PhD THESIS

Biotechnological design to expand the apple cider profile diversity

(SUMMARY OF THE DOCTORAL THESIS)

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SUMMARY

Apple cider is defined as alcoholic beverages with an alcohol content between 1.2% and 8.5% obtained by partial or complete fermentation of apple juice. Sometimes, in cider production, besides to the alcoholic fermentation process, malolactic fermentation is also involved, due to the activity of lactic bacteria. These yeasts and bacteria play a crucial role in the fermentation of fruit wines by producing flavoring substances.

Global cider production is constantly growing, but a small decrease occurred after 2020, but a rebound in 2022. The world's most important cider consumption areas are Western Europe (48.1%), Africa (19.1%), North America (9.7%) and Eastern Europe (8.6%). The latest data places Romania on the 3rd place in cider smaller markets, with an annual consumption of 165 thousand hL.

Cider is consumed for various reasons. Some consumers prefer the refreshing taste, which can range from sweet to dry depending on the type of apples used and the fermentation process. Others appreciate its lower alcohol content compared to many other alcoholic beverages, making it a suitable option for social gatherings, casual drinking or serving as a flavorful alternative to beer and wine. Additionally, cider is often associated with tradition and craft, especially in regions with a strong cider-making history like England, France, and parts of the United States.

The apple variety, fermentation process, and yeast strains significantly influence the chemical composition of apple cider. Volatile compounds are crucial for cider quality, sensory characteristics, and consumer purchasing decisions. Esters, formed by the reaction of higher alcohols with acids, contribute to the cider's fragrance and typicity even in small amounts, imparting flavors such as green apple, yellow apple, fruity, strawberry, banana, and caramel aromas. Phenolic compounds are also vital to cider quality, affecting its color, astringency, and bitterness, and their balance defines the drink's overall profile. Another group of compounds that affects cider aroma consists of organic acids, which consistently contribute to its sour taste. These acids play a crucial role in determining the cider's acidity, bitterness, and astringency. Their composition changes during fermentation, leading to the formation of other compounds.

Recently, researchers focused on identifying and selecting yeast strains with ideal traits for cider production has produced encouraging results. *Saccharomyces cerevisiae* and *Saccharomyces bayanus* are suggested as starter cultures for making cider. Furthermore, various non-*Saccharomyces* species have been recognized for their beneficial effects on sensory properties, such as increasing the complexity of aromas and volatile compounds in both co-fermentations and pure cultures.

The main objectives of this PhD thesis were:

The thesis aimed to design the alcoholic and malolactic fermentation processes by obtaining cider assortments from local apple cultivars. Also, considering the variety of yeasts and lactic acid bacteria involved in the fermentation processes, both

Saccharomyces yeasts, non-*Saccharomyces* yeasts, and lactic acid bacteria were used, in monocultures and mixed cultures. The innovative aspect of the current study consists both in its design and the research methods employed. Therefore, the profiles of carbohydrates, polyphenols, volatile compounds, organic acids and amino acids in apple juice were identified and their traceability during alcoholic and malolactic fermentation was followed. During malolactic fermentation, the influence of nutrients on the chemical composition of cider and sensory characteristics was also monitored.

Considering these aspects, the present study involved a complex analysis of the cider samples obtained, in order to identify and quantify the chemical compounds of interest, and also a sensory analysis carried out by a group of trained panellists, in order to correlate the chemical composition with the sensory profile.

To achieve the aim of this thesis, the following aspects were investigated and materialized into four objectives:

O.1. Influence of co-inoculation of *Saccharomyces* and non-*Saccharomyces* yeasts on the chemical composition of cider

O.2. Study of nutrient addition impact on malolactic fermentation efficiency

O.3. Improvement of fermentation dynamics and reduction of operational process time with yeast and lactic bacteria synergy

O.4. Assessment of sparkling cider obtained by the traditional method using encapsulated yeasts

The preliminary action for achieving the objectives was to select apple varieties whose chemical composition is suitable for cider production. A wide range of yeasts (seven strains of *Saccharomyces* and non-*Saccharomyces* yeasts from different producers) and LAB (two strains of *O. oeni* and one *L. plantarum*) were also selected. In accordance with the current studies, but also with the aim for innovation, the work plan was defined, by establishing the compatibility between microorganisms, setting fermentation conditions, monitoring fermentation processes, and performing chemical and sensory analyses.

