 ± 0.04 a
Ethyl lactate	0.13 ± 0.00 f	0.24 ± 0.00 c	0.24 ± 0.01 c	0.22 ± 0.00 c	0.17 ± 0.00 e	0.27 ± 0.02 b	0.29 ± 0.00 a	0.12 ± 0.00 f	0.20 ± 0.01 d	0.12 ± 0.00 f
Ethyl octanoate	6.86 ± 0.06 a	4.62 ± 0.22 b	2.69 ± 0.15 c	2.70 ± 0.03 c	2.54 ± 0.05 c	4.28 ± 0.54 b	1.45 ± 0.04 d	2.84 ± 0.14 c	1.51 ± 0.03 d	2.53 ± 0.19 c
Ethyl decanoate	0.36 ± 0.02 a	0.29 ± 0.02 b	0.29 ± 0.03 b	0.24 ± 0.01 c	0.32 ± 0.00 b	0.19 ± 0.03 d	0.19 ± 0.00 d	0.11 ± 0.00 e	0.15 ± 0.00 d	0.24 ± 0.00 c

Ethyl myristate	0.07 ± 0.00 c	0.06 ± 0.00 cd	0.31 ± 0.01 a	n.d.	0.05 ± 0.00 d	n.d.	n.d.	n.d.	n.d.	0.13 ± 0.00 b
Ethyl formate	0.07 ± 0.00 a	0.08 ± 0.00 a	0.08 ± 0.00 a	0.08 ± 0.00 a	0.02 ± 0.00 d	0.02 ± 0.00 d	0.04 ± 0.01 c	0.08 ± 0.01 a	0.06 ± 0.00 b	0.04 ± 0.00 c
Ethyl hexanoate	4.38 ± 0.16 a	3.37 ± 0.19 b	4.39 ± 0.40 a	2.78 ± 0.12 bc	1.81 ± 0.11 ef	3.33 ± 0.23 b	1.20 ± 0.03 f	2.67 ± 0.16 cd	2.08 ± 0.05 de	4.51 ± 0.37 a
Ethyl phenylacetate	0.09 ± 0.00 d	0.13 ± 0.00 b	0.14 ± 0.01 b	0.20 ± 0.01 a	0.11 ± 0.00 c	n.d.	n.d.	0.09 ± 0.00 d	n.d.	0.13 ± 0.00 b
Diethyl succinate	0.34 ± 0.01 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.12 ± 0.00 c	0.10 ± 0.00 c	0.37 ± 0.02 a
Isoamyl acetate	2.77 ± 0.08 a	2.55 ± 0.15 a	1.22 ± 0.06 d	0.94 ± 0.04 e	1.95 ± 0.06 c	1.98 ± 0.09 bc	0.78 ± 0.02 e	1.37 ± 0.12 d	2.17 ± 0.04 bc	2.21 ± 0.08 b
Propyl acetate	0.24 ± 0.03 cd	0.46 ± 0.01 ab	0.19 ± 0.00 de	0.49 ± 0.04 a	0.40 ± 0.01 b	0.11 ± 0.01 e	0.43 ± 0.09 ab	0.28 ± 0.01 c	0.28 ± 0.01 c	0.50 ± 0.01 a
Isobutyl acetate	0.13 ± 0.01 d	0.28 ± 0.01 a	n.d.	0.09 ± 0.00 e	n.d.	n.d.	0.14 ± 0.01 d	0.22 ± 0.01 c	0.23 ± 0.01 c	0.26 ± 0.01 b
Butyl acetate	0.26 ± 0.00 e	n.d.	n.d.	0.20 ± 0.00 f	0.46 ± 0.03 c	n.d.	n.d.	0.34 ± 0.00 d	0.59 ± 0.00 b	0.67 ± 0.03 a
Hexyl acetate	0.25 ± 0.01 b	0.16 ± 0.00 c	0.35 ± 0.02 a	0.12 ± 0.00 cd	0.02 ± 0.00 e	0.26 ± 0.04 b	0.12 ± 0.00 d	0.13 ± 0.00 cd	0.13 ± 0.00 cd	0.11 ± 0.00 d
2-Phenethyl acetate	0.17 ± 0.00 f	0.30 ± 0.02 d	0.72 ± 0.01 a	0.48 ± 0.01 c	0.21 ± 0.01 e	0.21 ± 0.01 ef	0.32 ± 0.00 d	0.54 ± 0.02 b	0.25 ± 0.01 e	0.56 ± 0.03 b
Phenylacetic acid	0.07 ± 0.00 e	0.25 ± 0.03 a	0.15 ± 0.00 d	0.23 ± 0.01 ab	0.25 ± 0.02 a	0.20 ± 0.00 bc	0.19 ± 0.00 c	0.20 ± 0.00 bc	0.22 ± 0.00 bc	0.14 ± 0.00 d
Limonene	0.07 ± 0.00 e	0.13 ± 0.00 d	0.20 ± 0.02 b	0.24 ± 0.01 a	0.15 ± 0.01 c	0.02 ± 0.01 f	0.07 ± 0.01 e	0.12 ± 0.00 d	0.08 ± 0.00 e	0.16 ± 0.00 c
Myrcene	0.02 ± 0.00 b	0.02 ± 0.00 b	0.02 ± 0.00 b	0.02 ± 0.00 b	0.02 ± 0.01 ab	0.02 ± 0.00 b	0.03 ± 0.00 a	0.02 ± 0.00 b	0.02 ± 0.00 ab	0.02 ± 0.00 b
Linalool	0.06 ± 0.00 d	0.07 ± 0.00 cd	0.08 ± 0.01 bc	0.09 ± 0.00 ab	0.05 ± 0.00 e	0.02 ± 0.00 f	0.05 ± 0.00 e	0.03 ± 0.00 f	0.10 ± 0.00 a	0.10 ± 0.00 a

Data are expressed as mean ± standard deviation; n.d.: not detected. Values with different letters in the same row are significantly different according to Tukey's HSD test ($p < 0.05$).

As a first approximation, it can be stated that the addition of nutrients to pear juice led to the generation of varied ester profiles. The findings indicate that the addition of any form of nutrient in the juice leads to a decrease in the levels of isoamyl acetate, ethyl octanoate, ethyl decanoate, and diethyl succinate. Consistent results for ethyl octanoate and ethyl decanoate were documented by Hernández-Orte et al. (2006b). In contrast, conflicting outcomes were presented by Torrea et al. (2011), who observed an increase in the concentration of these compounds irrespective of the type and concentration of nutrient supplement utilized, whether ammonium or a combination of amino acids and ammonium nitrogen. Concentrations of 2-phenethyl acetate and ethyl phenylacetate displayed a positive correlation with nutrient supplementation. Likewise, in the literature, it was reported that 2-phenethyl acetate levels increased in response to the supplementation of grape must with organic and inorganic nitrogen (Garde-Cerdán & Ancín-Azpilicueta, 2008; Torrea et al., 2011; Liu et al., 2017). In alignment with earlier research, the addition of nutrients had a negligible effect on the production of ethyl butyrate (Hernández-Orte et al., 2006b). The concentrations of the remaining esters, including ethyl acetate, ethyl propionate, ethyl lactate, ethyl myristate, ethyl hexanoate, propyl acetate, isobutyl acetate, butyl acetate, and hexyl acetate, were dependent on the specific type of nutrient added. Except for Nutrient 1 and 9, all other samples were characterized by a significant reduction in the amount of ethyl acetate produced. Ugliano et al. (2010) demonstrated that the patterns of ethyl acetate production were greatly influenced by the yeast strain. In fermentations with *S. bayanus*, reductions in ethyl acetate levels correlated with increased diammonium phosphate (DAP) concentrations, whereas the opposite trend was observed in *S. cerevisiae* fermentations where ethyl acetate levels rose with higher DAP concentrations. Generally, ethyl propionate, propyl acetate, and isobutyl acetate responded positively to nutrient supplementation of the juice, with up to 50% increases in some cases. Ethyl lactate showed an increase in the nutrient-treated samples, except for Nutrient 7 and 9, where a minor decrease was observed. Other studies confirm that different types of nitrogen sources trigger diverse ranges of ethyl lactate production (Hernández-Orte et al., 2005; Vilanova et al., 2012). In most samples, the levels of ethyl myristate, hexyl acetate, and ethyl hexanoate showed notable decreases relative to the Control. The study conducted by Torrea et al. (2011) presents contrasting results, indicating that supplementing must with either ammonium or a combination of amino acids and ammonium leads to an increase in the quantities of hexyl acetate and ethyl hexanoate in Chardonnay wines. The findings presented in Table 7 demonstrate that adding nutrients to the fermentation media significantly influences the volatile

profile of fruit spirits, even when provided in the form of complex mixtures and at varying concentrations.

Notably, treatments involving Nutrient 2 and Nutrient 9 were highlighted for their positive contributions to the fermentation process. Besides supporting a more rapid yeast population growth, these treatments also resulted in an enhanced complexity of the distillate's aroma profile.

5.3. Screening yeast strains for fruit spirit production

It is evident that *S. cerevisiae* reliably produces high ethanol yields and a consistent aroma profile in alcoholic fermentations. However, today, we are witnessing a shift in the alcoholic beverage industry, with a focus on producing distinctive and more aromatic products. Consequently, other yeast strains are being evaluated as possible fermentation agents, as each possesses unique characteristics that can significantly influence the final product's aroma and overall quality. Hence, the fermentation capacity of different non-*Saccharomyces* and hybrid yeasts and their influence on the aroma profile of fruit spirits was investigated.

5.3.1. Hybrid yeast strains

5.3.1.1. Fermentation performance of hybrid yeasts

The enological characteristics of hybrid yeasts and the reference strain *S. cerevisiae* (Uvaferm 228) are presented in Table 8, accompanied by Figures 13 and 14. Refraction values were measured throughout fermentation to monitor the progress of yeast strains (Figure 13A).

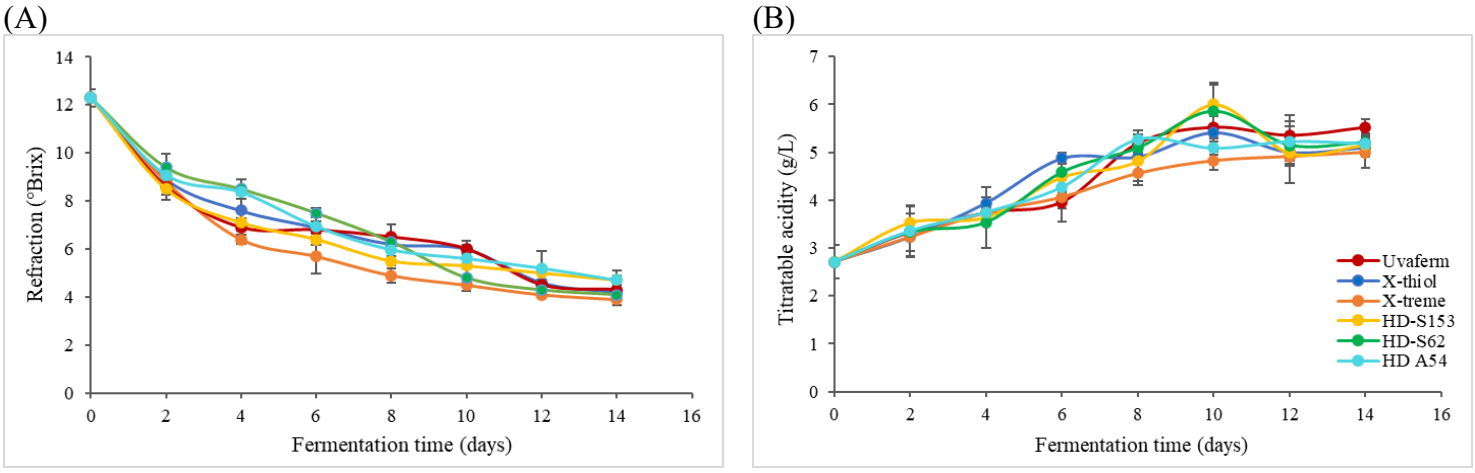


Figure 13. Changes in refraction (A) and titratable acidity (B) during fermentation

Overall, hybrid yeasts displayed a similar fermentative potential to that of the reference strain, *S. cerevisiae* (Uvaferm 228). A gradual reduction of soluble solids in apple mash was observed during fermentation, with a consistently higher reduction rate during the first week. HD S62 exhibited a slower rate of sugar consumption during the initial phase of fermentation; nevertheless, its potential became evident after the 8th day. On the other hand, the hybrid strain X-treme consistently surpassed other strains by displaying the most rapid fermentation kinetics. The titratable acidity of the mash experienced an increase ranging from 2.09 to 2.61 g/L, attributed to the biosynthesis of organic acids by yeast metabolism (Whiting, 1976). No significant variations in titratable acidity and pH were observed among the fermented samples (Figure 13B and Table 8).

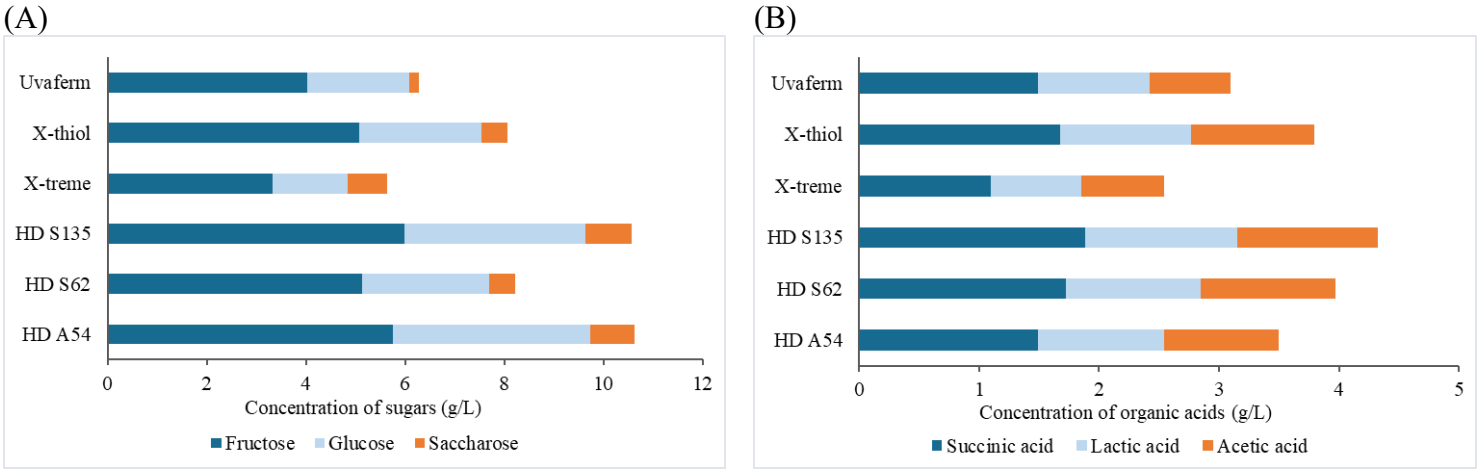


Figure 14. Concentration of sugars (A) and organic acids (B) in the fermented mashes

The fresh mash was characterized by the presence of 65.93 g/L fructose, 5.60 g/L saccharose, and 32.37 g/L glucose. Throughout fermentation, a comparable rate of sugar consumption was observed, varying from 91.64% (HD A54) to 93.54% (X-treme). As depicted in Figure 14A, the hybrid strain X-treme favored the consumption of fructose and glucose, resulting in the lowest remaining levels (3.33 g/L and 1.51 g/L), while the consumption of sucrose was more limited (0.80 g/L). The residual sugar contents in samples fermented with hybrid yeasts exceeded those in *S. cerevisiae* (Uvaferm 228). Initially, the fresh mash contained 0.99 g/L of succinic acid and 0.05 g/L of lactic acid. As fermentation progressed, their concentrations rose, with the highest levels observed in samples fermented with HD S135 (1.88 g/L and 1.27 g/L) and HD S62 (1.72 g/L and 1.13 g/L) (Figure 14B). Blazques Rojas et al. (2012) emphasized the potential of hybrid yeasts to generate higher lactic acid quantities compared to strains of *S. cerevisiae* and *S. bayanus* during wine fermentation. The

amount of acetic acid produced during fermentation depends on the yeast strain applied and, to a lesser extent, on the raw material used (Satora et al., 2008; Satora & Tuszynski, 2010). The presence of acetic acid is essential in the formation of acetate esters through esterification (Baena-Ruano et al., 2010). However, excessive acetic acid concentrations are undesirable in alcoholic beverages due to their potential to introduce a vinegar-like off-flavor (Li et al., 2011). The lowest concentration of acetic acid was noted in the sample X-treme (0.69 g/L), while the highest was in the sample HD S135 (1.17 g/L).

Table 8. Physico-chemical parameters of fresh and fermented apple mashes

	Reducing sugars (g/L)	Total sugars (g/L)	Sugars' consumption (%)	pH	Volatile acidity (g/L)	Ethanol (vol%)
Fresh apple mash	106.73 ± 5.25	107.29 ± 4.11	n.a.	3.10 ± 0.12	n.a.	n.a.
Fermented apple mash						
Uvaferm 228	8.34 ± 1.10 a	8.36 ± 0.72 a	92.19 ± 0.87 a	2.95 ± 0.08 a	0.43 ± 0.16 a	5.90 ± 0.16 a
X-thiol	8.64 ± 1.23 a	8.69 ± 0.89 a	91.88 ± 0.97 a	2.98 ± 0.12 a	0.40 ± 0.15 a	5.60 ± 0.10 ab
X-treme	6.85 ± 0.54 a	6.93 ± 0.52 a	93.54 ± 0.52 a	3.02 ± 0.02 a	0.38 ± 0.14 a	5.50 ± 0.10 b
HD-S135	8.84 ± 1.11 a	8.93 ± 0.94 a	91.67 ± 0.91 a	2.99 ± 0.09 a	0.41 ± 0.17 a	5.50 ± 0.17 b
HD-S62	7.85 ± 1.66 a	7.90 ± 0.83 a	92.63 ± 0.82 a	2.93 ± 0.14 a	0.41 ± 0.13 a	5.60 ± 0.10 ab
HD-A54	8.90 ± 1.17 a	8.96 ± 0.91 a	91.64 ± 0.91 a	2.96 ± 0.10 a	0.39 ± 0.14 a	5.40 ± 0.00 b

Data are expressed as mean ± standard deviation; n.a.: not analyzed. Values with different letters in the same column are significantly different according to Tukey's HSD test ($p < 0.05$).

A slight reduction of volatile acidity was noted in the mashes fermented with hybrid yeasts compared to the control, but it was not significant. Moreover, the ethanol content produced by hybrid yeasts (5.40–5.60%) was lower in comparison to *S. cerevisiae* (Uvaferm 228) (5.90%) (Table 8).

5.3.1.2. Production of volatile aroma compounds by hybrid strains

Metabolite differences between hybrids and the control were identified in distillates derived from fermented apple mashes (Table 9). These variations in metabolite levels could arise from various factors, including polyploidy (Hull-Sanders et al., 2009); the additive effect of an extra genome; synergistic genetic interactions (Mani et al., 2008); heterosis, which leads to the hybrid outperforming both parent varieties in terms of growth and yield (Lippman & Zamir, 2007); or variations in gene expression (Bellon et al., 2011).

The levels of higher alcohols were significantly influenced by the yeast strain employed during fermentation. Intriguingly, the hybrids produced noticeably lower concentrations of higher alcohols compared to *S. cerevisiae* (Uvaferm 228), although there were a few exceptions. X-thiol produced the

highest amounts of 1-propanol (1025.70 mg/L a.a.) and 1-hexanol (38.98 mg/L a.a.), whereas HD S135 was distinguished for the production of higher amounts of 1-butanol (61.16 mg/L a.a.) and benzyl alcohol (0.16 mg/L a.a.) compared to other strains. The concentration of 3-methyl-1-butanol in the spirits fermented with HD A54 reached 1466.74 mg/L a.a., surpassing the levels found in *S. cerevisiae* (Uvaferm 228) (1229.12 mg/L a.a.). Similarly, Gamero et al. (2011) noted elevated levels of 3-methyl-1-butanol in wines fermented with hybrid yeasts (*S. cerevisiae* x *S. bayanus*) compared to *S. cerevisiae*.

X-thiol was distinguished, among other strains, for producing significantly lower amounts of amyl alcohols (837.87 mg/L a.a.). Phenethyl alcohol levels in X-treme and HD S62 were 33% and 52% higher than in *S. cerevisiae* (Uvaferm 228), respectively. Blazquez Rojas et al. (2012) also reported increases in phenethyl alcohol content in wines fermented with interspecific *Saccharomyces* hybrids compared to those fermented with the parental *S. cerevisiae* strain. Trace amounts of cis-2-hexen-1-ol were produced by hybrid yeasts.

The ability of hybrids to reduce the higher alcohol content of spirits can be considered a favorable characteristic. As claimed by Saison et al. (2009), elevated concentrations of specific aroma compounds in the distillate create a more pronounced sensory impact but can also potentially mask less prominent aromas. Conversely, reducing the presence of a specific compound may unveil other positive aromas in the spirit that were previously subtle due to their very low concentration. The methanol content in the spirits ranged from 2591.30 mg/L a.a. (X-thiol) to 4857.23 mg/L a.a. (HD S135).

All yeast strains displayed distinctive ester profiles. Acetate esters are usually associated with pleasant aroma descriptions like fruity, perfume-like, and floral, with the exception of ethyl acetate, which is described as nail polish (Blazquez Rojas et al., 2012). Ethyl acetate emerged as the most abundant volatile ester in all the resulting spirits. It's worth noting that hybrids formed lower concentrations of ethyl acetate compared to the control. These findings align with prior research (Bellon et al., 2011; Blazquez Rojas et al., 2012). X-treme, HD S135, and HD A54 produced similar quantities of 2-phenethyl acetate, which were twice the amount found in *S. cerevisiae* (Uvaferm 228). HD A54 yielded higher concentrations of isoamyl acetate, whereas HD S62 formed higher levels of isobutyl acetate compared to other samples. In X-treme spirits, the highest amounts of butyl acetate (0.57 mg/L a.a.) and hexyl acetate (0.58 mg/L a.a.) were observed. Propyl acetate was only detected in spirits produced by HD S135 (0.24 mg/L a.a.) and X-treme (0.31 mg/L a.a.).

