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Controlled fermentation strategies to enhance the aroma profile of fruit spirits

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1. INTRODUCTION AND OBJECTIVES

Fruit spirits are popular alcoholic beverages that have been traditionally produced for ages. 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. 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., 2020; Moreno et al., 2023). Nevertheless, the search for new flavors and aromas, has shifted the attention towards new fermentation alternatives. 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 grow 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. 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).

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:
  - 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.
- Assess the impact of different commercially available nutrient supplements on the fermentation kinetics and the production of aroma compounds by *Saccharomyces cerevisiae*.
- Study new alternative yeasts that offer enhanced aroma compound production. In particular:
  - 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.
- 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.

2. MATERIAL AND METHODS

2.1. 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.

The yeast and bacterial strains used in the study included Uvaferm 228™ (*Saccharomyces cerevisiae*), Level2 Biodiva™ (*Torulaspora delbrueckii*), Viniflora Concerto™ (*Lachancea thermotolerans*), Laktia™ (*Lachancea thermotolerans*), Melody™ (mixed culture of 20% *Torulaspora delbrueckii*, 20% *Lachancea thermotolerans*, and 40% *Saccharomyces cerevisiae*), Oenoferm® X-treme (Hybrid *Saccharomyces cerevisiae*), Oenoferm® X-thiol (Hybrid *Saccharomyces cerevisiae*), SafŒno™ HD S135 (Hybrid *Saccharomyces cerevisiae* x *Saccharomyces bayanus*), SafŒno™ HD S62 (Hybrid *Saccharomyces cerevisiae* x *Saccharomyces bayanus*), SafŒno™ HD A54 (Hybrid *Saccharomyces cerevisiae* x *Saccharomyces bayanus*), Harvest LB-1 (*Lactiplantibacillus plantarum*), and WildBrew™ Sour Pitch (*Lactiplantibacillus plantarum*). The yeast and bacterial strains were provided by Chr. Hansen A/S (Hoersholm, Denmark), Kertrade Ltd. (Dunavarsány, Hungary), and Kokoferm Ltd. (Gyöngyös, Hungary).

The applied yeast nutrient supplements included VitaDrive F3, Vitaferm Ultra, Vitamon Combi, and Vitamon A produced by Erbslöh (Geisenheim, Germany). OptiMUM White and Uvavital produced by Lallemand Oenology (Montréal, QC, Canada). Genesis Fresh produced by Oenofrance (France) and V Starter Premium, Fosfoactiv Premium, and Booster Activ Premium by Enologica Vason (Verona, Italy).

Analytical grade standards and chemicals were supplied by Sigma-Aldrich (Steinheim, Germany).

2.2. Methods

2.2.1. Mashing and fermentation conditions

Upon delivery to the laboratory, apples were manually sorted, cleaned, and crushed with a centrifugal mill. 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. 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.

2.2.1.1. Acidification of the mash

The pH of the mash is typically adjusted to 3.0 to inhibit the growth of undesirable microorganisms. 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. Following the addition of the acid solutions, the mash from Gala apples was thoroughly mixed. Afterwards, the mash was supplemented with 40 g/100 kg of Uvavital^{TM} 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.

2.2.1.2. Nutrient supplementations

Lab-scale fermentations were conducted to evaluate the effect of nutrient supplements on the fermentation performance and the production of volatile compounds by *S. cerevisiae*. Nine different nutrient combinations (Table 1) were tested using pear juice (13 ºBrix) as the fermentation medium.

The juice was inoculated with 40 g/100 kg of *S. cerevisiae* (Uvaferm 228) yeast to initiate the fermentation process.

Table 1. Experimental design of nutrient supplementation during fermentation

	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)			

2.2.1.3. Hybrid yeasts

Lab-scale fermentations were performed to evaluate the fermentation potential of various hybrid yeasts in comparison to an industrial strain of *S. cerevisiae*. The pH of the Jonagold apple 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.

2.2.1.4. Non-*Saccharomyces* yeasts

Pilot-scale fermentations were carried out to assess the fermentation performance of non-*Saccharomyces* strains on Jonathan apple mash. 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.

2.2.1.5. 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 2.

Once the corresponding yeasts were inoculated, all the tanks/flasks 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 2. 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.

2.2.2. Distillation Process

When laboratory-scale fermentations were carried out, the distillation process was performed on glass distillation equipment with a capacity of 3 L. 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).