The results of this thesis were published in one review article (ISI indexed journal with IF 3.5 – in *Processes* Journal) and three original articles (one ISI indexed journal with IF 5.2 – in *Food Bioscience* Journal; the second and the third ISI indexed journal with IF 3.7 – in *Agronomy* Journal).

The studies and experiments described in this thesis were performed in the Faculty of Food Science and Technology from The University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, in the Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy, and in the Faculty of Chemistry and Chemical

Engineering, Babeș-Bolyai University, under the coordination of prof. dr. Elena Mudura and the supervision and guidance of the co-authors listed in the publication.

The PhD thesis is structured in two main parts: state-of-the art containing one literature review article (Chapter 1) and original research containing the working hypothesis/objectives (Chapter 2) and the general methodologies (Chapter 3), followed by the own research articles (Chapters 4-6), general conclusions and recommendations (Chapter 7), respectively the originality and innovative contributions of the thesis (Chapter 8).

In what concerns the **first part** (literature review article), the study was identified through electronic searches on PubMed, the Web of Science Core Collection, Scopus, and Google Scholar. Following the literature review presented the state of the art chapter on cider quality screening, it was found that the main classes of compounds influencing cider quality and sensory profile are: polyphenols, volatile compounds, organic acids and carbohydrates.

In what concern the **second part**, the experiments included complex processes of alcoholic fermentation, malolactic fermentation, and secondary fermentation, all carried out under controlled conditions. Fermentation and inoculation conditions for yeasts and lactic acid bacteria were established and optimized according to the literature. The raw material (apple juice) was kindly given by a local producer from Cluj-Napoca, which has all the modern equipment for processing fruit to obtain juice. The yeasts and nutrients used in the experiments in Chapter 4 were purchased from university funds, and the yeasts and lactic acid bacteria used in Chapters 5 and 6 were kindly given by Bevitech (Romania) and Chr. Hansen (Romania). The analyses and determinations were performed in triplicates for every individual study and analysis.

To identify the phenolic compounds cider samples were filtered through a 0.45 µm Chromafil Xtra nylon filter and 20 µl were injected into the HPLC system and comparing the retention time, UV–Vis absorption, and mass spectra with those of the standard compounds and literature data. For the volatile compounds, the extraction procedure, ultrasonic extraction was used instead of a magnetic stirrer. The separation and identification of volatile compounds were achieved by gas chromatographic analysis. Amino acids (AA) extracted from 100 µL of sample were analyzed by gas chromatography using the Phenomenex EZ: Faast™ kit. Data manipulation and processing were performed by Empower 2 software (Walters, Milford, MA, USA). For the separation and quantification of carbohydrates, lactic and acetic acids, the Agilent 1200 HPLC system was used, equipped with a quaternary pump, solvent degasser, manual injector coupled with a refractive index detector (RID). For the malic, pyruvic, succinic and citric acids determination, an Agilent 1200 HPLC system equipped with a quaternary pump, solvent degasser, manual injector and UV–Vis detector (VWD). The compounds were identified by comparing the retention times of the standards with those of the peaks from the juice and apple cider samples.

The statistical analyses were performed using SPSS 19.0 software (IBM, New York, NY, USA) and Tukey's honestly significant difference (HSD) test with a confidence interval of 95%. Pearson correlation was calculated for all data (Excel, Microsoft 365).

Principal component analysis (PCA) and Heat Maps Analysis (HMA) were carried out in order to observe the correlation between samples and the identified compounds (sugars, organic acids, polyphenols, amino acids, volatiles, and sensory attributes) by using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) and XLSTAT (Addinsoft, New York, NY, 414 USA).

Sensory analysis was conducted in University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, laboratory for Sensory Analysis of Foods. The trained participants ranged in age from 26 to 47. There were two persons working in the quality control of alcoholic beverages and one sommelier among the panelists. The selection criteria minimum 2 years of experience in sensory analysis of beverages. The sensory analysis followed the ethical guidelines of the laboratory.

Chapter 4 presents the physico-chemical and sensory evaluation of cider varieties obtained by single strain fermentation, co-fermentation and malolactic fermentation (with or without the addition of nutrients). Certainly, malolactic fermentation has a beneficial role in terms of sensory profile in cider making, and also co-fermentation between *Saccharomyces* and *Torulaspora* yeasts can lead to the formation of a specific volatile profile.

Chapter 5 presents the efficiency of malolactic fermentation, in tandem with alcoholic fermentation, which is a great advantage from an economic point of view, thus reducing the process time of the technological fermentation operation. The efficiency of co-fermentation of *Saccharomyces* with *Pichia* yeast has also been investigated, the latter being recently more widely studied and recognised for the advantages it offers to fermented beverages.