Table 9. Volatile aroma compounds of spirits fermented with *S. cerevisiae* and hybrid strains

Compounds (mg/L alcohol 100% v/v)	Uvaferm 228	X-treme	X-thiol	HD S135	HD S62	HD A54
Acetaldehyde	32.54 ± 1.34 cd	62.75 ± 4.12 a	61.59 ± 12.62 a	17.43 ± 1.24 d	36.02 ± 2.75 bc	49.97 ± 2.35 ab
Benzaldehyde	0.34 ± 0.02 a	0.31 ± 0.00 a	0.27 ± 0.00 b	n.d.	n.d.	0.11 ± 0.00 c
Methanol	3128.15 ±133.64 c	3114.21±130.54 c	2591.30 ±119.83d	4857.23 ±128.67a	4688.74 ±133.79a	4025.23 ±128.78b
1-Propanol	1006.46 ± 43.39 a	950.28 ± 37.49 ab	1025.70 ± 48.79 a	985.91 ± 48.16 a	766.99 ± 34.49 c	841.88 ± 48.57 bc
1-Butanol	59.86 ± 2.98 a	44.03 ± 4.20 b	54.51 ± 5.21 a	61.16 ± 2.60 a	30.48 ± 3.54 c	34.41 ± 3.02 bc
2-Butanol	20.58 ± 2.79 a	13.14 ± 1.72 b	13.64 ± 1.66 b	14.57 ± 1.04 b	13.98 ± 2.01 b	14.81 ± 1.70 b
1-Hexanol	29.89 ± 1.47 a	23.87 ± 0.04 c	38.98 ± 0.03 c	24.29 ± 1.66 b	30.25 ± 2.83 a	27.98 ± 0.52 ab
2-methyl-1-propanol	675.17 ± 28.63 a	473.06 ± 47.38 c	421.49 ± 52.75 c	400.99 ± 52.08 c	593.20 ± 24.99 ab	511.35 ± 36.00 bc
3-methyl-1-butanol	1229.12 ± 60.58 b	866.58 ± 44.28 cd	751.97 ± 52.42 d	883.74 ± 22.67 c	791.06 ± 44.29 cd	1466.74 ± 54.36 a
2-methyl-1-butanol	193.76 ± 26.72 a	128.12 ± 17.02 b	85.90 ± 5.32 b	104.85 ± 13.61 b	106.69 ± 11.65 b	184.30 ± 15.18 a
Trans-3-hexen-1-ol	0.05 ± 0.00 a	0.02 ± 0.01 c	0.02 ± 0.00 c	0.03 ± 0.01 bc	0.04 ± 0.00 ab	0.03 ± 0.01 bc
Cis-2-hexen-1-ol	n.d.	0.04 ± 0.01 b	0.07 ± 0.00 a	0.04 ± 0.00 b	n.d.	0.03 ± 0.00 b
Benzyl alcohol	0.11 ± 0.00 c	0.05 ± 0.00 e	0.14 ± 0.01 b	0.16 ± 0.00 a	0.11 ± 0.01 c	0.07 ± 0.00 d
Phenethyl alcohol	1.92 ± 0.35 c	2.88 ± 0.29 b	1.51 ± 0.03 cd	1.16 ± 0.39 d	4.01 ± 0.22 a	1.59 ± 0.04 cd
Ethyl propionate	0.12 ± 0.01 bc	0.17 ± 0.01 b	0.27 ± 0.02 a	0.25 ± 0.02 a	0.30 ± 0.04 a	0.10 ± 0.00 c
Ethyl butyrate	0.37 ± 0.01 c	1.04 ± 0.10 a	0.49 ± 0.03 bc	0.47 ± 0.01 bc	0.50 ± 0.00 b	0.94 ± 0.06 a
Ethyl lactate	0.30 ± 0.01 b	0.39 ± 0.04 ab	0.35 ± 0.04 b	0.32 ± 0.00 b	0.48 ± 0.08 a	0.49 ± 0.05 a
Ethyl benzoate	0.10 ± 0.00 c	0.31 ± 0.02 b	0.43 ± 0.01 a	0.11 ± 0.01 c	0.12 ± 0.00 c	0.29 ± 0.02 b
Ethyl octanoate	3.36 ± 0.19 d	5.86 ± 0.39 a	3.62 ± 0.26 cd	4.60 ± 0.28 b	4.30 ± 0.42 bc	3.60 ± 0.28 cd
Ethyl decanoate	0.40 ± 0.02 e	0.60 ± 0.01 c	0.96 ± 0.04 a	0.79 ± 0.04 b	0.50 ± 0.04 d	0.63 ± 0.00 c
Ethyl myristate	0.01 ± 0.00 d	0.07 ± 0.00 a	0.03 ± 0.01 c	0.05 ± 0.00 b	n.d.	0.05 ± 0.00 b
Ethyl formate	n.d.	0.51 ± 0.01 a	n.d.	1.11 ± 0.05 b	n.d.	n.d.
Ethyl hexanoate	3.27 ± 0.14 d	6.88 ± 0.39 a	6.39 ± 0.55 ab	6.56 ± 0.66 ab	4.84 ± 0.15 c	5.60 ± 0.59 bc
Diethyl succinate	0.12 ± 0.01 d	0.13 ± 0.00 d	0.56 ± 0.01 a	0.50 ± 0.03 b	0.25 ± 0.01 c	0.16 ± 0.01 d
Ethyl acetate	271.33 ± 14.39 a	202.45 ± 19.80 c	226.50 ± 17.54 bc	227.12 ± 18.99 bc	258.60 ± 16.71 ab	235.70 ± 8.69 abc
Ethyl phenylacetate	0.15 ± 0.01 a	0.16 ± 0.00 a	n.d.	n.d.	0.15 ± 0.01 a	0.16 ± 0.00 a
Isoamyl acetate	0.96 ± 0.06 ab	0.88 ± 0.04 b	0.94 ± 0.03 ab	0.64 ± 0.02 c	0.80 ± 0.07 bc	1.09 ± 0.13 a
Propyl acetate	n.d.	0.31 ± 0.01 a	n.d.	0.24 ± 0.01 a	n.d.	n.d.
Isobutyl acetate	0.03 ± 0.00 c	0.06 ± 0.00 b	n.d.	0.05 ± 0.00 b	0.09 ± 0.01 a	0.04 ± 0.00 c
Butyl acetate	0.17 ± 0.00 c	0.57 ± 0.03 a	0.30 ± 0.02 b	0.51 ± 0.07 a	0.28 ± 0.06 bc	0.31 ± 0.03 b
Hexyl acetate	0.38 ± 0.05 d	0.58 ± 0.04 a	0.47 ± 0.04 bc	0.43 ± 0.02 cd	0.39 ± 0.01 cd	0.52 ± 0.02 ab
2-Phenethyl acetate	0.16 ± 0.01 b	0.39 ± 0.02 a	0.21 ± 0.01 b	0.40 ± 0.06 a	0.23 ± 0.01 b	0.35 ± 0.06 a
Phenylacetic acid	0.02 ± 0.00 a	n.d.	n.d.	n.d.	0.03 ± 0.00 a	n.d.
Limonene	0.19 ± 0.00 c	0.28 ± 0.01 a	0.15 ± 0.00 d	0.24 ± 0.02 b	0.09 ± 0.00 e	0.13 ± 0.00 d
Myrcene	0.01 ± 0.01 b	0.03 ± 0.01 a	0.02 ± 0.00 a	0.01 ± 0.00 b	0.01 ± 0.00 b	0.01 ± 0.00 b
Linalool	0.11 ± 0.00 c	0.15 ± 0.00 b	0.06 ± 0.00 e	0.09 ± 0.01 d	0.14 ± 0.00 b	0.21 ± 0.00 a

Data are expressed as mean ± standard deviation; n.d.: not detected. Values with different letters in the same row are significantly different according to Tukey's HSD test ($p < 0.05$).

Short-chain ethyl esters are characterized by pleasant fruity, berry, and green apple aromas, while long-chain ethyl esters have a pleasant to soapy aroma (Blazquez Rojas et al., 2012). Hybrids HD S62 and X-thiol produced higher concentrations of ethyl propionate, 0.30 mg/L a.a. and 0.27 mg/L a.a., respectively. Ethyl lactate was detected at a concentration of 0.30 mg/L a.a. in the control variant (*S. cerevisiae* - Uvaferm 228), while the hybrids produced notably higher concentrations, ranging from 0.32 mg/L a.a. to 0.49 mg/L a.a. In contrast, Bellon et al. (2011) reported reductions of up to 50% in ethyl lactate levels in wines produced by hybrids compared to the parental *S. cerevisiae* strain. The hybrid strain X-treme exhibited significantly higher concentrations of ethyl butyrate (1.04 mg/L a.a.), ethyl octanoate (5.86 mg/L a.a.), and ethyl hexanoate (6.88 mg/L a.a.). Ethyl myristate was found in relatively low concentrations in all the spirits, ranging from 0.01 to 0.07 mg/L a.a., and was not detected in HD S62. Ethyl formate was only detected in spirits fermented with X-treme and HD S135. X-thiol stood out for its significant production of ethyl benzoate (0.43 mg/L a.a.), ethyl decanoate (0.96 mg/L a.a.), and diethyl succinate (0.56 mg/L a.a.) when compared to the control and other hybrid strains.

To gain an overview of the ester production abilities of different strains, we compared the total ester produced during alcoholic fermentation by all the strains. Additionally, ethyl acetate was excluded from the total esters because of its distinctive contribution to spirit aroma. Among the spirits produced, X-treme had the highest concentration of this group of fermentation-derived compounds (20.47 mg/L a.a.), followed by HD S135 (18.20 mg/L a.a.) and HD S62 (17.25 mg/L a.a.). On the other hand, X-thiol tended to exhibit lower ester concentrations (16.53 mg/L a.a.), although still higher than *S. cerevisiae* (Uvaferm 228) (11.83 mg/L a.a.). Several studies support our findings regarding the ability of hybrid yeasts to produce higher concentrations of esters in alcoholic beverages (Bellon et al., 2011; Blazquez Rojas et al., 2012; Ye et al., 2013; Pérez-Torrado et al., 2015; Wang et al., 2020; Pérez et al., 2022).

In short, hybrid yeasts exhibited similar enological characteristics to those of *S. cerevisiae* (Uvaferm 228). However, significant differences were observed in their secondary metabolism. Hybrid strains generated reduced amounts of higher alcohols and a wider array of esters. Among these hybrids, X-treme consistently stood out for its rapid fermentation kinetics and the ability to form numerous esters that impart positive sensory notes to the distillate.

5.3.2. Non-*Saccharomces* yeast strains

5.3.2.1. Fermentation performance of non-*Saccharomces* yeasts

The analytical profiles of the fresh and fermented mashes obtained from pure and mixed fermentations are reported in Table 10. The fresh apple mash was characterized by a high total sugar content (148.3 g/L), which included reducing sugars with a concentration of 133 g/L. After completion of fermentation, in the mashes that were inoculated with mixed cultures of *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) and *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228), lower concentrations of residual sugars were detected (10.6 and 11.3 g/L, respectively). This behavior highlights the high fermentation capacity of yeasts in mixed fermentation.

The total acidity of the fresh mash was 5.3 g/L. However, following fermentation, this parameter increased by 1.6–2.3 g/L, owing to the synthesis of certain organic acids as normal products of yeast metabolism. In contrast, in the study of Satora et al. (2016), a decreasing tendency of total acidity was shown in the plum mash after fermentation, which was probably a result of microbial activity. The co-inoculation *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) showed the lowest concentration of volatile acidity (0.33 g/L) compared with other samples. All mashes were characterized by a comparable consumption rate of sugars, 86.7-92.9%, whereas the highest ethanol production was observed in the fermentation with *S. cerevisiae* (Uvaferm 228) (6 vol%).

5.3.2.1.Analyzed sugars and organic acids profile during the fermentation process

The amounts of sugar in the mash depend on the variety of fruit, climatic conditions, and time of harvest (Satora et al., 2016). The apple mash was characterized by high initial concentrations of fructose (89.06 g/L), glucose (40.66 g/L), and sucrose (18.59 g/L). All yeast strains showed similar patterns of sugar utilization (Figure 15). A sharper decrease in carbohydrate content was recorded in the first week of fermentation, indicating a more vigorous utilization rate of sugars. The fastest rate of fermentable sugars utilization was detected in the co-inoculation *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228). No further decline in fermentable sugars content was observed after the 15th day, indicating the end of fermentation for all inoculum types. Similar decreasing trends in the concentration of sugars during the fermentation process were reported in the literature (Amorim et al., 2016; Satora et al., 2016).

Table 10. The main enological parameters of fresh and fermented apple mashes

	Refraction (w/w%)	Reducing sugars (g/L)	Total sugars (g/L)	Sugars' consumption (%)	pH	Titratable acidity (g/L)	Volatile acidity (g/L)	Ethanol (vol%)
Fresh apple mash	15.80 ± 0.50	133.01 ± 5.10	148.30 ± 6.20	n.a.	3.58 ± 0.12	5.30 ± 0.34	n.a.	n.a.
Fermented apple mash								
Uvaferm 228	5.20 ± 0.17 a	11.20 ± 2.30 a	11.80 ± 2.10 a	92.00 ± 0.81 ab	3.19 ± 0.12 a	7.60 ± 0.34 b	0.50 ± 0.08 a	6.00 ± 0.08 d
Biodiva	5.30 ± 0.18 ab	12.10 ± 1.50 a	13.30 ± 1.10 a	91.00 ± 0.22 b	3.17 ± 0.08 a	7.30 ± 0.19 ab	0.42 ± 0.11 a	5.20 ± 0.10 b
Biodiva+Uvaferm 228	5.10 ± 0.22 a	9.50 ± 2.20 a	10.60 ± 1.30 a	92.90 ± 0.84 a	3.10 ± 0.09 a	7.20 ± 0.23 ab	0.33 ± 0.09 a	5.60 ± 0.15 c
Concerto	5.70 ± 0.11 b	18.20 ± 2.40 b	19.70 ± 1.70 b	86.70 ± 0.65 c	3.16 ± 0.15 a	7.10 ± 0.17 ab	0.45 ± 0.05 a	4.80 ± 0.10 a
Concerto+Uvaferm 228	5.25 ± 0.20 ab	10.10 ± 1.20 a	11.30 ± 2.10 a	92.40 ± 0.45 ab	3.14 ± 0.10 a	6.90 ± 0.13 a	0.50 ± 0.10 a	5.70 ± 0.12 cd
Melody	5.20 ± 0.23 a	12.50 ± 2.50 a	13.50 ± 1.90 a	90.90 ± 0.89 b	3.15 ± 0.13 a	6.90 ± 0.25 a	0.36 ± 0.06 a	5.60 ± 0.20 c

Data are expressed as mean ± standard deviation; n.a.: not analyzed. Values with different letters in the same column are significantly different according to Tukey's HSD test ($p < 0.05$)

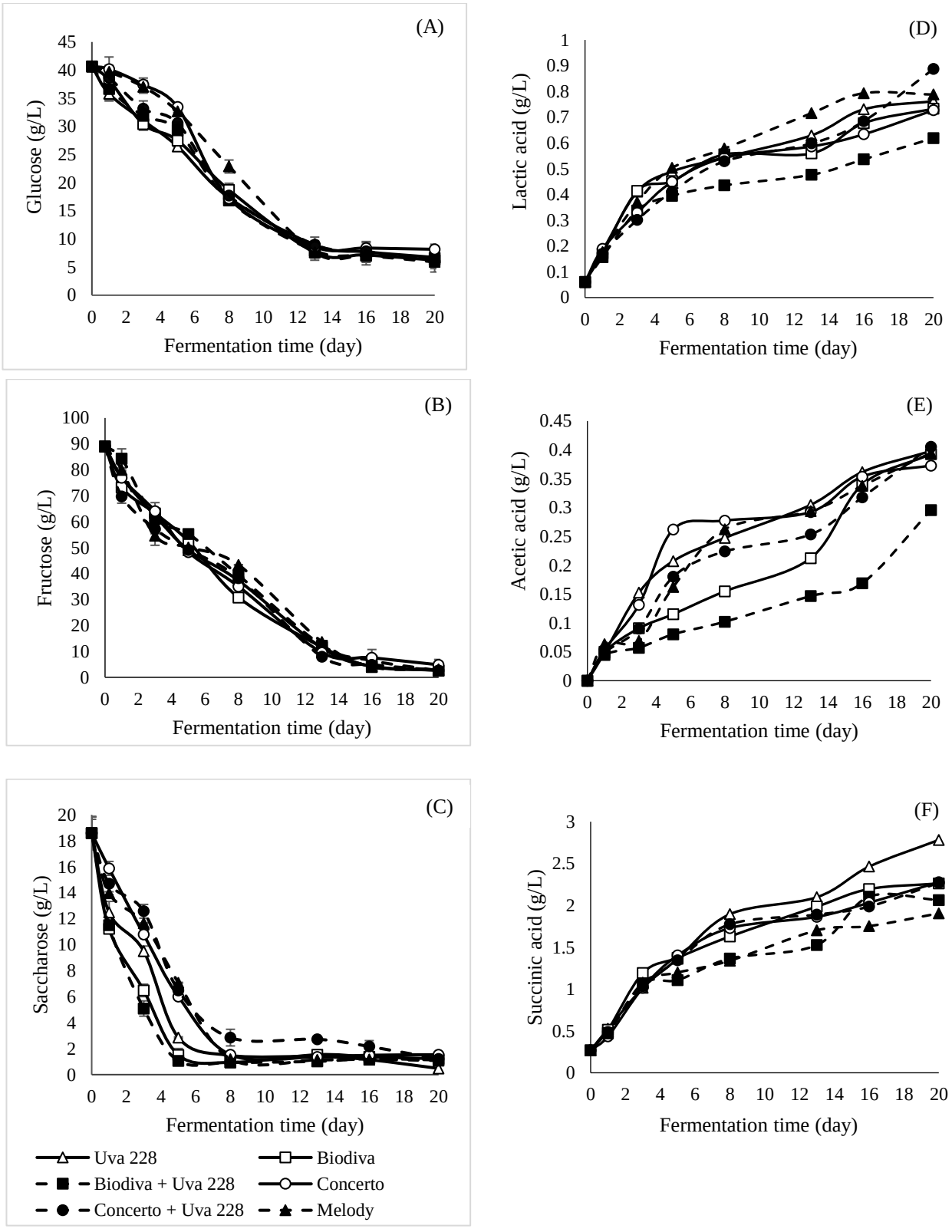


Figure 15. The concentrations of glucose (A), fructose (B), saccharose (C), lactic acid (D), acetic acid (E), and succinic acid (F) in apple mash during the fermentation process

Figure 15 shows the evolving profiles of the main organic acids during fermentation. As illustrated in Figure 15D, lactic acid was formed throughout the fermentation process, with the final concentration being the highest in the co-inoculation of *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) and Melody. Lactic acid is synthesized by the reduction of pyruvic acid during glycolysis or the transformation of malic acid. Succinic acid is another common metabolite formed from pyruvic acid via malic acid, fumaric acid, and the decomposition of some amino acids. The importance of succinic acid is not solely due to its presence in the fruit mash; it also readily reacts with other molecules to form esters (Ye et al., 2014c). Its changing profile is shown in Figure 15F. The initial concentration of succinic acid in the apple mash was 0.27 g/L. After fermentation, its content increased sharply, with a minimum value of 1.90 g/L (Melody) and a maximum of 2.78 g/L (*S. cerevisiae* - Uvaferm 228). Fluctuations in the concentration of acetic acid in mash were observed throughout the fermentation process, and the yeast strain used had a major influence on the observed differences. However, final concentrations were similar among all tested samples, with an exception in the case of the co-inoculum *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228), where the lowest concentration of acetic acid was detected (Figure 15E). The results were in agreement with previous studies (Herrero et al., 1999).

5.3.2.2. Analyzed volatile compounds in the apple distillates

The volatile composition of the distillates obtained is presented in Table 11. Esters, higher alcohols, and carbonyl compounds comprised the main volatile classes that make up their "fermentation bouquet". Acetaldehyde is an important carbonyl compound found in alcoholic beverages, and in small concentrations, it has a fresh, "fruity" odor (Urošević et al., 2014). The highest acetaldehyde concentration was noted in the sample fermented with *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) (199.32 mg/L a.a.), while in the other samples, the detected values were 125-152.34 mg/L a.a. Winterová et al. (2008) reported that the acetaldehyde content in apple brandies was in the range of 30-260 mg/L a.a.

Among the analyzed higher alcohols, isoamyl alcohol predominated. The highest concentrations of this compound were found in the spirits produced with *S. cerevisiae* (Uvaferm 228) (329.77 mg/L a.a.) and with *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) (297.40 mg/L a.a.), and the lowest with Concerto+Uvaferm 228 (209.87 mg/L a.a.). The other samples were characterized by a fairly uniform level of this compound (227.07-243.61 mg/L a.a.). Rusu Coldea et al. (2011) measured isoamyl alcohol values between 75.28 and 196.59 mg/100mL a.a. in different apple brandies. In

addition to isoamyl alcohol, high amounts of 1-propanol (157.32-206.10 mg/L a.a.) and 2-methyl-1-butanol (88.45-149.47 mg/L a.a.) were detected in the samples.

Table 11. Volatile aroma compounds identified in the apple distillates

Compounds (mg/L alcohol 100% v/v)	Uvaferm 228	Biodiva	Biodiva + Uvaferm 228	Concerto	Concerto + Uvaferm 228	Melody
Acetaldehyde	140.49 ± 17.23 a	133.82 ± 12.56 a	199.32 ± 15.67 b	152.34 ± 14.38 a	125.00 ± 7.45 a	125.49 ± 9.89 a
Methanol	1706.07 ±125.56 a	1710.03 ±115.62 a	1720.68 ± 134.25 a	1986.88 ± 142.85a	1933.19 ± 147.21 a	1944.73 ± 131.51 a
1-Propanol	157.32 ± 11.53 a	172.77 ± 17.78 a	206.10 ± 19.66 a	176.12 ± 10.34 a	167.73 ± 22.24 a	163.78 ± 13.23 a
1-Butanol	3.64 ± 0.56 a	3.38 ± 0.34 a	3.75 ± 0.37 a	4.10 ± 0.16 a	3.83 ± 0.35 a	3.97 ± 0.23 a
1-Hexanol	33.12 ± 2.46 ab	27.35 ± 3.04 a	34.23 ± 2.14 b	43.56 ± 3.56 c	47.62 ± 1.78 c	46.86 ± 4.16 c
2-Butanol	0.29 ± 0.03 a	n.d.	0.60 ± 0.05 c	n.d.	0.43 ± 0.02 b	0.75 ± 0.05 d
3-Methyl-1-butanol	329.77 ± 32.27 c	241.17 ± 27.49 ab	297.40 ± 21.91 bc	227.07 ± 22.63 ab	209.87 ± 18.02 a	243.61 ± 31.71 ab
2-Methyl-1-butanol	110.56 ± 9.14 ab	88.45 ± 7.52 a	116.89 ± 11.64 b	115.08 ± 12.44 b	149.47 ± 15.02 c	95.50 ± 8.34 ab
Trans-3-hexen-1-ol	0.04 ± 0.01 ab	0.02 ± 0.00 a	0.06 ± 0.01 bc	0.08 ± 0.00 c	0.15 ± 0.01 d	0.14 ± 0.01 d
Cis-2-hexen-1-ol	0.02 ± 0.00 a	0.02 ± 0.00 a	0.02 ± 0.00 a	0.02 ± 0.00 a	0.02 ± 0.00 a	0.02 ± 0.00 a
Benzyl alcohol	0.42 ± 0.02 cd	0.47 ± 0.06 d	0.37 ± 0.05 cd	0.12 ± 0.01 a	0.23 ± 0.03 b	0.34 ± 0.03 c
Phenethyl alcohol	22.44 ± 1.64 b	n.d.	13.24 ± 0.96 a	n.d.	29.84 ± 2.06 c	27.02 ± 1.87 c
Ethyl acetate	178.50 ± 10.35 bc	147.30 ± 25.46 ab	165.20 ± 12.26 abc	131.60 ± 10.67 a	167.40 ± 14.24 abc	195.50 ± 15.03 c
Ethyl butyrate	0.02 ± 0.00 a	0.05 ± 0.01 b	0.04 ± 0.00 b	0.05 ± 0.00 b	0.05 ± 0.01 b	0.05 ± 0.01 b
Ethyl benzoate	3.81 ± 0.64 b	4.47 ± 0.21 c	4.08 ± 0.34 bc	1.56 ± 0.14 a	2.27 ± 0.27 a	4.77 ± 0.34 cd
Ethyl octanoate	3.05 ± 0.34 bc	3.43 ± 0.55 c	2.96 ± 0.24 bc	2.13 ± 0.17 a	2.45 ± 0.12 ab	2.79 ± 0.31 ab
Ethyl formate	n.d.	n.d.	n.d.	0.56 ± 0.05 a	0.69 ± 0.07 b	0.63 ± 0.03 ab
Ethyl hexanoate	6.03 ± 0.35 c	3.89 ± 0.27 a	4.64 ± 0.47 ab	4.29 ± 0.56 ab	4.02 ± 0.36 ab	4.93 ± 0.28 b
Diethyl succinate	0.25 ± 0.05 a	0.41 ± 0.04 c	0.30 ± 0.03 ab	0.37 ± 0.02 bc	0.39 ± 0.03 c	0.38 ± 0.03 c
Isoamyl acetate	0.03 ± 0.01 ab	0.04 ± 0.01 bc	0.06 ± 0.01 c	0.02 ± 0.00 a	0.03 ± 0.00 ab	0.06 ± 0.01 c
Propyl acetate	n.d.	n.d.	n.d.	0.02 ± 0.00 a	0.01 ± 0.00 a	0.03 ± 0.00 b
2-Phenethyl acetate	0.04 ± 0.01 a	0.03 ± 0.01 b	0.06 ± 0.01 ab	0.04 ± 0.01 a	0.05 ± 0.01 ab	0.05 ± 0.0ab
Linalool	0.12 ± 0.02 ab	0.17 ± 0.02 bc	0.13 ± 0.02 ab	0.18 ± 0.03 c	0.15 ± 0.02 abc	0.11 ± 0.01 a

Data are expressed as mean ± standard deviation; n.d.: not detected. Values with different letters in the same row are significantly different according to Tukey's HSD test ($p < 0.05$).