2.2.3. Maturation Process

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 was diluted to an ABV of 60% v/v. Samples from both batches of each distillate were placed in 100 mL glass containers. These samples were stored at controlled temperatures of 10 °C and 25 °C for 24 weeks. For GC-FID analysis, samples were collected at three consecutive time points: 0, 12, and 24 weeks of maturation. The fresh distillates were used as a control.

2.2.4. Analytical methods

The fermentation processes were monitored continuously by measuring critical parameters including total soluble solids, pH, reducing sugar content, titratable acidity, ethanol content, volatile acid content, and yeast assimilable nitrogen (YAN). The quantities of sugars and organic acids in the mash were determined using HPLC, following the method described by Chinnici et al. (2005). Chromatographic analyses of selected volatile compounds were carried out using a GC-FID (Perichrom PR2100, Alpha MOS, Toulouse, France) and a HS-SPME/GC-MS system (Agilent Technologies, Santa Clara, CA, USA), according to the method outlined by Rodríguez Madrera and Valles

(2007). Organoleptic properties of the spirits produced with the involvement of non-*Saccharomyces* yeasts were evaluated using the 20-point scale test (MSZ ISO 11132, 2013), while the sensory evaluation of the spirits produced, as described in Section 2.2.1.5., was performed using QDA methodology.

2.2.5. Statistical Analysis

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% ($\alpha = 0.05$). To analyze the effect of the maturation process, the volatile compounds of apple distillates were compared by three-way repeated measures of ANOVA model, followed by Games-Howell's post hoc tests. Principal component analysis (PCA) was conducted to explore potential relationships between maturation conditions and the volatile compounds of apple spirits. The statistical analyses were performed using R software (version 4.2.2, R Core Team, Vienna, Austria). To analyze the effect of combined treatments, the 12 apple spirits and control samples were compared by a one-way MANOVA model. 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. 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. Heatmap analysis using normalized GC-MS data was used to illustrate the dynamic evolution of volatile compounds during the production process of spirits. PCA was performed to explore the contribution of volatile compounds to the aroma profile of apple spirits during different phases of the production process. The statistical analyses were performed using R software (version 4.3.1, 'Beagle Scouts', R Core Team, Vienna, Austria).

3. RESULTS AND DISCUSSION

3.1. Chemical and biological acidification of fruit mash

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. 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. Among the *Lactiplantibacillus plantarum* strains, LB-1 reduced the pH of the mash by 0.38 units, while Sour Pitch decreased it by 0.29 units. 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. The involvement of lactic acid-producing strains in the mash favored the production of ethyl lactate, with the highest concentrations produced by *Lb. plantarum* (LB-1) (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.). Administering phosphoric and lactic acids in a 70:30 ratio has yielded the most favorable enological and aromatic outcomes.

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

Several experiments were conducted to assess the impact of different nutrient supplements, either individually or in combination, on apple mash and

various fruit juices. Finally, 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. Nutrient 9 (a combination of Genesis Fresh and Vitamon Combi) was distinguished among other treatments for reducing the amount of higher alcohols and promoting ester synthesis by *S. cerevisiae* (Uvaferm 228).

3.3. Screening yeast strains for fruit spirit production

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. The sensory analysis results show that the fruitiness and high flavor intensity perceived by the panelists were highly appraised in the distillates produced from the mixed inoculums involving non-*Saccharomyces* strains.

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

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. In general, higher alcohols showed a decreasing tendency during the maturation period. These changes were primarily dependent on the alcohol content of the distillates. 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. Ester changes were diverse and highly correlated with all the tested factors. Ethyl lactate and diethyl succinate levels consistently decreased, while ethyl octanoate and ethyl myristate contents significantly increased. Under different maturation

conditions, ethyl acetate and isoamyl acetate concentrations varied widely. No relationship was found between temperature and variations of methanol, acetaldehyde, phenylacetic acid, and diethyl succinate. The PCA analysis revealed that these changes in the concentration of volatile compounds were primarily dependent on the alcohol content of the distillates, with higher alcohol content enabling greater changes.

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

Lastly, a GC-MS monitoring analysis was conducted to follow the changes in the aroma profile during different stages of the production process. The best variants of previously tested fermentation parameters, including acidification methods, nutrient supplements, and yeast strains, were combined to create twelve new alternatives (Table 2). 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. During fermentation, different yeast strains 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. Ultimately, the pleasant aroma and taste of Spirit 6 result from a complex combination of optimal volatile concentrations.

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.