1-Propanol has a pleasant, sweetish odor, but excessive concentrations will introduce solvent notes that mask all the positive notes in distillates (Tešević et al., 2009). The highest concentration of 1-propanol was observed in the sample made with *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) (206.10 mg/L a.a.), and the lowest in the sample with *S. cerevisiae* (Uvaferm 228) (157.3 mg/L a.a.). Nearly similar amounts of 1-propanol were measured in cherry (132-300 mg/L a.a.) and plum (166-303 mg/L a.a.) distillates (Tešević et al., 2009; Satora et al., 2016). In the case of *T. delbrueckii* (Biodiva) and Melody starter cultures, a 2-methyl-1-butanol content of less than 100 mg/L a.a. was detected. This compound showed the highest value (149.47 mg/L a.a.) in the sample of *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228). The quantities of the other higher alcohols were lower in the investigated samples. The shares of 1-hexanol, 2-phenylethanol, 1-butanol, 2-butanol, trans-3-hexen-1-ol, cis-2-hexen-1-ol, and benzyl alcohol accounted for less than 10% of the total amount of the higher alcohols. Among these, the largest quantities of 1-hexanol (47.62 mg/L a.a.) and 2-phenylethanol (29.84 mg/L a.a.) were detected in the distillate fermented with *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228). 1-Hexanol is not a fermentation product but most often originates from linolenic acid found in the green parts of plants and unripe fruits (Satora et al., 2016). Phenethyl alcohol has a positive influence on the aroma of the distillate and is derived from L-phenylalanine through the metabolic reaction of yeast during carbonic anaerobiosis (Tešević et al., 2009). This compound was not detected in the *T. delbrueckii* (Biodiva) and *L. thermotolerans* (Concerto) samples. All samples were characterized by low amounts of 1-butanol (3.38-4.09 mg/L a.a.). No significant differences in 1-butanol production were observed between strains. The compound 2-butanol was not detected in *T. delbrueckii* (Biodiva) and *L. thermotolerans* (Concerto) samples. Spaho et al. (2013) mentioned that the presence of 2-butanol in distillates is a result of bacterial action. The two aliphatic alcohols, 3-hexen-1-ol and cis-2-hexen-1-ol, originate from the process of crushing and macerating fruits. The highest concentration of 3-hexen-1-ol was measured in the samples fermented with *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) and Melody.

Regarding the esters, the most abundant compound was ethyl acetate. The analyzed samples were characterized by a diversified content of ethyl acetate, ranging from 131.60 mg/L a.a. in *L. thermotolerans* (Concerto) to 195.50 mg/L a.a. in Melody. Ethyl hexanoate supplies the aroma of fruit (banana, green apple, etc.), and its presence, along with other ethyl esters, is beneficial for the spirit (Tešević et al., 2009). The highest content of this compound was observed in sample *S. cerevisiae* (Uvaferm 228) (6.03 mg/L a.a.) and the lowest in *T. delbrueckii* (Biodiva) (3.89 mg/L

a.a.). In addition to ethyl hexanoate, significant amounts of ethyl octanoate and ethyl benzoate were measured in the samples. These compounds were present in higher amounts in the samples fermented with *T. delbrueckii* (Biodiva) and Melody. Phenylethyl acetate, isoamyl acetate, and propyl acetate were present in very low concentrations in the analyzed spirits. Furthermore, propyl acetate was not detected in three samples (*S. cerevisiae* (Uvaferm 228), *T. delbrueckii* (Biodiva), and *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228)). A similar result was observed for ethyl formate.

Methanol production is associated with the enzymatic degradation of the methoxy groups of pectin as well as the acidic degradation of pectin (Tešević et al., 2009). The methanol content in the analyzed samples ranged between 1706.07-1986.88 mg/L a.a. (the maximum legal limit is 12 g/L a.a.) (EC Regulation 2019/787). The linalool profile was similar in all distillates.

5.3.2.3. Sensory evaluation of apple distillates

The results of the sensory evaluations are provided in Table 12. The total scores ranged between 15.20 (Melody) and 18.90 (*L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228)). All samples received a maximal score for technological purity, indicating that the hearts were properly cut from head and tail fractions during the distillation process. The fruitiness and high flavor intensity perceived by the panelists were highly appraised, especially in the distillates produced from the mixed inoculums (*L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) and *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228)). The best-rated distillate was the one produced by the mixed culture *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228). This sample was characterized by a pleasant, delicate apple aroma (fresh fruit with a citrus-like, skin-spicy aroma) and a well-harmonized, refreshing, and pleasantly burning taste.

Table 12. Sensory analysis of apple spirits obtained from different starter cultures

	Technological purity (max 5 point)	Fruit character (max 5 point)	Mouthfeel (max 5 point)	Harmony (max 5 point)	Total (max 20 point)
Uvaferm 228	5.00 ± 0.00	3.93 ± 0.46	3.73 ± 0.59	3.46 ± 0.63	16.10 ± 1.24
Biodiva	5.00 ± 0.00	4.33 ± 0.61	4.27 ± 0.59	4.40 ± 0.63	18.00 ± 1.55
Biodiva + Uvaferm 228	5.00 ± 0.00	4.26 ± 0.59	3.93 ± 0.46	3.73 ± 0.46	16.90 ± 0.79
Concerto	5.00 ± 0.00	3.80 ± 0.56	3.80 ± 0.67	3.40 ± 0.73	16.00 ± 1.36
Concerto + Uvaferm 228	5.00 ± 0.00	4.86 ± 0.35	4.46 ± 0.52	4.60 ± 0.50	18.90 ± 1.03
Melody	5.00 ± 0.00	3.53 ± 0.64	3.46 ± 0.74	3.20 ± 0.77	15.20 ± 1.69

Data are expressed as mean ± standard deviation

The sensory analysis results could be correlated with the findings from the chemical characterization (Tables 10 and 11). The use of a mixed inoculum of *L. thermotolerans* (Concerto) and *S. cerevisiae* (Uvaferm 228) led to increased ester production (ethyl butyrate, ethyl formate, ethyl hexanoate, propyl acetate, etc.), providing a sweeter taste and fruity-floral aroma, along with moderate levels of higher alcohols contributing to coconut and honey notes (Amorim et al., 2016). Particularly, higher levels of phenethyl alcohol, which imparts a rose-like aroma, were present in this spirit. The good balance of the quantities of these volatiles led to a pleasant sensory perception.

The findings indicate that the sequential fermentation approach of *L. thermotolerans* (Concerto) and *T. delbrueckii* (Biodiva) with *S. cerevisiae* (Uvaferm 228) presents a better alternative compared to pure culture fermentations. While non-*Saccharomyces* strains do not possess the same fermentation capacity as *S. cerevisiae* (Uvaferm 228), they contribute additional metabolites that enhance the aroma complexity and diversity of the final product. In particular, sequential fermentations involving *L. thermotolerans* (Concerto) yielded distillates of superior sensory quality with highlighted fruity and floral notes.

5.4. Changes in the volatile composition of apple distillates during maturation under different conditions

Following the assessment of the fermentation potential of non-*Saccharomyces* yeasts and their positive impact on the aroma profile of apple distillates, the two most promising strains were used to produce apple spirits, which were then subjected to a maturation period. The aim was to evaluate the changes in the volatile compounds of apple distillates over the course of a 24-week maturation period. In addition, the influence of alcohol by volume (ABV) and temperature on volatile changes was investigated. Through these experiences, fruit spirit maturation conditions could be optimized.

5.4.1. Physicochemical characteristics of the mash during fermentation

Table 13 summarizes the main physicochemical properties of the mash. The apple mash was characterized by high initial concentrations of sugars (133.90 g/L), in particular reducing sugars (117.57 g/L). All fermentation trials showed similar patterns of sugar utilization. At the end of fermentation, the lowest amounts of fructose (3.51 g/L) and sucrose (0.53 g/L) were measured in the samples fermented by *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) and *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228), respectively. The fastest rate of glucose utilization was observed in the pure culture of *S. cerevisiae* (Uvaferm 228) (1.75 g/L). During fermentation, the total acidity increased by 2.5 to 3.19 g/L, due to the synthesis of certain organic acids by the yeasts. A

greater amount of acetic and succinic acid was produced by *S. cerevisiae* (Uvaferm 228) compared to mixed cultures. A higher amount of lactic acid was detected in the mash fermented by *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) (0.75 g/L), which could be linked to the well-known ability of *L. thermotolerans* to produce lactic acid (Joran et al., 2022).

Table 13. Physicochemical parameters of fresh and fermented apple mashes

Parameter	Fresh apple mash	Fermented apple mashes		
		S. cerevisiae	*L. thermotolerans* + *S. cerevisiae*	*T. delbrueckii* + *S. cerevisiae*
Refraction (w/w%)	13.80 ± 0.14	4.00 ± 0.14 a	3.80 ± 0.00 a	4.15 ± 0.07 a
Total Sugars (g/L)	133.90 ± 2.83	11.84 ± 0.40 a	11.31 ± 0.14 a	12.54 ± 0.77 a
Reducing Sugars (g/L)	117.57 ± 2.73	11.05 ± 0.09 ab	10.58 ± 0.54 b	12.00 ± 0.17 a
pH	3.45 ± 0.03	3.13 ± 0.00 a	3.10 ± 0.01 a	3.17 ± 0.03 a
Titratable Acidity (g/L)	4.26 ± 0.05	6.76 ± 0.15 b	7.45 ± 0.10 a	6.86 ± 0.10 b
Volatile Acidity (g/L)	n.a.	0.26 ± 0.01 b	0.29 ± 0.01 b	0.38 ± 0.01 a
Ethanol (vol%)	n.a.	5.80 ± 0.07 a	5.60 ± 0.14 a	5.50 ± 0.00 a
Sugars' Consumption (%)	n.a.	91.15 ± 1.07 a	91.55 ± 0.98 a	90.64 ± 1.02 a
HPLC results				
Glucose (g/L)	31.48 ± 0.76	1.75 ± 0.04 c	2.77 ± 0.01 b	4.85 ± 0.01 a
Fructose (g/L)	75.77 ± 0.15	3.95 ± 0.11 b	3.51 ± 0.14 b	4.43 ± 0.32 a
Sucrose (g/L)	16.32 ± 0.60	0.79 ± 0.03 a	0.73 ± 0.02 a	0.53 ± 0.03 b
Acetic acid (g/L)	n.d.	0.74 ± 0.03 a	0.73 ± 0.04 ab	0.59 ± 0.03 b
Succinic acid (g/L)	n.d.	0.74 ± 0.02 a	0.69 ± 0.03 a	0.47 ± 0.03 b
Lactic acid (g/L)	0.11 ± 0.01	0.29 ± 0.06 a	0.75 ± 0.14 b	0.50 ± 0.01 ab

Data are expressed as mean ± standard deviation; n.a.: not analyzed; n.d.: not detected. Values with different letters in the same row are significantly different according to Tukey's HSD test ($p < 0.05$).

5.4.2. Changes in the volatile composition of distillates during maturation

The within-subject time effect was significant for all compounds (min F value = 6.88 with Greenhouse-Geisser-corrected min df factor; df error = 1.21; 9.83, $p > 0.05$). Most of the two-way, three-way, and four-way interaction effects with time were also significant ($p < 0.05$). The between-subject temperature effect was not significant for acetaldehyde, methanol, 1-propanol, 2-butanol, 2-methyl-1-butanol, cis-2-hexen-1-ol, diethyl succinate, and phenylacetic acid (maxF(1; 8 or 24) = 3.12; $p > 0.09$) and for all the other compounds (minF(1;8 or 24) = 4.70; $p < 0.05$). The between-subject alcohol content effect was significant for all the compounds (minF(1;8 or 24) = 7.30; $p < 0.05$), except for acetaldehyde ($F(1;24) = 0.10$; $p = 0.76$). The majority of the two-way and three-way interaction effects of between-subject factors were also significant ($p < 0.05$).

Table 14. Changes in volatile compounds of apple distillates obtained by *S. cerevisiae* during maturation

Compounds (mg/L alcohol 100% v/v)	80.6% v/v						60% v/v					
	10 ˚C			25 ˚C			10 ˚C			25 ˚C		
	0	12 weeks	24 weeks	0	12 weeks	24 weeks	0	12 weeks	24 weeks	0	12 weeks	24 weeks
Acetaldehyde	6.75 ±0.63 Ba	4.82 ±0.22 Ba	3.58 ±0.44 Aa	6.75 ±0.63 Bb	4.91 ±0.44 Bb	3.62 ±0.26 Ba	5.02 ±0.23 Ab	**3.99 ±0.28 Aab**	2.81 ±0.24 Aa	5.02 ±0.23 Ab	**3.18 ±0.14 Aa**	2.69 ±0.43 Aa
Methanol	5397.72 ±25.72 Ba	**5797.28 ±99.99 Ba**	**6405.88 ±399.26 Bb**	5397.72 ±25.72 Ba	**6707.70 ±134.69 Bc**	**6421.76 ±94.73 Bb**	4018.16 ±20.13 Ab	**3872.86 ±32.61 Aa**	3069.03 ±239.48 Aab	4018.16 ±20.13 Ab	**3394.51 ±28.64 Aa**	2774.28 ±196.48 Aa
1-propanol	1166.40 ±40.83 Bb	**1171.26 ±1.57 Bb**	**770.22 ±22.72 Ba**	1166.40 ±40.83 Bb	**987.40 ±22.72 Bb**	**835.43 ±28.53 Ba**	868.29 ±20.61 Ab	**770.08 ±15.27 Ab**	**677.07 ±6.30 Aa**	868.29 ±20.61 Ab	**699.53 ±2.04 Aa**	**609.77 ±18.12 Aa**
1-butanol	92.42 ±0.41 Bb	79.73 ±3.39 Bab	52.86 ±5.31 Aa	92.42 ±0.41 Bb	76.88 ±5.31 Bab	59.70 ±0.19 Ba	68.80 ±1.26 Ac	63.17 ±2.03 Ab	**52.86 ±0.38 Aa**	68.80 ±1.26 Ab	56.63 ±4.68 Aab	**47.62 ±0.93 Aa**
2-butanol	0.97 ±0.19 Aa	1.22 ±0.29 Aa	**0.88 ±0.10 Ba**	0.97 ±0.19 Aab	1.15 ±0.23 Bb	**0.44 ±0.02 Aa**	0.82 ±0.00 Ab	**0.93 ±0.04 Ab**	**0.42 ±0.03 Aa**	0.82 ±0.00 Aa	**0.83 ±0.01 Aa**	**0.56 ±0.06 Ba**
2-methyl-1-propanol	458.78 ±13.27 Bb	**405.74 ±9.18 Bb**	**285.97 ±3.64 Aa**	458.78 ±13.27 Bc	**383.22 ±3.64 Bb**	**314.57 ±2.68 Ba**	341.52 ±11.28 Ab	298.80 ±12.77 Aa	269.27 ±20.51 Aa	341.52 ±11.28 Ab	273.15 ±12.77 Aab	239.40 ±2.84 Aa
2-methyl-1-butanol	179.89 ±4.97 Ba	156.14 ±16.39 Ba	104.44 ±26.07 Aa	179.89 ±4.97 Bc	154.15 ±0.39 Bb	118.42 ±2.62 Ba	133.91 ±4.65 Ab	**123.75 ±0.16 Ab**	**106.05 ±1.38 Aa**	133.91 ±4.65 Ac	**113.63 ±1.16 Ab**	**97.03 ±1.16 Aa**
3-methyl-1-butanol	984.71 ±23.73 Bc	866.77 ±10.89 Bb	**559.32 ±17.64 Aa**	984.71 ±23.73 Bc	845.99 ±17.64 Bb	**637.83 ±20.49 Ba**	733.04 ±53.83 Ab	**675.01 ±2.88 Ab**	**556.24 ±13.88 Aa**	733.04 ±53.83 Ab	**614.19 ±2.88 Ab**	**506.40 ±10.20 Aa**
1-hexanol	56.90 ±1.00 Bb	47.75 ±3.41 Bb	**26.18 ±0.69 Aa**	56.90 ±1.00 Bc	46.70 ±0.69 Bb	**31.11 ±0.16 Ba**	42.36 ±3.93 Aab	**40.17 ±1.29 Ab**	**30.34 ±0.24 Ba**	42.36 ±3.93 Ab	**35.19 ±1.29 Aab**	**27.61 ±0.97 Aa**
Phenethyl alcohol	0.77 ±0.30 Aab	2.24 ±0.53 Bb	**1.15 ±0.18 Aa**	0.77 ±0.30 Aa	1.59 ±0.04 Ba	**1.68 ±0.11 Ba**	0.57 ±0.04 Aa	**0.64 ±0.03 Aa**	**2.68 ±0.10 Bb**	0.57 ±0.04 Ab	**0.11 ±0.00 Aa**	**0.61 ±0.01 Ab**
Trans-3-hexen-1-ol	0.64 ±0.01 Bc	**0.42 ±0.01 Bb**	0.17 ±0.03 Aa	0.64 ±0.01 Bc	**0.34 ±0.03 Bb**	0.16 ±0.01 Aa	0.47 ±0.03 Ab	**0.23 ±0.01 Aa**	0.17 ±0.05 Aab	0.47 ±0.03 Ab	**0.18 ±0.00 Aa**	0.26 ±0.10 Aab
Cis-2-hexen-1-ol	0.30 ±0.04 Ba	0.27 ±0.09 Aa	**0.10 ±0.02 Aa**	0.30 ±0.04 Ba	0.24 ±0.05 Ba	**0.17 ±0.00 Ba**	0.22 ±0.00 Ac	**0.18 ±0.00 Ab**	0.14 ±0.00 Aa	0.22 ±0.00 Ac	**0.15 ±0.00 Ab**	0.13 ±0.00 Aa
Benzyl alcohol	0.24 ±0.01 Bb	**0.11 ±0.00 Ba**	0.19 ±0.02 Ab	0.24 ±0.01 Bb	**0.28 ±0.02 Bab**	0.19 ±0.00 Ba	0.18 ±0.01 Ab	**0.08 ±0.01 Aa**	**0.58 ±0.04 Bc**	0.18 ±0.01 Ab	**0.04 ±0.01 Aa**	**0.11 ±0.01 Ab**
Ethyl acetate	165.20 ±1.57 Ba	213.01 ±0.14 Bb	**312.63 ±44.68 Bab**	165.20 ±1.57 Bb	246.40 ±44.68 Bb	**126.92 ±7.11 Ba**	122.98 ±1.90 Ac	**62.90 ±2.16 Ab**	**47.51 ±0.12 Aa**	122.98 ±1.90 Ab	**136.53 ±1.81 Ab**	**39.92 ±0.44 Aa**
Ethyl lactate	0.61 ±0.16 Ab	0.44 ±0.05 Ab	0.09 ±0.01 Aa	0.61 ±0.16 Ab	0.42 ±0.09 Ab	0.06 ±0.01 Aa	0.59 ±0.08 Ac	0.33 ±0.08 Ab	**0.11 ±0.01 Aa**	0.59 ±0.08 Ab	0.41 ±0.01 Ab	**0.05 ±0.00 Aa**
Ethyl butyrate	0.38 ±0.01 Ba	**0.38 ±0.07 Ba**	**0.38 ±0.01 Ba**	0.38 ±0.01 Bc	**0.27 ±0.01 Ab**	**0.14 ±0.01 Aa**	0.28 ±0.01 Ab	0.22 ±0.01 Aab	**0.16 ±0.02 Aa**	0.28 ±0.01 Ab	0.24 ±0.02 Aab	**0.28 ±0.01 Ba**
Isoamyl acetate	1.99 ±0.11 Ba	**1.71 ±0.21 Ba**	**1.85 ±0.07 Ba**	1.99 ±0.11 Bb	**1.33 ±0.03 Ba**	**2.11 ±0.06 Bb**	1.19 ±0.01 Ab	**0.55 ±0.04 Aa**	**0.49 ±0.08 Aa**	1.19 ±0.01 Ab	**0.78 ±0.04 Aa**	**0.87 ±0.04 Aa**

Ethyl octanoate	6.62 ±0.93 Ba	9.33 ±0.08 Ba	12.03 ±0.87 Ba	6.62 ±0.93 Ba	9.69 ±0.87 Ba	11.88 ±0.14 Ba	4.93 ±0.21 Aa	**6.26 ±0.24 Ab**	**9.76 ±0.15 Ac**	4.93 ±0.21 Aa	**4.08 ±0.24 Aa**	**3.25 ±0.79 Aa**
Diethyl succinate	0.19 ±0.02 Bab	**0.21 ±0.01 Bb**	0.13 ±0.00 Aa	0.19 ±0.02 Ba	**0.14 ±0.01 Aa**	0.13 ±0.00 Ba	0.14 ±0.02 Aa	0.15 ±0.01 Aa	**0.12 ±0.01 Aa**	0.14 ±0.02 Aab	0.13 ±0.00 Ab	**0.11 ±0.00 Aa**
Ethyl myristate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-phenethyl acetate	0.21 ±0.01 Ba	0.19 ±0.00 Ba	**0.18 ±0.01 Ba**	0.21 ±0.01 Bb	0.20 ±0.02 Bb	**0.11 ±0.02 Aa**	0.15 ±0.01 Ac	0.12 ±0.00 Ab	0.09 ±0.00 Aa	0.15 ±0.01 Ab	0.11 ±0.01 Aab	0.09 ±0.02 Aa
Ethyl phenylacetate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Limonene	0.22 ±0.07 Ab	**0.07 ±0.01 Ab**	0.00 ±0.00 Aa	0.22 ±0.07 Ac	**0.01 ±0.00 Ab**	0.00 ±0.00 Aa	0.16 ±0.02 Ab	0.09 ±0.01 Bb	**0.03 ±0.00 Ba**	0.16 ±0.02 Ab	0.13 ±0.04 Bab	**0.09 ±0.00 Ba**
Linalool	0.45 ±0.10 Aa	**0.13 ±0.01 Aa**	**0.11 ±0.03 Aa**	0.45 ±0.10 Aa	**0.33 ±0.03 Aa**	**0.26 ±0.04 Aa**	0.34 ±0.01 Ab	0.29 ±0.01 Ba	0.23 ±0.01 Ba	0.34 ±0.01 Ab	0.28 ±0.01 Aab	0.23 ±0.01 Aa
Phenyl acetic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Data are expressed as mean ± standard deviation; n.d.: not detected. Different lower-case letters in a row show significant differences for the time effect; upper-case letters are for the ABV effect comparing 60% v/v and 80.6% v/v. Significant differences between the temperatures (10 °C and 25 °C) are in bold, with the significantly greater value marked with an underline (Games-Howell's post hoc test, $p < 0.05$).

Table 15. Changes in volatile compounds of apple distillates obtained by *L. thermotolerans* and *S. cerevisiae* during maturation

Compounds (mg/L alcohol 100% v/v)	84.8% v/v						60% v/v					
	10 ˚C			25 ˚C			10 ˚C			25 ˚C		
	0	12 weeks	24 weeks	0	12 weeks	24 weeks	0	12 weeks	24 weeks	0	12 weeks	24 weeks
Acetaldehyde	6.40 ±0.47 Bb	5.66 ±0.37 Aab	**4.75 ±0.34 Aa**	6.40 ±0.47 Ba	5.18 ±1.07 Aa	**3.80 ±0.37 Aa**	4.53 ±0.44 Aa	4.31 ±0.73 Aa	**4.84 ±0.26 Aa**	4.53 ±0.44 Aa	3.85 ±0.36 Aa	**5.87 ±0.15 Ba**
Methanol	7022.33 ±200.38 Ba	**6570.25 ±233.28 Ba**	6470.86 ±598.87 Ba	7022.33 ±200.38 Ba	**7532.00 ±450.92 Ba**	6242.26 ±406.49 Ba	4968.63 ±161.21 Ab	**3909.44 ±79.24 Aa**	3699.71 ±1011.65 Aab	4968.63 ±161.21 Ac	**2983.58 ±48.92 Aa**	3924.70 ±39.94 Ab
1-propanol	1018.89 ±45.65 Bb	847.25 ±14.97 Bab	853.40 ±22.34 Ba	1018.89 ±45.65 Ba	967.05 ±14.59 Ba	837.92 ±81.63 Ba	720.92 ±5.78 Ab	**557.88 ±12.24 Aa**	**515.79 ±21.38 Aa**	720.92 ±5.78 Ab	**662.16 ±34.42 Aab**	**562.83 ±8.87 Aa**
1-butanol	69.61 ±0.79 Ba	52.48 ±7.18 Ba	52.20 ±8.72 Ba	69.61 ±0.79 Bb	64.01 ±1.11 Bb	52.01 ±0.49 Ba	49.25 ±2.09 Ac	**37.09 ±0.49 Ab**	**33.90 ±0.34 Aa**	49.25 ±2.09 Ab	**49.49 ±1.87 Aab**	**37.69 ±1.40 Aa**
2-butanol	0.59 ±0.04 Bb	0.30 ±0.03 Aa	**0.16 ±0.04 Aab**	0.59 ±0.04 Ba	0.54 ±0.25 Aa	**0.34 ±0.04 Ba**	0.42 ±0.05 Aa	**0.44 ±0.01 Ba**	**0.46 ± 0.02 Ba**	0.42 ±0.05 Aab	**0.65 ±0.07 Ab**	**0.26 ±0.01 Aa**
2-methyl-1-propanol	164.19 ±3.16 Bb	**127.59 ±7.16 Ba**	131.81 ±12.85 Bab	164.19 ±3.16 Ba	**153.23 ±7.37 Ba**	130.09 ±6.97 Ba	116.17 ±1.36 Ab	**90.34 ±0.75 Aa**	**84.24 ±1.48 Aa**	116.17 ±1.36 Ab	**107.89 ±6.76 Aab**	**91.79 ±0.47 Aa**
2-methyl-1-butanol	73.12 ±0.42 Bb	54.51 ±4.70 Bab	52.80 ±1.47 Ba	73.12 ±0.42 Bb	66.67 ±9.37 Aab	53.30 ±0.45 Ba	51.74 ±0.37 Ab	**37.60 ±1.32 Aa**	**36.15 ±0.25 Aa**	51.74 ±0.37 Ab	**52.67 ±0.39 Ac**	**40.01 ±0.51 Aa**
3-methyl-1-butanol	405.24 ±12.40 Bb	**298.13 ±3.21 Ba**	285.87 ±69.89 Aab	405.24 ±12.40 Ba	**367.69 ±9.67 Aa**	290.34 ±57.71 Aa	286.73 ±5.44 Ab	**206.50 ±1.06 Aa**	**192.77 ±5.22 Aa**	286.73 ±5.44 Ab	**294.78 ±58.50 Aab**	**216.46 ±3.01 Aa**
1-hexanol	33.81 ±2.87 Ba	**23.19 ±0.61 Ba**	20.64 ±2.27 Ba	33.81 ±2.87 Ba	**29.35 ±1.08 Ba**	23.89 ±1.52 Ba	23.93 ±2.95 Ab	**16.22 ±1.13 Aa**	**13.91 ±1.16 Aab**	23.93 ±2.95 Aab	**19.03 ±0.40 Ab**	**16.57 ±0.88 Aa**
Phenethyl alcohol	2.02 ±0.19 Bc	1.00 ±0.13 Ab	**0.16 ±0.05 Ba**	2.02 ±0.19 Bc	1.05 ±0.12 Bb	**0.36 ±0.02 Aa**	0.95 ±0.04 Ab	**1.03 ±0.01 Ab**	**0.07 ±0.01 Aa**	0.95 ±0.04 Ac	**0.11 ±0.02 Aa**	**0.65 ±0.08 Bb**
Trans-3-hexen-1-ol	0.49 ±0.00 Bb	**0.29 ±0.02 Ba**	**0.29 ±0.03 Ba**	0.49 ±0.00 Ba	**0.52 ±0.01 Ba**	**0.55 ±0.02 Ba**	0.35 ±0.00 Ac	0.22 ±0.00 Ab	**0.10 ±0.00 Aa**	0.35 ±0.00 Ab	0.20 ±0.02 Aa	**0.28 ±0.00 Aa**
Cis-2-hexen-1-ol	0.13 ±0.00 Ba	**0.13 ±0.00 Ba**	0.12 ±0.01 Ba	0.13 ±0.00 Ba	**0.10 ±0.01 Ba**	0.08 ±0.03 Aa	0.09 ±0.02 Aa	**0.10 ±0.01 Aa**	**0.09 ±0.00 Aa**	0.09 ±0.02 Aa	**0.06 ±0.00 Aa**	**0.06 ±0.00 Aa**
Benzyl alcohol	0.32 ±0.00 Bb	**0.11 ±0.01 Ba**	**0.09 ±0.01 Aa**	0.32 ±0.00 Ba	**0.31 ±0.03 Ba**	**0.24 ±0.07 Aa**	0.22 ±0.01 Ab	**0.04 ±0.01 Aa**	**0.25 ±0.00 Bb**	0.22 ±0.01 Ac	**0.19 ±0.01 Ab**	**0.15 ±0.00 Aa**
Ethyl acetate	497.74 ±64.86 Bb	472.12 ±11.77 Bab	248.71 ±34.04 Aa	497.74 ±64.86 Ba	497.93 ±46.34 Ba	212.67 ±41.37 Aa	352.17 ±10.90 Aa	**397.19 ±1.45 Aa**	**364.26 ±12.80 Ba**	352.17 ±10.90 Ab	**251.41 ±28.86 Aab**	**262.44 ±5.41 Aa**
Ethyl lactate	0.61 ±0.16 Ab	0.44 ±0.05 Ab	0.09 ±0.01 Aa	0.61 ±0.16 Ab	0.42 ±0.09 Ab	0.06 ±0.01 Aa	0.59 ±0.08 Ac	0.33 ±0.08 Ab	**0.11 ±0.01 Aa**	0.59 ±0.08 Ab	0.41 ±0.01 Ab	**0.05 ±0.00 Aa**
Ethyl butyrate	0.05 ±0.01 Aa	**0.03 ±0.01 Aa**	0.02 ±0.01 Ba	0.05 ±0.01 Aa	**0.06 ±0.01 Ba**	0.03 ±0.02 Aa	0.03 ±0.01 Aab	**0.05 ±0.00 Bb**	**0.00 ±0.00 Aa**	0.03 ±0.01 Aab	**0.04 ±0.00 Ab**	**0.02 ±0.00 Aa**

Isoamyl acetate	0.56 ±0.02 Bb	0.19 ±0.01 Aa	**0.28 ±0.11 Bab**	0.56 ±0.02 Bb	0.23 ±0.03 Aa	**0.53 ±0.06 Bb**	0.40 ±0.04 Ab	**0.30 ±0.01 Bb**	**0.00 ±0.00 Aa**	0.40 ±0.04 Ab	**0.23 ±0.02 Ab**	**0.05 ±0.01 Aa**
Ethyl octanoate	3.82 ±0.59 Ba	5.35 ±0.32 Ba	7.37 ±0.74 Aa	3.82 ±0.59 Ba	5.75 ±0.64 Aab	7.68 ±0.02 Bb	2.70 ±0.01 Aa	4.66 ±0.17 Ab	**6.61 ±0.11 Ac**	2.70 ±0.01 Aa	4.92 ±0.23 Ab	**6.14 ±0.07 Ab**
Diethyl succinate	0.18 ±0.01 Bb	0.17 ±0.01 Bb	**0.13 ±0.01 Aa**	0.18 ±0.01 Bb	0.16 ±0.02 Aab	**0.10 ±0.01 Aa**	0.13 ±0.00 Aa	**0.13 ±0.01 Aa**	0.12 ±0.01 Aa	0.13 ±0.00 Aab	**0.16 ±0.01 Ab**	0.12 ±0.01 Ba
Ethyl myristate	0.03 ±0.00 Aa	0.05 ±0.00 Bb	**0.07 ±0.01 Bab**	0.03 ±0.00 Aa	0.08 ±0.06 Aab	**0.13 ±0.02 Bb**	0.02 ±0.01 Aa	0.03 ±0.01 Aa	0.03 ±0.01 Aa	0.02 ±0.01 Aa	0.02 ±0.01 Aa	0.06 ±0.01 Aa
2-phenethyl acetate	0.39 ±0.01 Ba	0.35 ±0.06 Ba	0.30 ±0.06 Ba	0.39 ±0.01 Bb	0.33 ±0.02 Bb	0.27 ±0.02 Ba	0.28 ±0.00 Ab	**0.24 ±0.00 Aa**	0.20 ±0.01 Aa	0.28 ±0.00 Ab	**0.26 ±0.01 Ab**	0.21 ±0.00 Aa
Ethyl phenylacetate	0.51 ±0.01 Bb	0.39 ±0.08 Bab	**0.11 ±0.02 Aa**	0.51 ±0.01 Ba	0.46 ±0.01 Ba	**0.23 ±0.05 Ba**	0.36 ±0.04 Ab	**0.26 ±0.00 Ab**	0.15 ±0.00 Ba	0.36 ±0.04 Ab	**0.34 ±0.05 Ab**	0.15 ±0.00 Aa
Limonene	0.55 ±0.09 Bb	0.12 ±0.00 Aa	**0.08 ±0.03 Ba**	0.55 ±0.09 Bc	0.12 ±0.02 Ab	**0.00 ±0.00 Aa**	0.39 ±0.02 Ac	**0.13 ±0.01 Ab**	0.00 ±0.00 Aa	0.39 ±0.02 Ac	**0.11 ±0.00 Ab**	0.00 ±0.00 Aa
Linalool	0.36 ±0.01 Bb	0.25 ±0.02 Ba	0.26 ±0.06 Bab	0.36 ±0.01 Ba	0.28 ±0.02 Ba	0.20 ±0.05 Ba	0.25 ±0.01 Ac	**0.09 ±0.02 Ab**	**0.04 ±0.01 Aa**	0.25 ±0.01 Ac	**0.19 ±0.01 Ab**	**0.07 ±0.01 Aa**
Phenyl acetic acid	0.19 ±0.02 Ba	**0.19 ±0.01 Ba**	**0.20 ±0.01 Ba**	0.19 ±0.02 Bb	**0.11 ±0.01 Aab**	**0.15 ±0.01 Ba**	0.13 ±0.01 Aa	**0.13 ±0.00 Aa**	**0.13 ±0.01 Aa**	0.13 ±0.01 Ac	**0.12 ±0.01 Ab**	**0.11 ±0.01 Aa**

Data are expressed as mean ± standard deviation. Different lower-case letters in a row show significant differences for the time effect; upper-case letters are for the ABV effect comparing 60% v/v and 84.8% v/v. Significant differences between the temperatures (10 °C and 25 °C) are in bold, with the significantly greater value marked with an underline (Games-Howell's post hoc test, $p < 0.05$).

The profile of volatile congeners with their means and standard deviations during maturation, completed with post hoc test results, is shown in Tables 14 and 15. A detailed evaluation of volatile changes during 24 weeks of maturation of spirits fermented with *S. cerevisiae* (Uvaferm 228) and *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) is given below. As the volatile changes during the maturation of the spirit produced by *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) showed similar tendencies with the other two spirits, the data for that spirit is not shown (but is provided in Table A2). The data for the spirit produced by *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) are reported only if a difference was encountered.

Acetaldehyde, an important carbonyl compound in alcoholic beverages, may be formed by yeast, acetic acid bacteria, or via auto-oxidation of ethanol and phenolic compounds (Liu & Pilone, 2000). Higher initial contents of acetaldehyde were detected in distillates produced by *S. cerevisiae* (Uvaferm 228) (6.75 mg/L a.a.) compared to *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) (6.55 mg/L a.a.) and *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) (6.40 mg/L a.a.). The majority of the samples showed a gradual decrease in acetaldehyde levels over time, probably as a result of acetaldehyde interactions with ethanol, resulting in the formation of acetal (Mangas et al., 1996). However, the acetaldehyde content of the 60% v/v ABV spirits, produced by both mixed cultures, slightly increased. In the variants that matured at 25 ºC, the increases were greater. This phenomenon is common during aging due to the chemical oxidation of ethanol (Flouros et al., 2003).

Esters are formed by yeast during fermentation by an interaction between acyl-CoA molecules and alcohols. Their synthesis may continue during aging through the processes of acidolysis, alcoholysis, and transesterification (Vyviurska et al., 2017). The predominant ester in the analyzed apple spirits was ethyl acetate. Large differences were observed in the concentration of ethyl acetate among the unmatured samples. Ethyl acetate content in the spirit produced by the monoculture of *S. cerevisiae* (Uvaferm 228) was 165.20 mg/L a.a.; higher values were measured in the spirits produced by the mixed cultures *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) (387.36 mg/L a.a.) and *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) (497.74 mg/L a.a.). At 10 ºC, samples with ABVs of 80.6% v/v and 84.8% v/v exerted opposing changing trends. In the first case, ethyl acetate levels increased by 50% to 312.63 mg/L a.a., whereas in the second case, ethyl acetate levels decreased by 50% to 248.71 mg/L a.a.. On the other hand, a gradual drop in ethyl acetate levels was observed in the samples stored at 25 ºC, regardless of their alcohol content. The only difference was experienced in the spirit produced by *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) (81.8% v/v), where ethyl acetate levels rose to 579 mg/L a.a.. Ethyl acetate changes were correlated

with the ABVs of the samples, storage time, and temperature ($p < 0.05$). A previous study (Flouros et al., 2003) has shown that ethyl acetate levels increased during the maturation of Tsipouro regardless of the type of bottle used (PET, PVC, or glass). Optimal concentrations of ethyl lactate stabilize the distillate's flavor and soften its harsh character (Spaho et al., 2021). In our study, ethyl lactate showed high dependence on the three factors studied, decreasing its content during maturation. Diethyl succinate levels decreased slightly over time, although the losses were not significant. Rodríguez Madrera et al. (2013) reported that ethyl acetate, ethyl lactate, and diethyl succinate decreased during the maturation of cider spirits in inert containers. Isoamyl acetate was present at low concentrations, amounting to 0.44 mg/L a.a. in the *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228), 0.56 mg/L a.a. in the *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228), and 1.99 mg/L a.a. in the *S. cerevisiae* (Uvaferm 228) fresh distillates. Two distinct changing behaviours of isoamyl acetate were observed during maturation, depending on all tested factors ($p < 0.05$). The samples with higher alcohol content experienced a decrease in the first 12 weeks and increased in the second phase of the maturation period, in some cases even exceeding the initial concentration. The increase was greatest in *S. cerevisiae* (Uvaferm 228) distillate, with an ABV of 80.6% v/v, stored at 25 ºC (2.11 mg/L a.a.). On the contrary, in the samples with 60% v/v alcohol content, isoamyl acetate concentrations consistently decreased, except in the samples from *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228), which experienced a threefold increase in isoamyl acetate concentrations. According to Rodríguez Madrera et al. (2011), those results are stimulated by a series of hydrolysis and esterification reactions in which isoamyl alcohol is involved. Furthermore, ethyl esters of fatty acids were also detected in the samples. The initial concentrations of ethyl butyrate and ethyl octanoate in the spirits produced by *S. cerevisiae* (Uvaferm 228) were 0.38 mg/L a.a. and 6.62 mg/L a.a., respectively, while they were 0.05 mg/L a.a. and 3.82 mg/L a.a. in the spirits produced by *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228). Ethyl myristate was only detected in the spirit produced by *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) with an initial concentration of 0.03 mg/L a.a. Generally, ethyl butyrate concentrations didn't experience any drastic changes during maturation. A slight increase of 0.03 mg/L a.a. in ethyl butyrate content was observed in the spirit from *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) (81.8% v/v) matured at 25 ºC. Ethyl octanoate increased significantly in the samples. Ethyl myristate followed the same increasing pattern during the investigated period. An increase in these esters might be fostered by the esterification of fatty acids. Similar results were detailed in the study of Rodríguez Madrera et al. (2011). Phenylacetic acid was only present in the distillates made from mixed cultures. The

concentration of this compound slightly increased in the sample of *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) with 81.8% v/v alcohol content stored at 25 ºC. Ethyl phenylacetate was noted in small amounts in the distillate produced by *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) but not in those made by *S. cerevisiae* (Uvaferm 228) or *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228).

Methanol is formed during fermentation by the enzymatic hydrolysis of pectin present in fruits (Różański et al., 2020). Although the presence of methanol does not affect the flavor of the spirit, it is subjected to restrictive control due to its high toxicity (Flouros et al., 2003). The apple spirit obtained from *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) was found to contain more methanol (7022.33 mg/L a.a.) than the spirit from *S. cerevisiae* (Uvaferm 228) (5397.72 mg/L a.a.). Unlike the results reported in the literature (Różański et al., 2020), the changes in methanol levels during maturation were highly dependent on alcohol content ($p < 0.05$). The concentration of methanol in the samples of *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) was observed to decrease in all cases; the loss was significantly higher in the samples with lower alcohol content (60% v/v). This trend was probably triggered by several concurrent processes, including oxidation to formaldehyde, esterification, and acetal transformation (Mangas et al., 1996). The same behaviour was observed in the samples of *S. cerevisiae* (Uvaferm 228). It should be emphasized that methanol contents never exceeded the maximum permitted limit (12 g/L alcohol 100% v/v) (EC Regulation 2019/787).

Eleven higher alcohols were detected in the apple distillates. The predominant higher alcohols were 1-propanol and amyl alcohols (3-methyl-1-butanol and 2-methyl-1-butanol). The amount of 1-propanol in fresh apple distillates was 1166.40 mg/L a.a. (*S. cerevisiae* (Uvaferm 228)) and 1018.89 mg/L a.a. (*L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228)). After the reduction of alcoholic strength to 60% v/v, the concentration of 1-propanol dropped to 868.29 mg/L a.a. and 720.92 mg/L a.a., respectively. During the 24 weeks of maturation, a continuous reduction of 1-propanol was recorded, especially in samples with a higher alcohol content (>80% v/v). Matias-Guiu et al. (2020) reported no significant differences in the concentration of 1-propanol after 1 year of spirit maturation. The concentration of amyl alcohols in the spirit obtained from *S. cerevisiae* (Uvaferm 228) was over two times higher compared to *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228) ($p < 0.05$). A significant decrease during maturation time was detected for both 2-methyl-1-butanol and 3-methyl-1-butanol concentrations. The factor influencing the above-mentioned changes of 2-methyl-1-butanol was alcohol content ($p < 0.05$) whereas the changes of 3-methyl-1-butanol were

also affected by temperature ($p < 0.05$). Additionally, abundant amounts of 2-methyl-1-propanol, 1-butanol, and 1-hexanol were detected. Their presence was significantly greater in the samples fermented by *S. cerevisiae* (Uvaferm 228) compared to *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228). Nevertheless, during maturation, the same diminishing behaviour was noted in all the maturation variants. The same tendency was also observed in the maturation of plum brandies (Balcerek et al., 2017a; Różański et al., 2020*).* Even though samples matured in the same conditions, the loss of 2-methyl-1-propanol, 1-butanol, and 1-hexanol was higher in the samples that belong to the *S. cerevisiae* (Uvaferm 228) group. The most drastic drop in those higher alcohols was observed in the spirit, with an ABV of 80.6% v/v matured at 10 ºC. After 24 weeks of maturation, the concentrations of 2-methyl-1-propanol, 1-butanol, and 1-hexanol decreased by 38%, 43%, and 54%, respectively. These concentration variations are influenced by all factors studied (time, temperature, and ABV) ($p < 0.05$). Likewise, the concentration of 2-butanol, phenethyl alcohol, benzyl alcohol, trans-3-hexen-1-ol, and cis-2-hexen-1-ol mainly decreased during the investigated time ($p < 0.05$). Those compounds exerted increasing trends in a few instances. Trans-3-hexen-1-ol concentrations of the spirits obtained from both mixed cultures increased during maturation in their original ABVs at 25 ºC. During maturation of the spirits obtained from *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) an increase in 2-butanol content was detected in two samples; the sample with 81.8% v/v alcohol content matured at 10 ºC, and the sample with 60% v/v alcohol content matured at 25 ºC. Trans-3-hexen-1-ol, cis-2-hexen-1-ol, and 1-hexanol are plant-derived compounds that may impart herbaceous odors to the spirits (Tsakiris et al., 2013). Significant differences in concentrations of phenethyl alcohol, benzyl alcohol, and trans-3-hexen-1-ol were found for the tested factors (temperature and ABV) ($p < 0.05$). No relationship was found between temperature and variations of 2-butanol and cis-2-hexen-1-ol ($p > 0.05$). According to Rodríguez Madrera et al. (2013), processes like evaporation, esterification, and oxidation may be the reason behind the decrease in higher alcohols during maturation.

Terpenes originate from fruits and are intensively released during fruit processing (Spaho et al., 2021). Trace amounts of limonene (0.22-0.55 mg/L a.a.) and linalool (0.36-0.45 mg/L a.a.) were identified in the fresh distillates. According to Januszek et al. (2020b), limonene and linalool concentrations in the brandies obtained from different apple cultivars were in the range of 0.21-0.39 mg/L a.a. and 0.33-0.37 mg/L a.a., respectively. Terpene profiles were strongly affected by maturation time, generally leading to a reduction. Limonene levels experienced a sharp drop, even

disappearing in the majority of samples. Changes in terpenes were significantly affected by temperature and alcohol content ($p < 0.05$).

5.4.3. Principal Component Analysis

Principal component analysis was conducted to explore potential relationships between maturation conditions and the volatile compounds of apple spirits. In the distillates obtained from *S. cerevisiae* (Uvaferm 228), the first two principal components explained 75.8% of the total variation (Figure 16A). PC1 was negatively correlated with 1-propanol, 2-butanol, ethyl butyrate, diethyl succinate, 1-butanol, acetaldehyde, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 1-hexanol, cis-2-hexen-1-ol, 2-phenethyl acetate, trans-3-hexen-1-ol, as well as ethyl lactate, explaining 57.1% of variation. PC2 was positively correlated with ethyl octanoate, ethyl acetate, phenethyl alcohol, isoamyl acetate, and methanol, while it was negatively correlated with limonene and linalool, explaining 18.7% of the variation. At the 12th week, maturation of distillates with an ABV of 80.6% v/v at temperatures of 10 ºC and 25 ºC had a significant impact especially on the changes in methanol, ethyl acetate, isoamyl acetate, and 2-phenethyl acetate (in Figure 16A, the brown and dark blue triangles are well separable from the red and light blue ones in the direction of these compounds). In the last phase of maturation, the mentioned maturation conditions had a notable impact on benzyl alcohol, phenethyl alcohol, and ethyl octanoate variations.

The two principal components accounted for 71.1% of the overall variance in the distillates obtained from *L. thermotolerans* (Concerto) + *S. cerevisiae* (Uvaferm 228). PC1 was negatively and strongly correlated with 1-hexanol, 2-methyl-1-butanol, 2-methyl-1-propanol, 1-butanol, 2-phenethyl acetate, linalool, 3-methyl-1-butanol, and 1-propanol, being responsible for 60.5% of variances. PC2 was positively correlated with 2-butanol, limonene, ethyl lactate, ethyl acetate, and phenethyl alcohol, while it was negatively correlated with ethyl octanoate, methanol, and trans-3-hexen-1-ol, being responsible for 10.6% of variation. As illustrated in Figure 16B, the maturation of distillates with 84.8% v/v ABV at temperatures of 10 ºC and more explicitly at 25 ºC on the 12th week showed a high contribution to the changes of methanol and the majority of higher alcohols. Additionally, maturation of samples under the aforementioned conditions on the 24th week had a profound effect on the variations of ethyl octanoate.

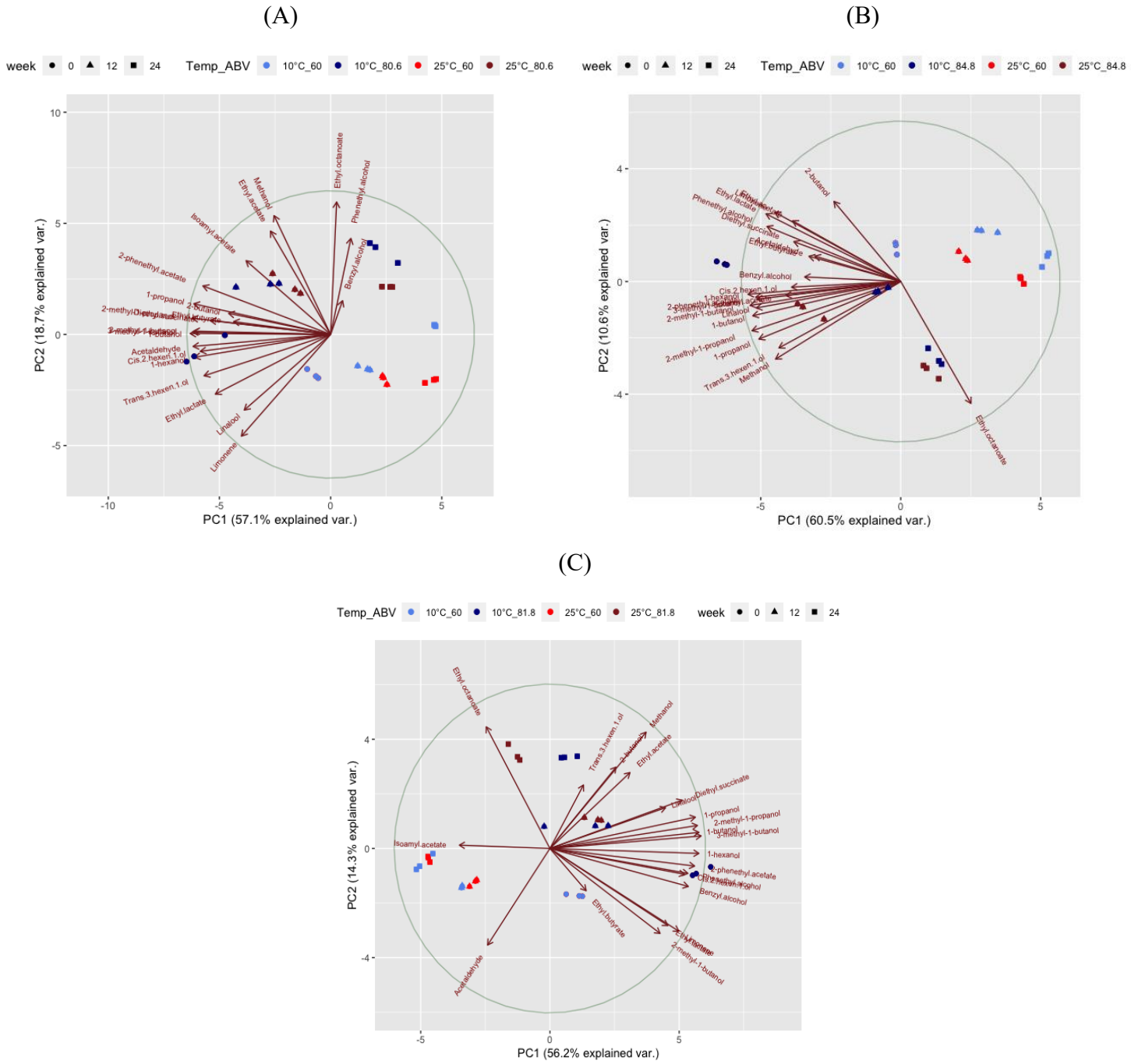


Figure 16. Principal component analysis (PCA) biplots for the observed variables of apple distillates obtained by (A) *S. cerevisiae*, (B) *L. thermotolerans* + *S. cerevisiae*, and (C) *T. delbrueckii* + *S. cerevisiae*. Brown arrows represent the measured variables (volatile compounds), and points of different shape and colour represent sampling points relative to the maturation time (0, 12, and 24 weeks), temperature (10 °C and 25 °C), and ABV (60% and >80%). Sampling points on the same side of a given variable should be interpreted as having a high contribution on it. The magnitude of the arrows shows the strength of their contribution to each PC. Arrows pointing in similar or opposite directions indicate positively or negatively correlated variables, while those close to be perpendicular indicate a low or no correlation.