4. CONCLUSION AND RECOMMENDATIONS

The results confirm that 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. Moreover, these strains, in sequential inoculation with *S. cerevisiae* (Uvaferm 228), produced apple distillates with unique aroma profiles, in particular increased production of ethyl lactate. Further studies, involving various LAB strains, are needed to expand our understanding of their metabolism.

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* in different nutritional media were not consistent, and the formation of secondary metabolites was influenced by the type of nutrient source available. 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 yeast strains are promising alternatives for use as fermentation agents in the production of fruit distillates. Among hybrid yeasts, the strain X-treme demonstrated the highest fermentation ability and supported the production of a number of esters that give the distillate positive characteristics. Moreover, the use of non-*Saccharomyces* yeasts in sequential fermentation with *S. cerevisiae* distinctly modulated the concentrations of specific fermentative volatiles, highlighting fruity and floral traits in the distillate. Further investigations are needed to understand interactions between *S. cerevisiae* and non-*Saccharomyces* yeasts in mixed or sequential fermentations and their impact on diverse fruit spirit quality.

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, highly influenced by the factors tested (time, temperature, and alcohol content). 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 (nutrient supplementation, acidification methods, and yeast strains) during fermentation and their effect on the production of aroma compounds and the overall sensory quality of fruit spirits. 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 the 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.

5. 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 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.

6. PUBLICATIONS

Journal articles

1. **Fejzullahu, F.,** Kiss, Z., Kun-Farkas, G., Kun, Sz. (2021). Influence of non-*Saccharomyces* strains on chemical characteristics and sensory quality of fruit spirit. Foods, 10 (6), 1336. DOI: 10.3390/foods10061336. (Q1, IF: 4.092)
2. **Fejzullahu, F.,** Ladányi, M., Kun-Farkas, G., Kun, Sz. Aroma profile of apple spirits fermented with non-*Saccharomyces* yeasts and their dynamic changes during maturation. Journal of the American Society of Brewing Chemists (Under revision) (Q2, IF: 2.0)

Book chapters

1. Kun, Sz., **Fejzullahu, F.,** Kun-Farkas, G. (2022). Nem-*Saccharomyces* élesztők hatása a gyümölcspárlatok kémiai tulajdonságaira és érzékszervi minőségre. In: Takács, L. (Eds.): Quintessence - 2022 : A pálinka világa. Ongai Kulturális Egyesület, 152-169 p.

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1. **Fejzullahu, F.,** Jókai, Zs., Uveges, M., Mórucz, I., Stefanovits-Bányai, É. (2018). Total phenolic content, antioxidant capacity and mineral content of selected fruits from northeastern Hungary. Proceedings of the 24th International Symposium on Analytical and Environmental Problems, Szeged, Hungary, 299-303 p. ISBN 978-963-306-623-2

Conferences

1. **Fejzullahu, F.,** Kun, Sz. Some aspects of Hungarian spirit (Pálinka) production. In: 1st International Conference & Exhibition “Spirit of Rakia”, Pula, Croatia (27-30th March, 2019).

2. **Fejzullahu, F.,** Pálvölgyi, V., Frey, T., Kun, Sz. Investigation of the metabolic activity of hybrid yeasts during fermentation of fruit mash. In: SZIEntific Meeting for Young Researchers Conference, Budapest, Hungary (9th December, 2019). ISBN 978-963-269-886-1
3. **Fejzullahu, F.,** Blakaj, D., Frey, T., Kun, Sz. Effects of nutrient supplementation on fermentation process and Pálinka quality. In: Chemical Engineering Day'21 Conference, MKN2021, Veszprém, Hungary (21st April, 2021).
4. **Fejzullahu, F.,** Németh, V., Frey, T., Kun, Sz. The impact of nutrients on yeast fermentation and Pálinka quality. In: 4th International Conference on Biosystems and Food Engineering, Budapest, Hungary (4th June, 2021). ISBN 978-963-269-878-6
5. **Fejzullahu, F.,** Blaskó E., Frey, T., Kun, Sz. A comparative study on the fermentation performance of hydrib yeasts in fruit mash. In: Lippay János-Ormos Imre-Vas Károly Scientific Congress (ITT Conference), Budapest, Hungary (29th November 2021). ISBN 978-963-269-988-2
6. **Fejzullahu, F.,** Ujszászi, R., Csernus, O., Kun, Sz. New insights into the acidification techniques of fruit mash during fermentation process. In: FoodConf - 4th International Conference on Food Science and Technology, Budapest, Hungary (9-11th June, 2022). ISBN 978-615-01-5422-0

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