The two principal components explained 70.5% of the total variation (56.2% and 14.3%, respectively) in the distillates obtained from *T. delbrueckii* (Biodiva) + *S. cerevisiae* (Uvaferm 228) (Figure 16C). PC1 was negatively correlated with isoamyl acetate and positively correlated with 1-hexanol, 2-phenethyl acetate, phenethyl alcohol, cis-2-hexen-1-ol, benzyl alcohol, 3-methyl-1-butanol, 2-methyl-1-propanol, 1-butanol, 1-propanol, diethyl succinate, limonene, ethyl lactate, and linalool. PC2 was positively correlated with ethyl octanoate, methanol, 2-butanol, and trans-3-hexen-1-ol, and negatively correlated with acetaldehyde, 2-methyl-1-butanol, and ethyl butyrate. Maturation of distillates with 81.8% v/v ABV under temperatures of 10 ºC and 25 ºC showed a high contribution to the changes of methanol, linalool, diethyl succinate, ethyl acetate, ethyl octanoate, 2-butanol, and trans-3-hexen-1-ol. Whereas the maturation of distillates with 60% v/v ABV influenced the changes of acetaldehyde and isoamyl acetate.

Overall, maturation has a significant effect on the changes in the volatile composition of the distillate, whereas maturation conditions govern the changing patterns. These changes are primarily dependent on the alcohol content of the distillates. Regardless of temperature, higher alcohol content (>80% v/v) in distillates during maturation leads to more intense and pronounced changes in volatiles.

5.5. Assessment of the combinatorial impacts of nutrient treatment, acidification technique and yeast strain on fermentation performance and aroma production in apple mash

After individually testing the parameters (acidification method, nutrient supplements, and yeast strains), the most promising variants were selected and combined in a new experiment. This comprehensive study aimed to investigate how optimized fermentation conditions and parameters influence the evolution of individual aroma components from the fruit through fermentation to the end of the distillation process.

5.5.1. Physicochemical characteristics of the mash during fermentation

The overall MANOVA resulted in highly significant differences among the samples (Wilk's lambda < 0.001, $p < 0.001$), which were detected in every parameter but sugars' consumption ($F(11;24) > 6.47$ with $p < 0.001$) and for sugars' consumption $F(11;24) = 0.23$ with $p = 0.99$. The chemical composition of the fresh and fermented apple mashes together with the post hoc test results are provided in Table 16. The fresh apple mash contains approximately 135.85 g/L of total sugars, with 118.59 g/L being reducing sugars. The high content of total sugars in the apples used holds technological advantages, as it indicates the potential for high ethanol yields (Pielech-Przybylska et

al., 2016). The high fermentation efficiencies and sugar consumption rates (88.08 to 91.00%) validate the proper progression of all the processes. Variations were noted in terms of residual sugars, which fell within the range of 7.63 (Apple mash 5) to 11.69 g/L (Apple mash 8). The ethanol content in the fermented mashes ranged from 5.10% (Apple mash 3 and Apple mash 9) to 5.90% (Apple mash 1).

During fermentation, the titratable acidity increased by 0.73 to 2.50 g/L. The pH levels in Apple mash 4, 5, 6, 10, 11, and 12 were slightly higher in comparison to the remaining six samples. However, effective acidification was achieved in these samples through the action of *Lb. plantarum* (LB-1), resulting in the reduction of spoilage bacteria populations and facilitating yeast growth (Santos et al., 2013; Li et al., 2021b). Apple Mash 1 and 2 displayed the lowest volatile acidity concentration at 0.15 g/L, while the highest level was observed in Apple Mash 12 at 0.41 g/L.

The initial YAN content of the fresh apple mash was 94.73 mg/L. Following the addition of the first dose of nutrient Vitamon A to apple mashes 1-6 and nutrient Genesis Fresh to apple mashes 7-12, the YAN concentrations increased to 333.68 mg/L and 220.52 mg/L, respectively. Noticeable variations in YAN utilization rates were evident among the first 6 samples, all of which received the same nutrient treatment (Vitamon A + Vitamon Combi). These differences may be due to distinct metabolic requirements of the yeast strains employed. Samples Apple mash 4, 5, and 6 exhibited a higher nitrogen utilization, potentially attributed to the pre-fermentation with *Lb. plantarum* (LB-1) in addition to the yeast strains. Among them, Apple Mash 4, inoculated with *S. cerevisiae* (Uvaferm 228), displayed the highest YAN utilization. A similar pattern was noted in the remaining 6 samples that received the second nutrient treatment (Genesis Fresh + Vitamon Combi). In this case, the YAN content in Apple mash 10, 11, and 12 was notably lower in comparison to Apple mash 7, 8, and 9. Among this group, Apple Mash 11, which was inoculated with the hybrid yeast X-treme, stood out for having the lowest remaining YAN levels at 68.63 mg/L.

Table 16. Physico-chemical parameters of fresh and fermented apple mashes

	Reducing sugars (g/L)	Total sugars (g/L)	Sugars' consumption (%)	pH	Titratable acidity (g/L)	Volatile acidity (g/L)	Ethanol (vol%)	YAN (mg/L)
Fresh apple mash	118.59 ± 3.94	135.85 ± 4.37	n.a.	3.52 ± 0.07	4.96 ± 0.07	n.a.	n.a.	94.73 ± 3.53
Fermented apple mash								
Apple mash 1	7.67 ± 0.44 c	8.28 ± 0.45 c	90.71 ± 2.80 a	3.06 ± 0.04 d	6.72 ± 0.07 b	0.15 ± 0.00 f	5.90 ± 0.10 a	216.86 ± 5.04 b
Apple mash 2	8.78 ± 0.67 bc	8.90 ± 0.67 bc	90.27 ± 3.58 a	3.15 ± 0.04 bcd	5.90 ± 0.03 e	0.15 ± 0.00 f	5.30 ± 0.00 cd	260.78 ± 5.02 a
Apple mash 3	8.50 ± 0.39 c	8.94 ± 0.36 bc	90.24 ± 2.8 a	3.19 ± 0.04 abc	5.81 ± 0.05 e	0.24 ± 0.02 cd	5.10 ± 0.10 d	260.78 ± 10.06 a
Apple mash 4	8.86 ± 0.99 bc	9.00 ± 0.98 bc	90.20 ± 2.43 a	3.29 ± 0.01 a	6.30 ± 0.08 cd	0.26 ± 0.00 bc	5.30 ± 0.10 cd	140.00 ± 5.12 e
Apple mash 5	11.69 ± 0.58 a	11.99 ± 0.58 a	88.08 ± 3.52 a	3.26 ± 0.04 ab	6.34 ± 0.11 c	0.24 ± 0.01 cd	5.30 ± 0.10 cd	178.43 ± 2.18 d
Apple mash 6	8.75 ± 1.29 bc	8.87 ± 1.29 c	90.29 ± 2.22 a	3.24 ± 0.04 ab	7.43 ± 0.05 a	0.24 ± 0.01 cd	5.40 ± 0.00 bc	194.90 ± 7.54 c
Apple mash 7	7.90 ± 0.10 c	8.23 ± 0.12 c	90.75 ± 3.04 a	3.10 ± 0.04 cd	6.56 ± 0.07 bc	0.21 ± 0.02 de	5.20 ± 0.00 cd	129.02 ± 5.00 ef
Apple mash 8	7.63 ± 0.17 c	7.88 ± 0.15 c	91.00 ± 3.01 a	3.04 ± 0.04 d	6.57 ± 0.13 bc	0.18 ± 0.01 ef	5.60 ± 0.10 b	131.76 ± 2.17 ef
Apple mash 9	7.80 ± 0.30 c	8.11 ± 0.29 c	90.83 ± 3.30 a	3.09 ± 0.05 cd	5.69 ± 0.32 e	0.24 ± 0.03 cd	5.10 ± 0.20 d	123.53 ± 3.05 f
Apple mash 10	8.28 ± 0.18 c	8.52 ± 0.18 c	90.54 ± 3.23 a	3.26 ± 0.08 ab	5.94 ± 0.07 de	0.24 ± 0.01 cd	5.30 ± 0.00 cd	71.37 ± 2.75 h
Apple mash 11	10.33 ± 0.40 ab	10.60 ± 0.40 ab	89.06 ± 2.84 a	3.30 ± 0.03 a	6.42 ± 0.03 bc	0.29 ± 0.01 b	5.40 ± 0.00 bc	68.63 ± 2.08 h
Apple mash 12	8.66 ± 0.04 bc	9.37 ± 0.05 bc	89.94 ± 3.14 a	3.24 ± 0.03 ab	7.46 ± 0.14 a	0.41 ± 0.04 a	5.40 ± 0.10 bc	87.84 ± 1.98 g

Data are expressed as mean ± standard deviation; n.a.: not analyzed. Values with different letters in the same column are significantly different according to Tukey's HSD test ($p < 0.05$).

5.5.2. Analyzed volatile compounds in the apple distillates (GC-FID)

The overall MANOVA resulted in highly significant differences among the samples (Wilk's lambda < 0.001, $p < 0.001$), which were detected in every compound ($F(11;24) > 11.37$ with $p < 0.001$). The concentrations of volatile aroma compounds identified and quantified in apple distillates together with the post hoc test results are presented in Table 17. The concentration of carbonyl compounds was strongly correlated with the type of nutrient used. Apple Spirits 7 to 12, which received Nutrient 2 supplementation, showed higher levels of acetaldehyde and benzaldehyde compared to Apple Spirits 1 to 6, which received Nutrient 1. The second dose of Nutrient 1 was added on the fourth day of fermentation, whereas samples treated with Nutrient 2 received their second dose on the second day. According to Beltran et al. (2005), the later the nitrogen addition, the lower the concentration of acetaldehyde. Additionally, the presence of *Lb. plantarum* (LB-1) during fermentation led to an increase in acetaldehyde content in apple spirits. However, benzaldehyde levels were generally higher in chemically acidified samples, except for Apple Spirit 9 and 10. The highest methanol content was detected in Apple Spirit 4, reaching 4484.26 mg/L a.a., while the lowest was found in Apple Spirit 10, with 3089.38 mg/L a.a.

The predominant alcohols in spirits are the linear-chain alcohols, including 1-propanol, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol (Stanzer et al., 2023). Regardless of the acidification technique used and yeast strain chosen for fermentation, a lower level of 1-propanol was observed in the Apple Spirits 7 to 12 treated with Nutrient 2 (ranging from 732.79 to 1857.96 mg/L a.a.) compared to the other alternative treatment. A significantly higher amount of 1-propanol was consistently produced by the hybrid yeast X-treme in all circumstances. However, the Nutrient 2 combination appears to stimulate a higher production of 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and cis-2-hexen-1-ol, compared to the Nutrient 1 combination. A significantly higher amount of 2-methyl-1-propanol was produced by *S. cerevisiae* (Uvaferm 228), irrespective of mash treatments. Likewise, *S. cerevisiae* (Uvaferm 228) produced the highest amounts of amyl alcohols, with the exception of Apple Spirit 5, where X-treme stood out among the samples that underwent biological acidification and were treated with Nutrient 1. The production of higher levels of these alcohols can be associated with the presence of specific amino acids in the nutrient treatment (Genesis Fresh), indicating their direct catabolic formation via the Ehrlich pathway (Barbosa et al., 2012).

1-Hexanol and 1-butanol constitute the second group of prevalent higher alcohols (Stanzer et al., 2023). The hybrid yeast X-treme produced the most 1-hexanol in Apple Spirits 2 and 5, where

Nutrient 1 was used as a supplement. On the other hand, when Nutrient 2 was added to the mash, *S. cerevisiae* (Uvaferm 228) produced the highest levels of 1-hexanol in Apple Spirits 7 and 10. The highest levels of phenethyl alcohol (rose-like aroma) were produced through the sequential inoculation of *L. thermotolerans* (Concerto) and *S. cerevisiae* (Uvaferm 228), particularly evident in Apple Spirit 6 (3.01 mg/L a.a.). This particular spirit had been acidified by *Lb. plantarum* (LB-1) and treated with inorganic nitrogen (Nutrient 1). The overall increase in phenethyl alcohol in mixed fermentations appeared to result from the synergistic interaction of these two yeast species (*L. thermotolerans* and *S. cerevisiae*), as in pure cultures, both of these yeasts are low phenethyl alcohol producers (Gobbi et al., 2013; Zhang et al., 2023).

Ethyl acetate was the dominant ester in all the samples, with its levels ranging from 268.41 mg/L a.a. in Apple Spirit 10 to 965.59 mg/L a.a. in Apple Spirit 9. The type of yeast strain used during fermentation significantly affected the production of this compound. Apple Spirits fermented with *S. cerevisiae* (Uvaferm 228) generated the highest amounts of ethyl acetate, whereas spirits fermented with the mixed culture of *L. thermotolerans* (Concerto) and *S. cerevisiae* (Uvaferm 228), as well as the hybrid strain X-treme, significantly reduced the levels of ethyl acetate. These findings are consistent with previous research conducted on hybrids and non-*Saccharomyces* yeasts. (Bellon et al., 2011; Blazquez Rojas et al., 2012; Dutraive et al., 2019; Zhang et al., 2023). The sequential fermentations, including *L. thermotolerans* (Concerto), produced elevated levels of ethyl propionate and propyl acetate in Apple Spirits 3, 6, 9, and 12, when compared to other yeast strains. The production of ethyl butyrate was favored by the addition of Nutrient 1.

The presence of lactic acid-producing strains in the mash promoted the synthesis of ethyl lactate. The co-fermentations involving *L. thermotolerans* (Concerto) and *Lb. plantarum* (LB-1) led to the highest levels of ethyl lactate in Apple Spirits 6 (1.18 mg/L a.a.) and 12 (0.74 mg/L a.a.). Previous studies have also reported elevated concentrations of ethyl lactate in co-fermentations of *Saccharomyces* with *L. thermotolerans* and/or *Lb. plantarum* (Gobbi et al., 2013; Morata et al., 2019; Hranilovic et al., 2021; Urbina et al., 2021). The hybrid yeasts were distinguished for the production of high levels of ethyl octanoate (4.17 mg/L a.a.) and butyl acetate (0.15 mg/L a.a.) in Apple Spirit 2; ethyl hexanoate (2.57 mg/L a.a.) and isoamyl acetate (11.44 mg/L a.a.) in Apple Spirit 5; and hexyl acetate (0.24 mg/L a.a.) in Apple Spirit 8. Ethyl myristate was only detected in Apple Spirit 4 and 7. Comparable amounts of 2-phenethyl acetate were detected in Apple Spirit 6 and 7, at 0.27 and 0.29 mg/L a.a., respectively.

Table 17. Aroma composition of apple spirits produced from different yeast strains under different mash treatments

Compounds (mg/L alcohol 100% v/v)	Apple Spirit 1	Apple Spirit 2	Apple Spirit 3	Apple Spirit 4	Apple Spirit 5	Apple Spirit 6	Apple Spirit 7	Apple Spirit 8	Apple Spirit 9	Apple Spirit 10	Apple Spirit 11	Apple Spirit 12
Acetaldehyde	19.17 ±0.24 ab	19.26 ±0.91 a	19.06 ±0.61 a	24.17 ±0.94 b	25.56 ±2.04 ab	25.68 ±1.41 ab	24.87 ±2.32 ab	24.63 ±1.50 ab	25.80 ±1.12 ab	26.84 ±1.68 ab	30.44 ±2.09 b	28.24 ±4.15 ab
Benzaldehyde	n.d.	1.44 ±0.10 c	1.02 ±0.09 b	0.30 ±0.00 a	1.03 ±0.49 abc	0.92 ±0.07 b	1.65 ±0.12 d	1.88 ±0.11 d	2.19 ±0.38 abcd	2.50 ±0.59 abcd	2.48 ±0.60 abcd	1.55 ±0.40 abcd
Methanol	3484.85 ±194.10 ab	3249.67 ±147.38 a	3134.37 ±137.63 a	4484.26 ±81.83 e	4098.86 ±104.62 cde	4313.42 ±207.74 de	3805.50 ±199.21 bc	3362.43 ±108.15 a	3277.24 ±90.60 a	3089.38 ±111.05 a	3992.61 ±100.07 cd	3352.57 ±156.05 a
1-Propanol	1370.33 ±55.41 d	2005.01 ±76.19 efg	1457.67 ±31.36 d	1657.17 ±24.10 ef	3683.96 ±49.60 h	2249.83 ±76.24 g	938.69 ±16.23 c	1394.03 ±93.67 cde	789.16 ±23.85 ab	862.09 ±22.12 b	1857.96 ±82.18 efg	732.79 ±10.78 a
1-Butanol	217.29 ±24.19 abc	203.75 ±7.90 a	186.48 ±4.91 a	257.12 ±4.95 b	406.13 ±16.36 e	283.37 ±3.30 c	348.71 ±29.51abcde	237.95 ±27.34abcd	308.33 ±12.80 bcd	359.34 ±10.82 de	288.11 ± 8.50 bc	266.31 ± 10.34 bc
1-Hexanol	26.80 ±1.85 ab	30.64 ±0.96 b	23.05 ±0.68 a	40.62 ±0.40 cd	103.54 ±1.88 g	44.22 ±2.74 c	103.06 ±10.21 fg	42.90 ±1.33 c	76.39 ±1.72 ef	62.01 ±4.55 de	52.99 ±7.19 bcde	46.47 ±2.41 cd
2-Methyl-1-propanol	1536.94 ±134.89 de	613.78 ±36.93 a	689.52 ±2.91 ab	905.92 ±12.19bcd	728.32 ±7.94 ab	902.96 ±56.60 bcd	2352.37 ±49.96 f	786.68 ±32.70 bc	1195.06 ±74.13 cd	2124.17 ±113.65 ef	740.81 ±20.07 ab	1292.23 ±88.36 d
3-Methyl-1-butanol	1397.02 ±17.48 d	853.53 ±26.21 a	879.55 ±8.35 a	989.16 ±4.42 bc	1336.48 ±56.87 d	977.29 ±15.52 b	3493.04 ±124.65 g	1296.55 ±52.21 cd	1915.62 ±35.98 e	2559.48 ±59.71 f	1347.23 ±47.25 d	1479.19 ±74.19 d
2-Methyl-1-butanol	373.73 ±13.88 fg	214.13 ±15.51 ab	177.16 ±13.07 a	260.62 ±4.66 bc	279.11 ±20.42 cd	247.02 ±7.36 bc	762.86 ±47.44 i	319.81 ±28.89 de	401.76 ±22.12 g	592.58 ±7.95 h	326.02 ±27.91 def	347.32 ±19.53 ef
Trans-3-hexen-1-ol	n.d.	0.16 ±0.05 ab	0.01 ±0.00 a	0.59 ±0.01 c	0.01 ±0.00 a	n.d.	n.d.	0.01 ±0.01 ab	0.05 ±0.00 b	n.d.	0.01 ±0.00 a	0.02 ±0.00 a
Cis-2-hexen-1-ol	0.25 ±0.01 a	0.31 ±0.00 b	0.21 ±0.10 abcd	0.25 ±0.05 abc	0.34 ±0.04 abc	0.31 ±0.04 abc	0.72 ±0.02 d	0.36 ±0.00 c	0.65 ±0.01 d	0.47 ±0.01 c	0.36 ±0.05 abc	0.40 ±0.01 cd
Benzyl alcohol	n.d.	n.d.	n.d.	n.d.	0.06 ±0.00 a	n.d.	n.d.	n.d.	n.d.	0.10 ±0.00 b	0.17 ±0.01 c	0.07 ±0.00 a
Phenethyl alcohol	0.69 ±0.02 b	0.79 ±0.01 b	1.30 ±0.17 bcdef	1.06 ±0.04 de	0.89 ±0.02 c	3.01 ±0.81 bcdef	0.07 ±0.01 a	0.94 ±0.10 bcdef	1.39 ±0.02 f	0.06 ±0.00 a	0.98 ±0.03 d	1.30 ±0.01 ef
Ethyl acetate	762.11 ±15.28 g	499.33 ±2.14 cd	506.82 ±18.98 d	543.99 ±21.20 de	365.79 ±10.21 b	532.82 ±14.04 de	965.59 ±20.81 i	443.71 ±29.98 c	574.16 ±25.72 e	886.77 ±24.89 h	268.41 ±5.34 a	656.89 ±30.72 f
Ethyl propionate	0.55 ±0.03 ef	0.43 ±0.03 d	0.65 ±0.05 f	0.22 ±0.07 a	0.20 ±0.01 a	0.47 ±0.01 de	0.22 ±0.06 a	0.21 ±0.04 a	0.33 ±0.02 bc	0.23 ±0.01 ab	0.23 ±0.01 ab	0.37 ±0.03 cd
Ethyl butyrate	0.38 ±0.02 e	0.18 ±0.01 cde	0.19 ±0.00 d	0.12 ±0.01 b	0.19 ±0.07 abcde	0.26 ±0.03 bcde	0.15 ±0.02 abcd	0.12 ±0.01 b	0.10 ±0.01 ab	0.10 ±0.00 ab	0.08 ±0.01 a	0.12 ±0.00 bc
Ethyl lactate	n.d.	0.15 ±0.01 b	0.20 ±0.00 c	n.d.	0.32 ±0.07abcde	1.18 ±0.14 f	n.d.	0.08 ±0.00 a	0.54 ±0.02 ef	n.d.	0.41 ±0.02 d	0.74 ±0.07 ef
Ethyl benzoate	n.d.	0.09 ±0.00 a	n.d.	n.d.	n.d.	n.d.	n.d.	0.11 ±0.02 ab	n.d.	n.d.	n.d.	0.13 ±0.00 b
Ethyl octanoate	0.91 ±0.07 a	4.17 ±0.04 h	0.92 ±0.01 a	1.23 ±0.07 bcd	1.34 ±0.08 cd	2.96 ±0.10 f	1.09 ±0.20 abc	4.00 ±0.12 gh	1.91 ±0.11 e	1.39 ±0.09 d	3.84 ±0.16 g	0.94 ±0.04 ab
Ethyl decanoate	1.06 ±0.05 a	2.29 ±0.21 bcde	1.25 ±0.05 ab	1.45 ±0.01 b	1.71 ±0.02 c	2.91 ±0.14 f	4.51 ±0.14 g	2.84 ±0.34abcdef	2.00 ±0.02 de	2.79 ±0.15 ef	1.49 ±0.19 abcd	1.03 ±0.00 a

Ethyl myristate	n.d.	n.d.	n.d.	0.43 ±0.01 b	n.d.	n.d.	0.13 ±0.02 a	n.d.	n.d.	n.d.	n.d.	n.d.
Ethyl formate	n.d.	0.20 ±0.00 c	0.10 ±0.01 ab	n.d.	0.12 ±0.01 b	0.06 ±0.01 a	n.d.	0.09 ±0.00 b	0.69 ±0.02 f	0.85 ±0.01 g	0.27 ±0.00 d	0.55 ±0.03 e
Ethyl hexanoate	0.94 ±0.09 d	1.10 ±0.01 de	0.37 ±0.03 a	0.77 ±0.03 c	2.57 ±0.05 i	1.02 ±0.09 fg	0.95 ±0.05 d	1.28 ±0.03 ef	0.59 ±0.01 b	1.91 ±0.10 h	1.51 ±0.13 g	0.56 ±0.02 b
Diethyl succinate	n.d.	n.d.	n.d.	n.d.	0.23 ±0.02 b	n.d.	0.25 ±0.02 b	n.d.	0.16 ±0.01 a	0.21 ±0.02 b	n.d.	n.d.
Isoamyl acetate	5.08 ±0.57 b	2.29 ±0.56 a	8.86 ±0.15 e	9.58 ±0.43 e	11.44 ±0.50 f	6.42 ±0.67 bcd	6.68 ±0.32 cd	5.32 ±0.80 bc	5.36 ±0.54 bc	5.45 ±0.46 bc	5.60 ±0.09 bc	7.15 ±0.55 d
Propyl acetate	0.11 ±0.01 de	0.08 ±0.01 abc	0.11 ±0.02abcde	0.04 ±0.00 a	0.09 ±0.00 cd	0.15 ±0.00 e	0.05 ±0.00 ab	0.04 ±0.01 ab	0.09 ±0.01 cd	0.04 ±0.00 a	0.06 ±0.00 b	0.08 ±0.01 abcd
Isobutyl acetate	0.03 ±0.00 fg	0.01 ±0.00 e	0.02 ±0.00 f	0.01 ±0.00 d	0.01 ±0.00 e	0.03 ±0.01 g	0.01 ±0.00 d	0.005 ±0.00 c	0.005 ±0.00 c	0.01 ±0.00 cd	0.003 ±0.00 b	0.002 ±0.00 a
Butyl acetate	0.14 ±0.01 e	0.15 ±0.02 cde	0.07 ±0.00 d	0.08 ±0.00 d	0.03 ±0.00 b	0.004 ±0.00 a	0.14 ±0.02 bcde	0.11 ±0.00 e	0.04 ±0.00 c	0.03 ±0.00 b	n.d.	0.11 ±0.00 e
Hexyl acetate	0.15 ±0.01 b	0.20 ±0.05 abcd	n.d.	0.02 ±0.00 a	0.21 ±0.01 cd	n.d.	0.08 ±0.01 a	0.24 ±0.02 d	n.d.	0.04 ±0.01 a	0.13 ±0.03 abc	n.d.
2-Phenethyl acetate	n.d.	n.d.	n.d.	n.d.	0.14 ±0.00 a	0.27 ±0.05 b	0.29 ±0.01 b	n.d.	n.d.	n.d.	n.d.	n.d.
Phenylacetic acid	n.d.	0.26 ±0.15 c	n.d.	0.06 ±0.01 a	0.18 ±0.01 b	0.07 ±0.00 a	0.88 ±0.02 f	0.31 ±0.00 d	0.85 ±0.02 f	0.63 ±0.02 e	0.28 ±0.02 cd	0.96 ±0.01 g
Limonene	0.07 ±0.01 d	0.04 ±0.00 c	n.d.	0.08 ±0.00 d	0.06 ±0.01 cd	0.07 ±0.01 d	0.06 ±0.01 bcd	0.01 ±0.00 a	0.01 ±0.00 a	0.08 ±0.02 abcd	n.d.	0.02 ±0.00 b
Myrcene	0.03 ±0.00 ab	0.04 ±0.00 bc	0.05 ±0.01 bcde	0.04 ±0.01abcde	0.03 ±0.00 a	0.07 ±0.01 de	0.07 ±0.00 e	0.04 ±0.00 bc	0.06 ±0.00 d	0.06 ±0.00 de	0.05 ±0.01abcde	0.05 ±0.00 c
Linalool	n.d.	n.d.	0.22 ±0.09 ab	0.13 ±0.00 a	n.d.	0.70 ±0.02 c	n.d.	n.d.	n.d.	0.45 ±0.04 b	n.d.	1.84 ±0.05 d

Data are expressed as mean ± standard deviation; n.d.: not detected. Values with different letters in the same row are significantly different ($p < 0.05$) (Tukey's post hoc test or Games-Howell's post hoc test).

Spirits contained a trace amount of terpenes. Linalool levels were higher in the samples fermented by mixed cultures. The same tendency was observed in the study of Zhang et al. (2023). The highest contents of limonene were noted in apple spirits obtained from *S. cerevisiae* (Uvaferm 228). Comparable myrcene levels in apple spirits were reported in the literature (Januszek et al., 2020b).

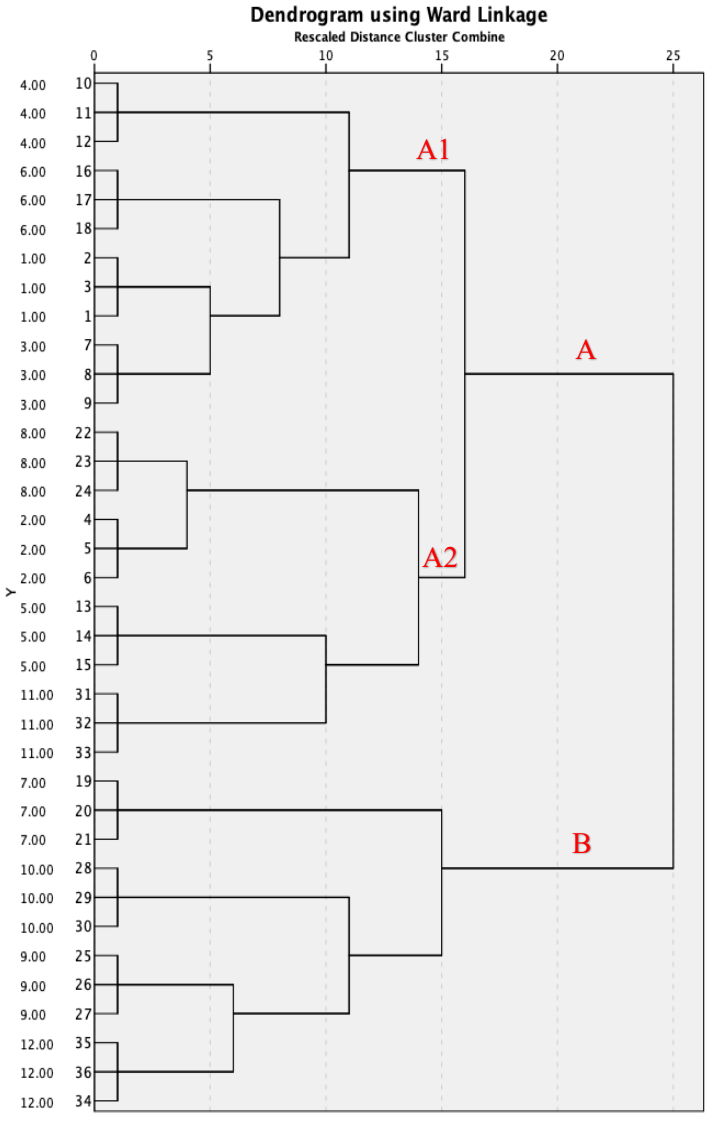


Figure 17. Dendrogram from Hierarchical Cluster Analysis (HCA) on the similarity of volatile profiles of obtained spirits using Ward linkage method

The dendrogram of the HCA (Figure 17) shows the tendency of the samples to gather in clusters based on their similarities in the volatile composition. In the dendrogram, two major clusters can be identified. The first cluster (A) is further divided into two sub-clusters. The first sub-cluster (A1)

consists of the spirits fermented with pure and mixed cultures of *S. cerevisiae* (Uvaferm 228) and *L. thermotolerans* (Concerto), all of which were treated with Nutrient 1. The second sub-cluster (A2) is composed of all the spirits fermented with the hybrid yeast X-treme, regardless of the treatment received. Whereas, the last cluster (B) encompasses the spirits fermented with pure and mixed cultures of *S. cerevisiae* (Uvaferm 228) and *L. thermotolerans* (Concerto) that were treated with Nutrient 2. The volatile profiles of the spirits within one cluster are similar. With increasing Euclidean distance, the similarity decreases. These clustering patterns indicate that the yeast strain dictates the volatile profile of the final product. This statement is especially valid for hybrid yeast. Nevertheless, different nutrient treatments added to the mash can result in entirely distinct spirits, even when fermented by the same yeast, as seen in clusters A1 and B.

5.5.3. The evolution of volatile aroma compounds during the production process of apple spirits (GC-MS)

The overall MANOVA resulted in highly significant differences among the samples (Wilk's lambda < 0.001, $p < 0.001$), which were detected in all 75 compounds ($p < 0.001$), and also compared the samples at different stages of the fermentation process ($p < 0.001$). Figure 18 illustrates the evolution of 75 volatile compounds during the apple spirit production process. These compounds fall into five chemical classes: alcohols, esters, acids, aldehydes, and acetals (Table 18). This monitoring analysis allows us to assess how the aroma profile transforms from the raw material, through fermentation, and ultimately during distillation.

The volatile composition of apples (primary aroma) is influenced by various factors, including the apple cultivar, cultural practices, climate conditions, and fruit maturity (Dixon & Hewett, 2000). The apples used as the raw material in this study were characterized by the presence of various volatiles, including 4 aldehydes, 4 acids, 8 alcohols, and 33 esters. These compounds have all been identified in the literature among different apple varieties (Ferreira et al., 2009; Wu et al., 2022; Pontesegger et al., 2023). The dominant volatiles in the analyzed apples included 1-hexanol, 2-hexen-1-ol, 1-octanol, hexanal, 2-hexenal, benzaldehyde, butyl acetate, butyl hexanoate, butyl octanoate, propyl acetate, hexyl acetate, hexyl hexanoate, and 2-methylbutyl acetate. Certain alcohols and carbonyl compounds, specifically 1-hexanol, 2-hexenal, and 2-hexen-1-ol, contribute to the herbaceous aroma of various fruits (Ferreira et al., 2009), while esters are responsible for the characteristic fruity and floral aromas (Plotto et al., 1999). Studies have demonstrated that in apples from the Gala cultivar, the esters 2-methylbutyl acetate, hexyl acetate, and butyl acetate have the highest impact on aroma and flavor (Plotto et al., 2000; Both et al., 2014).

Table 18. List of volatile compounds detected by GC-MS. ID: identification percentage according to NIST software, t_R: retention time, RI: retention index.

Compound name	CAS number	ID (%)	t_R (min)	Kovats RI (literature)	Kovats RI (measured)	RI difference
Methanol	67-56-1	76.16	0.87	381	n.c.	-
Ethanol	64-17-5	95.00	0.92	448	n.c.	-
1-Propanol	71-23-8	86.18	1.07	548	n.c.	-
Acetic acid	64-19-7	85.74	1.11	602	604.85	2.85
Ethyl acetate	141-78-6	94.91	1.20	628	626.54	1.46
2-methyl-1-propanol	78-83-1	91.42	1.25	626	636.09	10.09
1-Butanol	71-36-3	92.80	1.39	676	668.67	7.33
Ethyl propanoate	105-37-3	79.58	1.75	714	720.42	6.42
Propyl acetate	109-60-4	75.19	1.80	715	725.38	10.38
Acetal	105-57-7	96.81	1.84	725	729.37	4.37
3-Methyl-1-butanol	123-51-3	97.06	1.90	730	734.26	4.26
2-Methyl-1-butanol	137-32-6	96.22	1.95	743	739.50	3.50
Isobutyl acetate	110-19-0	79.24	2.49	781	790.68	9.68
Hexanal	66-25-1	80.32	2.74	803	806.22	3.22
Ethyl butanoate	105-54-4	82.02	2.72	805	805.58	0.58
Butyl acetate	123-86-4	90.39	2.95	814	814.63	0.63
2-Hexenal	505-57-7	92.83	3.74	847	846.18	0.82
Ethyl 2-methylbutanoate	7452-79-1	79.69	3.80	849	848.92	0.08
2-Hexen-1-ol	928-95-0	78.90	4.09	865	860.52	4.48
1-Hexanol	111-27-3	96.87	4.18	867	864.10	2.90
3-Methylbutyl acetate	123-92-2	94.19	4.43	876	874.19	1.81
2-Methylbutyl acetate	624-41-9	88.27	4.44	878	874.31	3.69
Benzaldehyde	100-52-7	82.98	7.92	961	949.50	11.50
Ethyl hexanoate	123-66-0	97.62	11.01	998	1004.69	6.69
Hexyl acetate	142-92-7	88.10	11.78	1017	1024.02	7.02
Butyl 2-methylbutanoate	15706-73-7	80.78	13.01	1041	1054.87	13.87
1-Octanol	111-87-5	84.72	14.02	1068	1080.17	12.17
Ethyl heptanoate	106-30-9	80.72	14.83	1097	1100.70	3.70
Nonanal	124-19-6	87.76	14.93	1105	1105.17	0.17
Heptyl acetate	112-06-1	82.62	15.21	1112	1117.77	5.77
Phenethyl alcohol	60-12-8	96.27	15.08	1114	1112.15	1.85
Methyl octanoate	111-11-5	82.71	15.47	1128	1129.65	1.65
Hexyl isobutanoate	2349-07-7	81.43	16.02	1152	1154.79	2.79
Isobutyl hexanoate	105-79-3	79.45	16.06	1156	1156.66	0.66
Ethyl benzoate	93-89-0	76.04	16.41	1170	1172.46	2.46
1-Nonanol	143-08-8	78.06	16.50	1176	1176.56	0.56
Butyl hexanoate	626-82-4	91.44	16.88	1189	1193.53	4.53
Hexyl butanoate	2639-63-6	87.97	16.90	1191	1194.49	3.49
Octanoic acid	124-07-2	90.54	16.72	1191	1186.17	4.83

Ethyl octanoate	106-32-1	96.67	17.05	1201	1201.35	0.35
Octyl acetate	112-14-1	93.20	17.28	1215	1215.13	0.13
Hexyl 2-methylbutanoate	10032-15-2	94.86	17.71	1239	1240.33	1.33
Ethyl phenylacetate	101-97-3	75.93	17.83	1252	1247.45	4.55
Isopentyl hexanoate	2198-61-0	89.53	17.92	1254	1253.14	0.86
2-Phenethyl acetate	103-45-7	82.57	18.03	1255	1259.10	4.10
Propyl octanoate	624-13-5	88.15	18.61	1295	1293.80	1.20
Ethyl nonanoate	123-29-5	88.71	18.68	1298	1297.58	0.42
Nonyl acetate	143-13-5	87.63	18.90	1309	1312.45	3.45
Methyl decanoate	110-42-9	92.15	19.10	1328	1326.19	1.81
Isobutyl caprylate	5461-06-3	90.27	19.45	1348	1350.14	2.14
Decanoic acid	334-48-5	87.58	19.76	1373	1371.65	1.35
Hexyl hexanoate	6378-65-0	83.12	20.00	1384	1387.95	3.95
Ethyl 9-decenoate	67233-91-4	85.15	20.11	1389	1395.31	6.31
Octanoic acid, 2-butyl ester	5458-61-7	80.71	20.01	1390	1388.80	1.20
Ethyl decanoate	110-38-3	96.56	20.17	1398	1399.34	1.34
Isopentyl octanoate	2035-99-6	84.94	20.82	1448	1448.70	0.70
Propyl decanoate	30673-60-0	81.70	21.39	1493	1491.65	1.35
Octyl decanoate	2306-92-5	84.32	21.39	n.d.	1491.89	-
Methyl dodecanoate	111-82-0	83.07	21.80	1527	1526.61	0.39
Isobutyl decanoate	30673-38-2	80.94	22.05	1545	1548.43	3.43
Dodecanoic acid	143-07-7	82.03	22.22	1565	1563.13	1.87
Hexyl octanoate	1117-55-1	84.97	22.45	1585	1584.02	0.98
Butyl decanoate	30673-36-0	89.68	22.50	1590	1587.85	2.15
Ethyl dodecanoate	106-33-2	95.37	22.59	1597	1596.19	0.81
Isopentyl decanoate	2306-91-4	83.27	23.09	1646	1647.19	1.19
Hexyl decanoate	10448-26-7	82.09	24.29	1784	1781.66	2.34
Butyl laurate	106-18-3	81.94	24.34	n.d.	1787.47	-
Isobutyl laurate	37811-72-6	81.14	24.34	n.d.	1786.52	-
Ethyl myristate	124-06-1	89.07	24.41	1795	1794.73	0.27
Isopropyl myristate	110-27-0	86.22	24.66	1827	1826.86	0.14
2-Phenethyl octanoate	5457-70-5	85.44	24.87	1842	1854.24	12.24
Hexadecanoic acid	57-10-3	76.97	25.64	1964	1956.55	7.45
Ethyl-E-11-hexadecanoate	n.d.	80.47	25.76	1974	1973.44	0.56
Ethyl palmitate	628-97-7	93.16	25.92	1994	1994.14	0.14
Ethyl stearate	111-61-5	86.01	27.87	2192	n.c.	-

n.d.: no data found in NIST libraries or literature. n.c.: not calculated

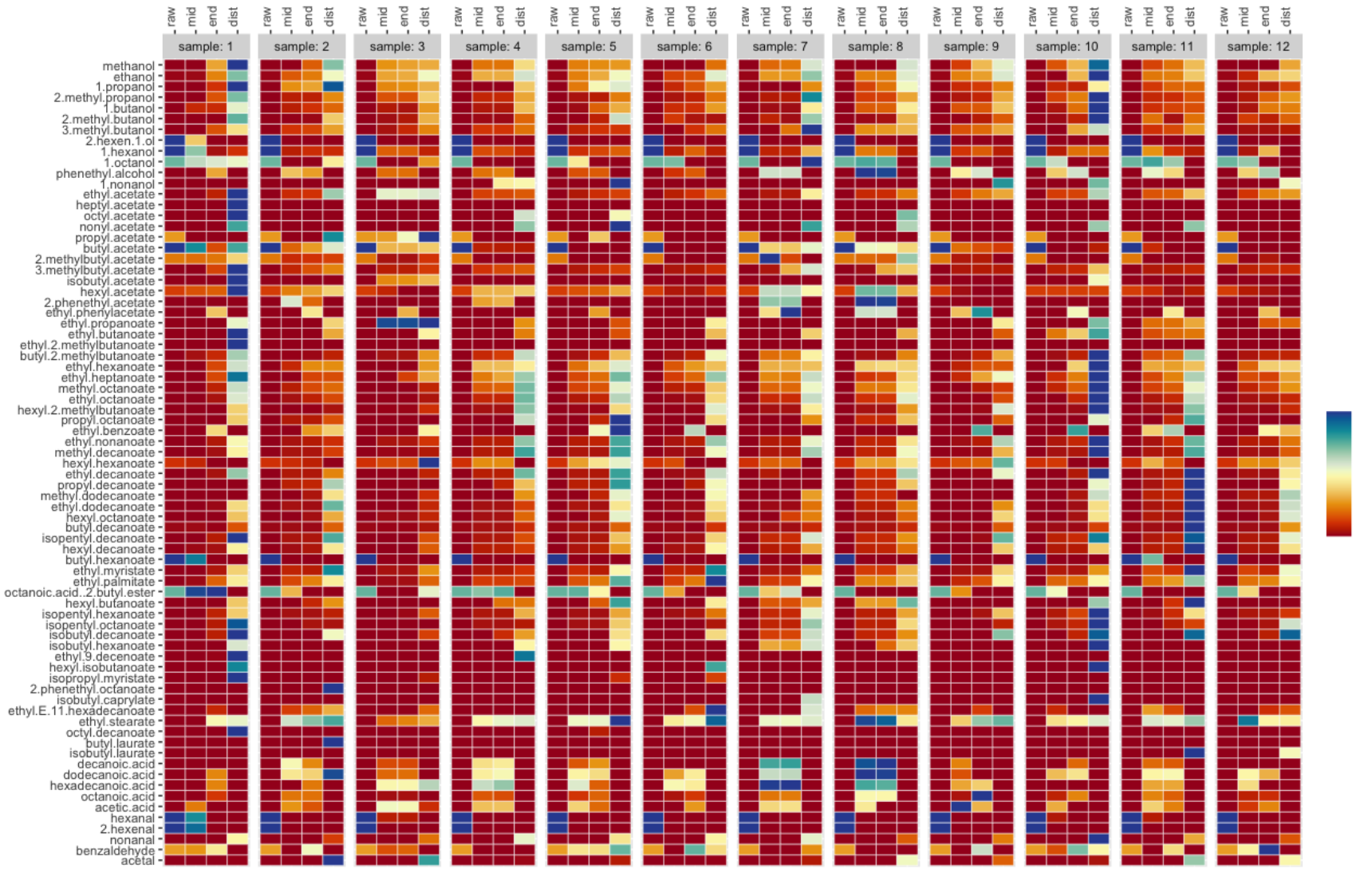


Figure 18. Heatmap visualization of the dynamic change in the concentration of the detected 75 volatile compounds during apple spirit production process. Each row on the heatmap represents the transformed concentration of an individual volatile compound (three replicates). Each column represents a particular phase during the production (raw: raw material; mid: middle of fermentation; end: end of fermentation; dist: the distillate). The colors from red to blue represent the concentrations from low to high for each volatile.

The volatile bouquet undergoes several transformations during the production process. During fermentation, the secondary (fermentative) aroma arises due to new compounds formed by the yeast metabolism. These molecules are produced from substrates such as sugars, proteins, and lipids (Ascrizzi et al., 2022). One of the main groups of compounds produced by yeast are higher alcohols (Cioch-Skoneczny et al., 2021). They are synthesized during fermentation from oxo-acids having their origin in amino acids and sugar metabolism (Mangas et al., 1994). 1-propanol, 2-methyl propanol, and 3-methyl butanol are newly formed during fermentation. 1-butanol and 2-methyl butanol were already present in the raw material; however, their levels increased steadily during fermentation as a result of yeast activity. According to the literature, the production of 2-methyl propanol, 2-methyl butanol, 3-methyl butanol, and 1-propanol is related to the presence of valine, isoleucine, leucine, and threonine in the fermentation medium (Reazin et al., 1973; Lambrechts & Pretorius, 2000). On the other hand, 1-butanol is considered a primary aroma compound in apples, and its concentration is influenced by the apple cultivar and its level of maturity, rather than the processing technology (Vidrih & Hribar, 1999). The production of higher alcohols was influenced by the yeast strain and mash treatments. In general, a more intensive production of higher alcohols was observed in samples 1, 7, and 10, all of which were fermented by *S. cerevisiae* (Uvaferm 228). The involvement of non-*Saccharomyces* yeasts, respectively *L. thermotolerans* (Concerto), resulted in the formation of lower concentrations of higher alcohols (2-methyl propanol and amyl alcohols) compared to the *S. cerevisiae* strain. These results align with previous studies (Balikci et al., 2016). Nevertheless, it's worth noting that there was a decrease in 1-hexanol levels throughout the fermentation in all samples. 1-Hexanol is not a fermentation product but originates from linolenic acid found in plants (Satora et al., 2016). Phenethyl alcohol levels, on the other hand, were notably higher during the second part of fermentation. However, these levels decreased during distillation, likely due to esterification, to form 2-phenylethylacetate in conjunction with acetyl-CoA through the action of alcohol acetyltransferase (Yoshioka & Hashimoto, 1981). Methanol was formed during fermentation as a result of its liberation from pectin by means of pectin methyl esterase (Vidrih & Hribar, 1999).

Fatty acids such as octanoic acid, decanoic acid, dodecanoic acid, and hexadecanoic acid were present at the highest levels in samples 8 and 7. These particular samples were treated with Nutrient 2 and acidified using organic acids. Fatty acids and their ethyl esters (e.g., ethyl octanoate, ethyl decanoate, ethyl dodecanoate, and ethyl hexadecanoate) have been experimentally confirmed to be produced during fermentation (Bardi et al., 1999; Diaz-Maroto et al., 2005). As depicted in Figure 18, the concentration of these ethyl esters of fatty acids rose slightly during fermentation, but a more

pronounced increase was evident after distillation. On the other hand, medium- and long-chain fatty acids were not detected in the distillates because they typically appear at the end of distillation and are collected in the tail fraction (Xiang et al., 2020).

Various esters were formed during fermentation, and the analyzed samples were characterized by unique ester profiles. Their production was significantly influenced by the yeast strain. A total of 19 esters were present in all distillates, but varying in concentration. Among them, ethyl acetate, isoamyl acetate, isobutyl decanoate, and isopentyl decanoate were found in the highest concentration in distillate 1. Distillate 10 stood out with the highest levels of hexyl 2-methylbutanoate, butyl 2-methylbutanoate, ethyl hexanoate, methyl octanoate, ethyl octanoate, ethyl nonanoate, methyl decanoate, ethyl heptanoate, and isopentyl hexanoate. The formation of metabolites by *S. cerevisiae* (Uvaferm 228) in distillates 1 and 10 was significantly influenced by nutrient supplementation and acidification method used. Distillate 11 contained the most ethyl dodecanoate, hexyl octanoate, butyl decanoate, hexyl decanoate, and ethyl myristate. Ethyl stearate and ethyl palmitate were most abundant in distillates 5 and 6.

Several volatiles, including acetal, nonanal, 1-nonanol, ethyl 2-methylbutanoate, heptyl acetate, hexyl isobutanoate, octyl acetate, nonyl acetate, isopropyl myristate, 2-phenethyl octanoate, isobutyl caprylate, butyl laurate, isobutyl laurate, and octyl decanoate, were exclusively identified in the distillates. Esterification reactions may occur throughout the distillation process and could explain the increase of esters observed (Awad et al., 2017).

5.5.3.1. Principal Component Analysis

Principal component analysis (PCA) was employed to assess the contribution of volatile compounds to the overall aroma profiles of the 12 apple spirits. Figure 19 displays biplots for the first two principal components at three distinct stages of the production process: mid-fermentation, end-fermentation, and distillation. The plots clearly demonstrate differentiation among the 12 samples throughout the production process.

PCA analysis (Figure 19A) revealed that during mid-fermentation, the first two components explained 53.9% of the total variance, with contributions of 40.4% and 13.5%, respectively. PC1 was negatively correlated with dodecanoic acid, hexyl hexanoate, heptyl acetate, octanoic acid, hexyl 2-methylbutanoate, ethyl heptanoate, octyl decanoate, phenethyl alcohol, hexyl decanoate, phenethyl alcohol, hexyl decanoate, ethyl myristate, hexyl octanoate, methyl decanoate, propyl decanoate, decanoic acid, and isobutyl decanoate. PC2 was positively correlated with butyl acetate, hexyl acetate,

benzaldehyde, hexanal, 1-hexanol, 2-methylbutyl acetate, and 2-phenethyl acetate, while it was negatively correlated with 3-methylbutyl acetate, 3-methyl butanol, ethyl butanoate, and 1-propanol.

At the end of fermentation (Figure 19B), the two principal components explained 49.2% of the overall variance in the samples. PC1, responsible for 35% of the variance, was negatively correlated with methyl decanoate, methyl octanoate, ethyl octanoate, hexyl decanoate, dodecanoic acid, isopentyl decanoate, butyl decanoate, ethyl hexanoate, hexyl 2-methylbutanoate, and ethyl dodecanoate. On the other hand, PC2, responsible for 14.2% of the variation, was positively correlated with hexadecanoic acid, isobutyl hexanoate, 2-phenethyl acetate, butyl acetate, and hexyl acetate, while it was negatively correlated with ethyl benzoate, benzaldehyde, 1-butanol, 2-methyl-1-butanol, ethyl nonanoate, and ethyl palmitate.

The two principal components explained 50.2% of the total variation (32.2% and 18%, respectively) in the distillates obtained (Figure 19C). PC1 was negatively correlated with ethyl heptanoate, methyl octanoate, butyl 2-methylbutanoate, ethyl hexanoate, ethyl octanoate, nonanal, isopentyl hexanoate, isobutyl hexanoate, 2-methyl butanol, 1-butanol, 2-methyl propanol, and isopentyl octanoate. PC2 was positively correlated with butyl acetate, 3-methylbutyl acetate, and isobutyl acetate and negatively correlated with methyl dodecanoate, propyl decanoate, hexyl octanoate, and isobutyl laurate. The apple spirits are characterized by a wealth of chemical compounds, which distinguish them from one another.

(A) Mid-fermentation (B) End-fermentation (C) Distillation

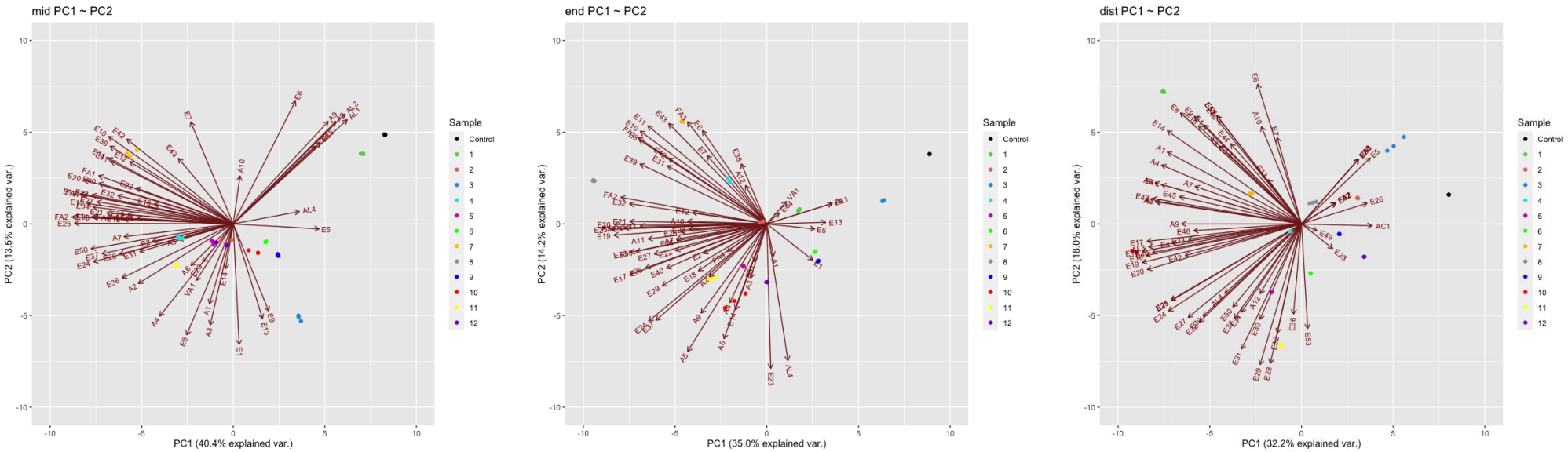
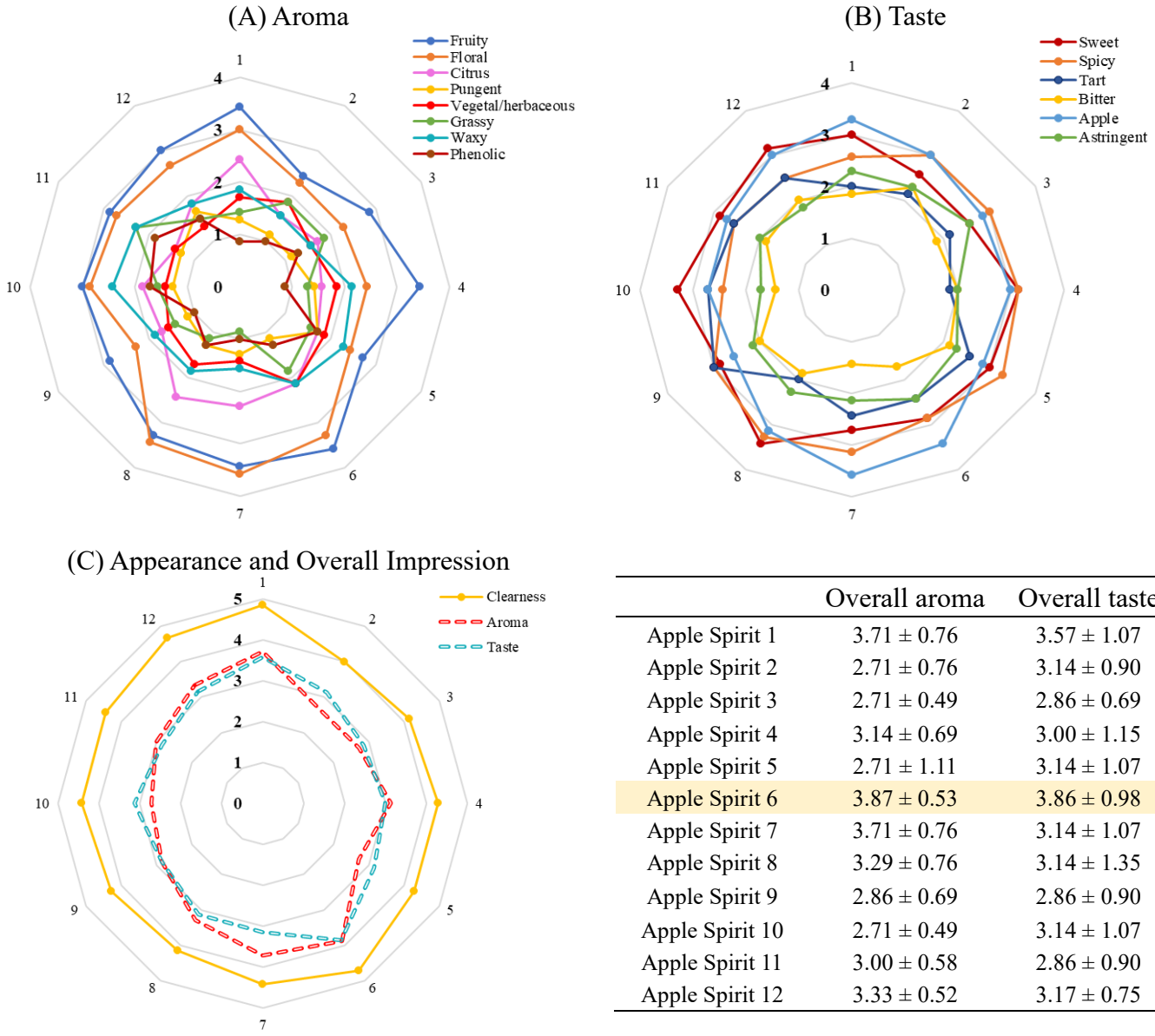


Figure 19. Principal Component Analysis (PCA) biplots for the observed variables (volatile compounds) during the production of apple spirits. Brown arrows represent the different compounds and points of different colors represent the apple spirit samples. A1: methanol, A2: ethanol, A3: 1-propanol, A4: 2-methyl-1-propanol, A5: 1-butanol, A6: 2-methyl-1-butanol, A7: 3-methyl-1-butanol, A8: 2-hexen-1-ol, A9: 1-hexanol, A10: 1-octanol, A11: phenethyl alcohol, A12: 1-nonanol, FA1: decanoic acid, FA2: dodecanoic acid, FA3: hexadecanoic acid, FA4: octanoic acid, VA1: acetic acid, AL1: hexanal, AL2: 2-hexenal, AL3: nonanal, AL4: benzaldehyde, AC1: acetal, E1: ethyl acetate, E2: heptyl acetate, E3: octyl acetate, E4: nonyl acetate, E5: propyl acetate, E6: butyl acetate, E7: 2-methylbutyl acetate, E8: 3-methylbutyl acetate, E9: isobutyl acetate, E10: hexyl acetate, E11: 2-phenethyl acetate, E12: ethyl phenylacetate, E13: ethyl propanoate, E14: ethyl butanoate, E15: ethyl 2-methylbutanoate, E16: butyl 2-methylbutanoate, E17: ethyl hexanoate, E18: ethyl heptanoate, E19: methyl octanoate, E20: ethyl octanoate, E21: hexyl 2-methylbutanoate, E22: propyl octanoate, E23: ethyl benzoate, E24: ethyl nonanoate, E25: methyl decanoate, E26: hexyl hexanoate, E27: ethyl decanoate, E28: propyl decanoate, E29: methyl dodecanoate, E30: ethyl dodecanoate, E31: hexyl octanoate, E32: butyl decanoate, E33: isopentyl decanoate, E34: hexyl decanoate, E35: butyl hexanoate, E36: ethyl myristate, E37: ethyl palmitate, E38: octanoic acid, 2-butyl ester, E39: hexyl butanoate, E40: isopentyl hexanoate, E41: isopentyl octanoate, E42: isobutyl decanoate, E43: isobutyl hexanoate, E44: ethyl 9-decenoate, E45: hexyl isobutanoate, E46: isopropyl myristate, E47: 2-phenethyl octanoate, E48: isobutyl caprylate, E49: ethyl E-11-hexadecanoate, E50: ethyl stearate, E51: octyl decanoate, E52: butyl laurate, E53: isobutyl laurate.

5.5.4. Sensory analysis of apple spirits

The results of the sensory evaluations are visualized in Figure 20. The highest intensity for the aroma descriptors ‘fruity’ and ‘vegetal’ was noted in Spirit 6. Spirits 6, 7, and 8 had the most pronounced ‘floral’ aroma. Spirits 1 and 8 scored highest in the ‘citrus’ note, while Spirit 11 had the highest score for the attributes ‘grassy’ and ‘phenolic’. ‘Waxy’ notes were perceived in Spirit 10.



	Overall aroma	Overall taste
Apple Spirit 1	3.71 ± 0.76	3.57 ± 1.07
Apple Spirit 2	2.71 ± 0.76	3.14 ± 0.90
Apple Spirit 3	2.71 ± 0.49	2.86 ± 0.69
Apple Spirit 4	3.14 ± 0.69	3.00 ± 1.15
Apple Spirit 5	2.71 ± 1.11	3.14 ± 1.07
Apple Spirit 6	3.87 ± 0.53	3.86 ± 0.98
Apple Spirit 7	3.71 ± 0.76	3.14 ± 1.07
Apple Spirit 8	3.29 ± 0.76	3.14 ± 1.35
Apple Spirit 9	2.86 ± 0.69	2.86 ± 0.90
Apple Spirit 10	2.71 ± 0.49	3.14 ± 1.07
Apple Spirit 11	3.00 ± 0.58	2.86 ± 0.90
Apple Spirit 12	3.33 ± 0.52	3.17 ± 0.75

Figure 20. Sensory evaluation of the apple spirits fermented by different yeast strains under different mash treatments. Data were standardized to make them comparable.

The taste descriptors with the highest perceptibility were ‘sweet’ and ‘apple’, particularly pronounced in Spirit 8 and 7, respectively. In general, the spirits were described as moderate in terms of ‘spicy’ and ‘tart’ tastes, and weak in terms of ‘bitterness’ and ‘astringency’. All the spirits were considered to have high clarity, with spirits 1, 6, and 12 receiving the highest scores. Overall, the aroma and taste of Spirit 6 received high praise, with the best performance, earning total scores of 3.87 and 3.86, respectively.

Spirit 6 is characterized by the presence of numerous esters, including ethyl decanoate, propyl decanote, ethyl butanoate, butyl 2-methyl butanoate, hexyl 2-methyl butanoate, ethyl myristate, ethyl palmitate, ethyl stearate, ethyl hexadecanoate, hexyl isobutanoate, ethyl hexanoate, and ethyl nonanoate, which contribute to ‘fruity’ and ‘floral’ notes. It's worth noting that having higher esters concentrations, as experienced in spirits 10, 11, 1, and 5 (Figure 18), doesn't necessarily translate to better flavor, as excessive amounts can mask their positive nuances and impart ‘waxy’ and other undesirable notes. Compared to other samples, Spirit 6 exhibited lower concentrations of higher alcohols and ethyl acetate, thereby avoiding the harsh and pungent notes associated with elevated levels of these compounds. The descriptor ‘apple’ can be correlated with the presence of esters like 2-methylbutyl acetate, hexyl acetate, and butyl acetate (Plotto et al., 2000; Both et al., 2014). The ‘vegetal’ and ‘grassy’ nuances are attributed to alcohols with six carbon atoms (Lukić et al., 2012), which are also perceived in Spirit 6. Ultimately, the pleasant aroma and taste of Spirit 6 result from a complex combination of optimal volatile concentrations.

6. CONCLUSIONS AND RECOMMENDATIONS

In recent years, research efforts around the world have been aligned to improve the quality of alcoholic beverages, including wine, beer, and fruit spirits. The production of high-quality fruit spirits relies on the successful completion of alcoholic fermentation. This work presents promising approaches that ultimately lead to an improved fermentation process and enhanced aroma complexity.

The results confirm that the acidification of the mash has a significant effect on the fermentation performance of *S. cerevisiae* (Uvaferm 228) and the resulting fermentation bouquet. Administering phosphoric and lactic acids in a 70:30 ratio has yielded the most favorable enological and aromatic outcomes among chemically acidified samples. Inoculating the mash with *L. thermotolerans* (Laktia) and *Lb. plantarum* strains (LB-1 and Sour Pitch) has been effective in achieving proper mash acidification, primarily through the production of lactic acid. These strains exhibit bioregulatory effects, as *L. thermotolerans* (Laktia) reduced the pH of the mash by 0.4 units, while *Lb plantarum* strains, LB-1 by 0.38 units, and Sour Pitch by 0.29 units. Moreover, these strains, in sequential inoculation with *S. cerevisiae* (Uvaferm 228), produced apple distillates with unique aroma profiles. *L. thermotolerans* (Laktia) promoted the formation of higher alcohols and esters. *Lb. plantarum* strains reduced the levels of higher alcohols and ethyl acetate and contributed to higher amounts of esters. In particular, *Lb. plantarum* (LB-1) produced higher levels of diethyl succinate and ethyl myristate, whereas *Lb. plantarum* (Sour Pitch) produced higher levels of ethyl butyrate, ethyl octanoate, and isobutyl acetate. All three strains were characterized by the production of ethyl lactate. These features make the utilization of *L. thermotolerans* and *Lb. plantarum* in spirit production of technological interest. Further studies, involving various LAB strains and fruit types, are needed to expand our understanding of their metabolism. Additionally, fermentation trials under industrial conditions will be essential.

The most common reason for poor fermentative performance is an imbalance in the mash nutrients. It was found that the supplementation of the fermentation medium with different nutrients promoted *S. cerevisiae* (Uvaferm 228) cell growth and fermentation kinetics. However, the growth profiles of *S. cerevisiae* (Uvaferm 228) in different nutritional media were not consistent, and the formation of secondary metabolites was influenced by the type of nutrient source available. Of particular practical relevance is the fact that the addition of any form of nutrient to the medium, did not affect ethanol and volatile acid production. However, these supplements induced specific changes in volatile composition, including increased concentrations of higher alcohols such as 1-propanol, 2-methyl-1-propanol, and phenethyl alcohol, as well as various esters that improve the fruity intensity

of the spirit. Conversely, isoamyl acetate, ethyl octanoate, ethyl decanoate, and diethyl succinate displayed a negative correlation with nutrient supplementation. This study serves as a starting point for further investigation into the use of different nutrient mixtures as supplements in the fruit spirit industry and their impact on yeast physiology, fermentation performance, and product quality.

The findings in this thesis suggest that, in addition to *S. cerevisiae* strains, other yeasts, including non-*Saccharomyces* and hybrid strains, are promising alternatives for use as fermentation agents in the production of fruit distillates. The use of hybrid yeasts resulted in a satisfactory conversion of fermentable sugars into ethanol, although the ethanol content produced was slightly lower than that of *S. cerevisiae* (Uvaferm 228). Each hybrid strain displayed its own unique fermentation pattern, resulting in distinctive aroma profiles for each apple spirit. The hybrids were characterized by lower production of higher alcohols and a greater diversity of esters. The hybrid strain X-treme demonstrated the highest fermentation ability and supported the production of a number of esters that give the distillate positive characteristics, including ethyl butyrate, ethyl octanoate, ethyl myristate, ethyl hexanoate, propyl acetate, butyl acetate, and hexyl acetate. These hybrid strains, can serve as a new tool to introduce flavor and aroma diversity to fruit spirits.

Analyzed byproduct concentrations, efficient sugar utilization, and reduced volatile acidity confirm that non-*Saccharomyces* yeasts in sequential fermentation with *S. cerevisiae* bring suitable enological characteristics to fruit distillates. The increase in ester and higher alcohol content demonstrates that non-*Saccharomyces* yeasts distinctly modulate the concentrations of specific fermentative volatiles, highlighting fruity and floral traits in the distillate. These findings are further supported by sensory analysis results, where panelists largely preferred the distillates produced by *L. thermotolerans* (Concerto) and *T. delbrueckii* (Biodiva) in mixed fermentation with *S. cerevisiae* (Uvaferm 228). Further investigations are needed to gain a deeper understanding of the specific interactions taking place between *S. cerevisiae* and non-*Saccharomyces* yeasts in mixed/sequential fermentations and their impact on the quality of other fruit spirits.

A 24-week maturation period significantly influenced the chemical composition of the distillates produced from the fermentation of apple mash using pure and mixed cultures of *S. cerevisiae* (Uvaferm 228), *L. thermotolerans* (Concerto), and *T. delbrueckii* (Biodiva). Patterns of volatile evolution during maturation were rather complex and appeared to cluster according to different trends. The concentrations of higher alcohols showed a gradual decrease during the investigated period. These alterations were primarily dependent on the alcohol content of the distillates. However, temperature showed a significant effect on the changes in 1-butanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 1-

hexanol, phenethyl alcohol, benzyl alcohol, and trans-3-hexen-1-ol. Ester changes were quite versatile and highly correlated with all the tested factors. Ethyl acetate and isoamyl acetate concentrations were diverse. Ethyl lactate and diethyl succinate levels were characterized by a consistent drop, whilst ethyl octanoate and ethyl myristate contents increased significantly. No relationship was found between temperature and variations of methanol, acetaldehyde, phenylacetic acid, and diethyl succinate. Moreover, it was confirmed that regardless of temperature, high alcohol contents enable greater changes in apple distillates. Further long-term studies are required to confirm the impact of different maturation conditions on the final analytical spirit composition.

The last part of this study aimed to provide a more comprehensive understanding of the interactions among various factors during fermentation and their effect on the production of aroma compounds and the overall sensory quality of fruit spirits. These factors included nutrient supplementation, acidification methods, and yeast strains. The contributions and strong interactions between the tested factors were evident. The patterns of volatile compound production were significantly influenced by the type of nutrient treatment and yeast strain. The acidification technique employed had a less prominent yet significant impact. Apple spirit 6, produced through sequential fermentation of *L. thermotolerans* (Concerto) and *S. cerevisiae* (Uvaferm 228), where *Lb. plantarum* (LB-1) was inoculated to induce acidification and Nutrient Treatment 1 (Vitamon A+Vitamon Combi) was supplemented to the mash, received the highest sensory praise due to its more pronounced fruity and floral notes. These sensory qualities are due to a very complex ester profile and reduced levels of higher alcohols. The results show that such tailored fermentation strategies allow the manipulation of the aroma profiles of fruit spirits towards desired sensory outcomes. Additionally, they help mitigate the risk of incomplete fermentation and undesirable aroma production, thanks to proper nitrogen supplementation practices, mash acidification, and the selection of specific yeast strains.

7. NEW SCIENTIFIC RESULTS

1. I have proved that *Lachancea thermotolerans* (Laktia) and *Lactiplantibacillus plantarum* (LB-1) possess acidifying potential and can serve as effective biotools for the protection of Gala apple mash during fermentation. Through their outstanding lactic acid production (1.22–1.26 g/L), they were able to reduce the pH of the mash by 0.38-0.40 pH units. Additionally, they enrich the ester content (e.g., ethyl lactate) of the fruit distillate.

2. I have demonstrated that the addition of complex nutrients (VitaDrive F3, Vitaferm Ultra, Vitamon Combi, Vitamon A, OptiMUM White, Uvavital, Genesis Fresh, V Starter Premium, Fosfoactiv Premium, and Booster Activ Premium) to the pear mash (made from fruit concentrate) promoted yeast growth, fermentation kinetics, and the synthesis of secondary metabolites. Regardless of the nutrient combination used in the mash, increases in the levels of 1-propanol, 2-methyl-1-propanol, and phenethyl alcohol were observed. In contrast, isoamyl acetate, ethyl octanoate, ethyl decanoate, and diethyl succinate displayed a negative correlation with nutrient supplementation. The specific Nutrient 9 (a combination of Genesis Fresh and Vitamon Combi) triggered distinctive responses in the production patterns of volatile compounds, in particular reducing the amount of higher alcohols and promoting ester synthesis by *Saccharomyces cerevisiae* (Uvaferm 228).

3. I have found that the tested hybrid yeasts (X-thiol, X-treme, HD S135, HD S62, and HD A54) exhibit similar fermentation potential to *Saccharomyces cerevisiae* (Uvaferm 228) in Jonagold apple mash; however, they are generally characterized by reduced production of higher alcohols and a greater diversity of esters. The hybrid strain X-treme showed particularly excellent fermentation capacity (rate of sugar consumption: 93.54%; lowest remaining fructose and glucose levels: 3.33 g/L and 1.51 g/L) and metabolic activity in fruit mash, contributing to the production of various esters, including ethyl butyrate, ethyl octanoate, ethyl myristate, ethyl hexanoate, propyl acetate, butyl acetate, and hexyl acetate, that impart positive notes to the distillate.

4. I have proved that *Lachancea thermotolerans* (Concerto) and *Torulaspora delbrueckii* (Biodiva), as non-*Saccharomyces* yeasts, when used in sequential inoculation with *Saccharomyces cerevisiae* (Uvaferm 228), are novel and promising alternatives in the fermentation of Jonathan apple mash for the production of fruit spirits. Their valuable potential to synthesize a diverse array of flavor-active compounds, especially esters, plays a significant role in enriching the aroma profile of the distillates. *L. thermotolerans* (Concerto) promotes the formation of ethyl lactate, along with specific

volatiles like ethyl myristate and ethyl phenylacetate. *T. delbrueckii* (Biodiva) exhibits an enhanced synthesis of 2-phenethyl acetate, diethyl succinate, and hexyl acetate. Sequential fermentations involving *L. thermotolerans* (Concerto) resulted in distillates of superior sensory quality, with highlighted fruity and floral notes.

5. I have demonstrated that 24 weeks of maturation had a significant effect on the chemical composition of the distillates produced by the fermentation of Golden Delicious apple mash with pure and mixed cultures of *Saccharomyces cerevisiae* (Uvaferm 228), *Lachancea thermotolerans* (Concerto), and *Torulaspora delbrueckii* (Biodiva).

- It was confirmed that the higher alcohol content (>80% v/v) of distillates enables greater changes in the volatiles during maturation.
- The concentrations of higher alcohols gradually decreased during the investigated period, depending on the alcohol content of the distillates.
- Temperature showed a significant effect on the following higher alcohols: 1-butanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 1-hexanol, phenethyl alcohol, benzyl alcohol, and trans-3-hexen-1-ol.
- Ester changes were versatile and highly correlated with all the tested factors (time, temperature, and alcohol content).

6. I developed and implemented an innovative fermentation technology in Gala apple mash that incorporated all three tested factors (acidification technique, nutrient supplementation, and novel yeasts). The alternative that yielded the most outstanding results was the combination of *Lactiplantibacillus plantarum* (LB-1), *Lachancea thermotolerans* (Concerto), *Saccharomyces cerevisiae* (Uvaferm 228), and the nutrient treatment 1 (Vitamon A+Vitamon Combi). In this complex scenario, *Lb. plantarum* (LB-1) effectively provides mash acidification and microbial stability. *L. thermotolerans* (Concerto) together with *S. cerevisiae* (Uvaferm 228) produce a variety of secondary metabolites, while *S. cerevisiae* (Uvaferm 228) simultaneously ensures the proper completion of the alcoholic fermentation. Nutrient supplements support the metabolism of yeasts and LAB. The compatibility and interactions observed between these species enhance the fermentation efficiency and positively contribute to the complexity of the spirit's aroma profile and overall sensory quality.

8. SUMMARY

Given the advent of consumer and producer demands for innovative and distinctive products, both traditional methods and innovative technologies applied in distilled beverage production are focusing on their quality improvement. The unique and complex flavor of fruit distillates depends on the quantity and quality of volatile aroma compounds. These aroma constituents originate from diverse sources, including the raw material and the employed technological process. Fermentation is a crucial step in fruit spirit production, that generates a variety of volatile compounds through yeast metabolism. Although humankind has exploited the fermentative activities of yeasts for millennia, many aspects of their metabolism remain poorly understood. Selected strains of *Saccharomyces cerevisiae* are typically used in alcoholic fermentation to ensure consistent flavor and predictable quality. Nevertheless, the search for new flavors and aromas, has shifted the attention towards new fermentation alternatives. The complexity of aroma development during fermentation can be manipulated and modulated by various fermentation parameters. The principal goal of this work was to explore the individual impact and the complex interactions between a number of contributing factors; namely nutrient source, yeast strain, and mash acidification method. Ultimately, this study aims to increase our knowledge and understanding of the factors that affect the fermentation performance and aroma production capability of different yeast strains.

Fermentations were conducted on both laboratory and pilot scales using apple mash or concentrated juice as substrate. To gain a deeper understanding of the interactions taking place during and after fermentation, various enological parameters were examined. Sugar consumption and organic acid production were analyzed using HPLC. The volatile organic compounds produced during the fermentation trials were assessed using GC-FID and GC-MS.

Effective mash acidification methods were developed using microorganisms as bioregulators and acidifying agents. The results show that *L. thermotolerans* (Laktia) and *Lb. plantarum* strains (LB-1 and Sour Pitch) can naturally acidify the mash, causing a rapid pH drop primarily through the production of lactic acid and other organic acids as part of their metabolism. No significant differences were observed among samples concerning the dynamics of refraction changes, sugar utilization, and volatile acid production. The co-inoculation of *S. cerevisiae* (Uvaferm 228) with bioregulators resulted in a slight reduction in alcohol content (0.7% vol), because their metabolism was focused on secondary metabolite formation. *L. thermotolerans* (Laktia) promoted the formation of higher alcohols and esters. *Lb. plantarum* strains (LB-1 and Sour Pitch) reduced the levels of higher alcohols and ethyl acetate and contributed to higher amounts of esters.

Nine nutrient treatments were designed and introduced into the mash in order to increase yeast assimilable nitrogen (YAN) and other nutrient availability. Besides consistently supporting fermentation performance and yeast population growth, these treatments also led to unexpected aroma outcomes. The specific type of nutrient added to the mash played a significant role in these outcomes. Certain nutrient treatments constantly result in substantial increases or decreases in the concentrations of specific aroma compounds, which can be categorized as nutrient treatment-dependent. Generally, ester production responded positively to nutrient supplementation. Other aroma compounds were produced similarly across all nutrient treatments and can be designated as nutrient treatment-independent. For instance, increases in the concentrations of 1-propanol, 2-methyl-1-propanol, and phenethyl alcohol, as well as decreases in isoamyl acetate, ethyl octanoate, ethyl decanoate, and diethyl succinate levels, were observed.

This study also emphasizes the importance of untapping the hidden wealth of hybrids and non-conventional yeast species in fruit spirit production. Each yeast strain displayed its own unique fermentation pattern, resulting in distinctive aroma profiles for each apple spirit.

The enological characteristics of hybrid yeasts were similar to those of *S. cerevisiae* (Uvaferm 228). Nevertheless, significant differences were observed in their secondary metabolism. Hybrid strains displayed reduced production of higher alcohols and a greater diversity of esters. Among these hybrids, X-treme demonstrated the highest fermentation ability and supported the production of numerous esters that give the distillate positive sensory notes, including ethyl octanoate, ethyl myristate, ethyl butyrate, ethyl hexanoate, butyl acetate, propyl acetate, and hexyl acetate.

The findings indicate that the sequential fermentation approach of non-*Saccharomyces* yeasts presents a better alternative compared to pure culture fermentations. Non-*Saccharomyces* strains do not possess the same alcoholic fermentation capacity as *Saccharomyces* yeasts but contribute additional metabolites that enhance flavor and aroma profiles; thus, offering the distillates a higher sensory praise. *T. delbrueckii* (Biodiva) positively influenced the synthesis of 2-phenethyl acetate, diethyl succinate, and hexyl acetate. On the other hand, *L. thermotolerans* (Concerto) promoted the formation of ethyl lactate, ethyl myristate, and ethyl phenylacetate.

We followed the variations in the volatile composition of apple distillates during a 24-week maturation period, focusing on the influence of alcohol content and maturation temperature. Remarkable changes in aroma constituents during maturation were observed, influenced by all tested factors (maturation time, temperature, and alcohol content). The PCA analysis revealed that these alterations were primarily dependent on the alcohol content of the distillates, with higher alcohol

content enabling greater changes. Nevertheless, temperature showed a significant effect on the changes in 1-butanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 1-hexanol, phenethyl alcohol, benzyl alcohol, and trans-3-hexen-1-ol.

Lastly, a GC-MS monitoring analysis was conducted to follow the changes in the aroma profile during different stages of the production process. We combined the best options of previously tested fermentation parameters, including the acidification methods, nutrient supplements, and yeast strains, to create twelve new alternatives. The volatile composition of the raw material (apples) was dominated by volatiles such as 1-hexanol, 2-hexen-1-ol, 1-octanol, hexanal, 2-hexenal, benzaldehyde, butyl acetate, butyl hexanoate, butyl octanoate, propyl acetate, hexyl acetate, hexyl hexanoate, and 2-methylbutyl acetate. All the tested factors and their interactions significantly influenced the formation of various volatiles during fermentation and distillation. Different yeast strains during fermentation formed numerous new compounds, primarily higher alcohols and esters. Additionally, fatty acids like octanoic acid, decanoic acid, dodecanoic acid, and hexadecanoic acid were generated. These fatty acids served as intermediates in the formation of ethyl esters and were not part of the final distillates. Distillation, being the final step, led to the separation and concentration of existing volatiles. Moreover, it influenced the formation of new volatiles, such as acetal, nonanal, 1-nonanol, ethyl 2-methylbutanoate, heptyl acetate, hexyl isobutanoate, octyl acetate, nonyl acetate, isopropyl myristate, 2-phenethyl octanoate, isobutyl caprylate, butyl laurate, isobutyl laurate, and octyl decanoate. The sensory evaluation revealed that the most favored apple spirit resulted from sample 6, which was scored with the highest fruity and floral notes. In sample 6, the apple mash was supplemented with nutrient treatment 1 (Vitamon A + Vitamon Combi), acidified by *Lb. plantarum* (LB-1), and sequentially fermented by *L. thermotolerans* (Concerto) and *S. cerevisiae* (Uvaferm 228). This combination triggered the formation of numerous secondary metabolites during fermentation. The aroma profile of Apple Spirit 6 was rich in various esters and contained reduced levels of higher alcohols, especially amyl alcohols. In total, 32 esters were present in moderate concentrations in the distillate, including ethyl decanoate, propyl decanote, ethyl butanoate, butyl 2-methyl butanoate, hexyl 2-methyl butanoate, ethyl myristate, ethyl palmitate, ethyl stearate, ethyl hexadecanoate, hexyl isobutanoate, ethyl hexanoate, and ethyl nonanoate.

The results in this thesis strongly suggest that it is possible to modulate the aroma by employing different yeasts and mash treatments in order to create novel fruit spirits with distinctive aromatic notes and styles.

9. APPENDICES

9.1. Bibliography

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9.2. Additional information

Table A1. Preliminary experimental design of nutrient supplementation during fermentation process

	Day 0	Day 1	Day 2	Day 4	Day 7	Day 10
Control (no nutrients)						
N1	Vitamon A (55 g/hL)					
N2	Vitamon A (30 g/hL)			Vitamon A (20 g/hL)		Vitamon A (20 g/hL)
N3	Vitamon A (55 g/hL)			Vitamon Combi (65 g/hL)		
N4	Vitamon Combi (50 g/hL)			Vitamon A (40 g/hL)		
N5	Vitamon Combi (30 g/hL)			Vitamon Combi (30 g/hL)		
N6	Vitaferm Ultra F3 (35 g/hL)			Vitaferm Ultra F3 (35 g/hL)		
N7	Vitadrive F3 (35 g/hL) + Vitaferm Ultra F3 (35 g/hL)			Vitaferm Ultra F3 (35 g/hL)		
N8	Vitadrive F3 (30 g/hL) + Vitamon Combi (30 g/hL)			Vitamon Combi (30 g/hL)		
N9	Vitadrive F3 (50 g/hL) + Vitamon Combi (50 g/hL)			Vitamon A (40 g/hL)		
N10	Uvavital (40 g/hL)					
N11	Uvavital (20 g/hL)			Uvavital (20 g/hL)		Uvavital (10 g/hL)
N12	Optimum White (30 g/hL)		Uvavital (10 g/hL)			
N13	V Starter Premium (20 g/hL)	Fosfoactiv (20 g/hL)			Booster Activ (10 g/hL)	
N14	V Starter Premium (20 g/hL)	Fosfoactiv (20 g/hL)		Fosfoactiv (20 g/hL)		
N15	Genesis Fresh (30 g/hL)		Uvavital (10 g/hL)			
N16	Genesis Fresh (30 g/hL)		Vitamon Combi (30 g/hL)			
N17	Vitadrive F3 (30 g/hL) + Vitamon A (30 g/hL)			Vitamon A (40 g/hL)		

Table A2. Changes in volatile compounds of apple distillates obtained by *T. delbrueckii* and *S. cerevisiae* during maturation

Compounds (mg/L alcohol 100% v/v)	81.8% v/v						60% v/v					
	10 ˚C			25 ˚C			10 ˚C			25 ˚C		
	0	12 weeks	24 weeks	0	12 weeks	24 weeks	0	12 weeks	24 weeks	0	12 weeks	24 weeks
Acetaldehyde	6.56 ±0.43 Bb	4.42 ±0.61 Aab	**3.48 ±0.29 Aa**	6.56 ±0.43 Bb	4.43 ±0.06 Aa	**4.70 ±0.41 Aab**	5.53 ±0.43 Aa	6.69 ±0.21 Ba	**7.98 ±0.14 Ba**	5.53 ±0.43 Aa	6.31 ±0.57 Bab	**8.99 ±0.03 Bb**
Methanol	5388.95 ±189.23 Ba	6028.52 ±136.89 Ba	**6181.09 ±260.88 Ba**	5388.95 ±189.23 Ba	6193.37 ±25.11 Ba	**7184.81 ±15.04 Bb**	3952.78 ±293.16 Ab	**2326.18 ±58.00 Aa**	**3011.35 ±4.43 Ab**	3952.78 ±293.16 Ac	**3234.97 ±128.35 Ab**	**1798.28 ±14.00 Aa**
1-Propanol	1512.58 ±60.23 Ba	1015.41 ±200.60 Aa	**1314.96 ±20.62 Ba**	1512.58 ±60.23 Bb	1213.87 ±88.63 Bab	**1083.23 ±7.10 Ba**	1109.47 ±94.45 Ab	**717.86 ±6.53 Aa**	770.84 ±10.60 Ab	1109.47 ±94.45 Ab	**899.63 ±0.55 Ab**	766.30 ±7.90 Aa
1-Butanol	86.26 ± 2.98 Ba	58.84 ±6.84 Ba	**72.28 ±4.68 Ba**	86.26 ±2.98 Bc	67.84 ±2.38 Bb	**56.79 ±2.34 Ba**	63.27 ±0.83 Ab	**43.56 ±0.78 Aa**	42.42 ±3.61 Aa	63.27 ±0.83 Ac	**51.65 ±0.48 Ab**	47.18 ±0.39 Aa
2-Butanol	0.82 ±0.11 Bab	**0.50 ±0.06 Aa**	**1.15 ±0.10 Bb**	0.82 ±0.11 Ba	**0.73 ±0.00 Ba**	**0.70 ±0.12 Aa**	0.60 ±0.00 Ab	**0.61 ±0.02 Bb**	**0.38 ±0.02 Aa**	0.60 ±0.00 Aab	**0.48 ±0.03 Aa**	**0.77 ±0.11 Ab**
2-Methyl-1-propanol	392.77 ±25.85 Bb	**264.20 ±11.95 Ba**	**337.20 ±23.66 Bab**	392.77 ±25.85 Bb	**312.55 ±12.61 Bb**	**268.49 ±12.30 Ba**	288.10 ±2.62 Ab	**200.80 ±0.71 Aa**	200.36 ±11.33 Aa	288.10 ±2.62 Ab	**235.36 ±1.48 Aa**	194.95 ±15.52 Aa
2-Methyl-1-butanol	120.01 ±0.46 Bb	94.67 ±2.63 Aa	**100.93 ±2.59 Ba**	120.01 ±0.46 Bb	90.40 ±3.79 Aa	**78.71 ±11.39 Aab**	110.42 ±1.16 Ac	**98.32 ±0.96 Ab**	**95.37 ±0.48 Aa**	110.42 ±1.16 Ac	**92.96 ±0.39 Ab**	**86.03 ±0.16 Aa**
3-Methyl-1-butanol	792.40 ±32.68 Bb	619.56 ±10.76 Ba	**656.12 ±10.87 Bab**	792.40 ±32.68 Bb	622.35 ±49.08 Bb	**506.46 ±22.19 Ba**	581.22 ±2.34 Ab	**423.23 ±0.42 Aa**	**402.31 ±5.50 Aa**	581.22 ±2.34 Ac	**476.72 ±1.35 Ab**	**442.03 ±0.06 Aa**
1-Hexanol	48.65 ±0.92 Ba	37.31 ±2.70 Ba	**37.96 ±2.04 Ba**	48.65 ±0.92 Bb	37.39 ±0.29 Ba	**28.24 ±2.44 Aa**	35.69 ±1.22 Ab	**25.08 ±1.54 Aa**	**21.82 ±0.33 Aa**	35.69 ±1.22 Ab	**29.04 ±0.91 Aab**	**28.95 ±0.32 Aa**
Phenethyl alcohol	1.30 ±0.19 Bb	0.72 ±0.18 Bab	**0.31 ±0.02 Ba**	1.30 ±0.19 Bb	0.89 ±0.03 Bb	**0.09 ±0.02 Ba**	0.36 ±0.07 Aab	**0.23 ±0.01 Ab**	**0.18 ±0.01 Aa**	0.36 ±0.07 Ab	**0.34 ±0.01 Ab**	**0.00 ±0.00 Aa**
Trans-3-hexen-1-ol	0.31 ±0.15 Aab	**0.58 ±0.06 Bb**	**0.24 ±0.01 Ba**	0.31 ±0.15 Aa	**0.37 ±0.04 Ba**	**0.43 ±0.06 Aa**	0.22 ±0.01 Ab	**0.09 ±0.01 Aa**	**0.10 ±0.01 Aa**	0.22 ±0.01 Ab	**0.14 ±0.01 Aa**	**0.49 ±0.01 Ac**
Cis-2-hexen-1-ol	0.28 ±0.03 Bab	0.27 ±0.05 Bb	0.12 ±0.03 Aa	0.28 ±0.03 Bb	0.20 ±0.02 Bb	0.12 ±0.02 Aa	0.21 ±0.01 Ab	0.07 ±0.01 Aa	**0.08 ±0.01 Aa**	0.21 ±0.01 Ab	0.10 ±0.01 Aa	**0.12 ±0.01 Aa**
Benzyl alcohol	0.30 ±0.01 Bb	**0.12 ±0.01 Aa**	**0.11 ±0.00 Ba**	0.30 ±0.01 Bb	**0.22 ±0.02 Bb**	**0.15 ±0.00 Ba**	0.22 ±0.00 Ac	**0.13 ±0.00 Ab**	**0.06 ±0.01 Aa**	0.22 ±0.00 Ac	**0.12 ±0.00 Ab**	**0.02 ±0.00 Aa**
Ethyl acetate	387.36 ±12.66 Bb	396.25 ±4.87 Bb	**189.39 ±17.04 Aa**	387.36 ±12.66 Ba	401.54 ±28.38 Bab	**579.00 ±39.85 Bb**	226.25 ±8.92 Ab	**148.87 ±1.61 Aa**	**267.86 ±7.17 Bb**	226.25 ±8.92 Ac	**143.94 ±1.33 Ab**	**21.37 ±0.88 Aa**
Ethyl lactate	0.49 ±0.01 Ab	0.24 ±0.06 Aab	**0.08 ±0.01 Ba**	0.49 ±0.01 Ab	0.27 ±0.08 Bb	**0.03 ±0.01 Aa**	0.35 ±0.10 Aab	**0.20 ±0.00 Ab**	0.04 ±0.00 Aa	0.35 ±0.10 Ab	**0.07 ±0.00 Aab**	0.04 ±0.01 Aa
Ethyl butyrate	0.36 ±0.14 Aa	**0.17 ±0.02 Aa**	**0.03 ±0.01 Aa**	0.36 ±0.14 Aa	**0.49 ±0.01 Ba**	**0.39 ±0.06 Ba**	0.32 ±0.02 Aab	0.33 ±0.05 Bb	**0.26 ±0.05 Ba**	0.32 ±0.02 Ab	0.35 ±0.05 Ab	**0.13 ±0.00 Aa**
Isoamyl acetate	0.44 ±0.01 Bb	**0.22 ±0.14 Aab**	**0.27 ±0.02 Aa**	0.44 ±0.01 Ba	**0.76 ±0.01 Bb**	**0.69 ±0.09 Aab**	0.32 ±0.04 Aa	0.50 ±0.02 Aa	**0.95 ±0.01 Bb**	0.32 ±0.04 Aa	0.57 ±0.06 Aa	**1.08 ±0.02 Bb**
Ethyl octanoate	3.92 ±0.16 Bb	**3.08 ±0.04 Aa**	7.96 ±0.40 Ac	3.92 ±0.16 Ba	**4.77 ±0.04 Bb**	8.32 ±0.26 Bc	2.88 ±0.18 Aa	**3.52 ±0.01 Bab**	6.94 ± 1.07 Ab	2.88 ±0.18 Aa	**4.69 ±0.02 Ab**	6.51 ±0.24 Ac
Diethyl succinate	0.31 ±0.02 Ba	**0.22 ±0.05 Aa**	0.26 ±0.02 Ba	0.31 ±0.02 Ba	**0.34 ±0.02 Ba**	0.23 ±0.02 Ba	0.22 ±0.00 Ab	**0.16 ±0.01 Aa**	0.15 ±0.03 Aab	0.22 ±0.00 Ab	**0.13 ±0.00 Aa**	0.15 ±0.02 Aab

Ethyl myristate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-Phenethyl acetate	0.44 ±0.01 Ab	0.35 ±0.10 Bab	**0.18 ±0.01 Ba**	0.44 ±0.01 Ab	0.30 ±0.06 Bab	**0.15 ±0.00 Ba**	0.32 ±0.07 Ab	**0.15 ±0.01 Ab**	0.00 ±0.00 Aa	0.32 ±0.07 Ab	**0.00 ±0.00 Aa**	0.00 ±0.00 Aa
Ethyl phenylacetate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Limonene	0.34 ±0.03 Bc	**0.16 ±0.03 Bb**	0.00 ±0.00 Aa	0.34 ±0.03 Bc	**0.08 ±0.01 Ab**	0.00 ±0.00 Aa	0.25 ±0.00 Ac	0.06 ±0.00 Ab	0.00 ±0.00 Aa	0.25 ±0.00 Ab	0.05 ±0.04 Aab	0.00 ±0.00 Aa
Linalool	0.30 ±0.02 Ba	**0.27 ±0.04 Ba**	0.25 ±0.05 Ba	0.30 ±0.02 Bb	**0.13 ±0.01 Aa**	0.19 ±0.02 Bb	0.14 ±0.01 Aa	**0.18 ±0.01 Ab**	0.11 ±0.02 Aab	0.14 ±0.01 Ab	**0.12 ±0.01 Aa**	0.12 ±0.00 Aab
Phenyl acetic acid	0.12 ±0.01 Bb	0.10 ±0.01 Ab	**0.01 ±0.00 Aa**	0.12 ±0.01 Bab	0.12 ±0.02 Aa	**0.15 ±0.02 Bb**	0.09 ±0.00 Aa	**0.08 ±0.01 Aa**	0.09 ±0.03 Ba	0.09 ±0.00 Aa	**0.11 ±0.01 Aa**	0.09 ±0.01 Aa
Hexyl acetate	0.54 ±0.07 Ba	0.53 ±0.06 Ba	**0.52 ±0.02 Ba**	0.54 ±0.07 Bab	0.61 ±0.01 Bb	**0.34 ±0.03 Ba**	0.40 ±0.00 Aa	**0.28 ±0.04 Aa**	0.16 ±0.07 Aa	0.40 ±0.00 Ac	**0.16 ±0.00 Aa**	0.16 ±0.00 Ab
Myrcene	0.12 ±0.09 Aa	0.06 ±0.04 Aa	**0.04 ±0.00 Aa**	0.12 ±0.09 Aa	0.05 ±0.01 Aa	**0.02 ±0.00 Aa**	0.09 ±0.01 Ab	0.08 ±0.01 Aab	0.07 ±0.01 Ba	0.09 ±0.01 Aa	0.07 ±0.01 Ba	0.06 ±0.00 Ba

Data are expressed as mean ± standard deviation; n.d.: not detected. Different lower-case letters in a row show significant differences for the time effect; upper-case letters are for the ABV effect comparing 60% v/v and 81.8% v/v. Significant differences between the temperatures (10 °C and 25 °C) are in bold, with the significantly greater value marked with an underline (Games-Howell's post hoc test, $p < 0.05$)

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