Chapter 6 presents the advantages of using encapsulated yeast in the production of sparkling cider by the traditional method, both technologically and in terms of the chemical and sensory characteristics of the product. Secondary fermentation and the production of sparkling cider results in the formation or degradation of some compounds. These compounds are closely related to the chemical composition of the apple juice, respectively the base cider, but the study showed that secondary fermentation resulted in a significant increase in polyphenols, but in contrast, some esters were decomposed.

The general conclusions were:

1. Technological perspectives:
   - To achieve balanced cider flavors it is crucial to understand the diverse composition and aroma of traditional apple varieties. The blending of apple juice from different varieties, together with technological optimization and selection of microorganisms are the key to produce high-quality natural beverages.
   - Alcoholic fermentation in tandem with malolactic fermentation, under the action of *Saccharomyces* yeast and LAB *L. plantarum* and *O. oeni*

have the technological effect of reducing fermentation time, and also facilitated the formation of lactic acid which led to increased the complexity of sensory profile.

- The use of encapsulated yeasts in the production of sparkling cider has many technological advantages: high cell densities, product yield improvement, lowered risk of microbial contamination, better control and reproducibility of the processes, easier biomass separation and potential cost savings.

2. Sensory profile insights:
   - Malolactic fermentation induced by *O. oeni* facilitated the increase of lactic acid, in some cases even a threefold rise (from 2.27 g/L in AF3 to 6.96 in MF3A and 7.15 g/L in MF3B), having a significant impact on sensory characteristics.
   - Cider obtained by co-inoculation of *T. delbrueckii* and *S. cerevisiae*, recorded significantly higher concentrations of volatile compounds, and moreover, the apple flavour was perceived most intensely in these samples.
   - The ester content increased following alcoholic fermentation in both single strain and co-fermentation, compared to apple juice, and even in low concentration, they provide fruity, floral and caramel notes in cider.
   - The apple cider co-fermented with *Pichia* and *Saccharomyces* stood out for its sweet flavor, strong apple aroma, and fruity notes, due to the benefit of *Pichia* yeast to increase the variety of esters.
   - The single strain *S. cerevisiae* fermented cider, obtained from "Topaz" and "Red topaz" apple juice, had the highest quantity of phenolic compounds (522.99 mg/L) and was characterised by a strong bitter taste.
   - The production of cider using various types of yeasts and LAB in co-fermentations results in an improved sensory profile, forming compounds that contribute to the complexity of its aroma and flavor.

3. Improvement of the bioactive profile:
   - At the end of the secondary fermentation of sparkling cider, the amount of polyphenols was higher in all samples compared to the base cider. Moreover, in one of the samples it doubled. In addition to the organoleptic qualities they confer, polyphenols could potentially contribute to the improvement of the bioactive profile due to their antioxidant, anti-inflammatory and free-radical scavenging.

- The classes of polyphenols known for their strong anti-inflammatory and antiplatelet effects along with antioxidant and metal chelating are benzoic acid and hydroxycinnamic acids which also contribute to the flavor of cider, and procyanidins which are responsible for the bitterness and astringency of apple juices and ciders.

Future perspectives:

1. Further analyses can be conducted to observe the influence of cider storage on the chemical compounds, or to identify and study other compounds with an effect on the taste and aroma of cider

2. Future work can investigate many other numerous *Saccharomyces*, non-*Saccharomyces* yeasts and lactic acid bacteria, taking into account several variants of co-inoculation, or different fermentation conditions, as they each have an impact on the sensory profile and chemical content of the cider.

3. Studies using encapsulated yeasts may include other, more economically efficient, methods of making sparkling cider, as well as obtaining cider with residual sugar.

4. Future work could investigate more local species of apple varieties in order to obtain cider, which would help both cider producers and apple growers.

Originality and personal contributions

The results presented in this thesis can be considered helpful for the scientific community in the food and biotechnology fields. The studies presented can be considered comprehensive for the valorisation of apples in the fermented beverage industry via biotechnological processes with the final aim to be integrated in large-scale cider production.

Identifying and integrating the performance of traditional apple beverages fermented with selective cultures of yeasts and LAB (mono- and co-cultures) can increase the aroma and taste characteristics. The selective cultures represent a possibility of creating a specific fermentation mix to obtain target compounds (esters, higher alcohols, acids) that contribute to future applications. Also, this thesis represents a comprehensive report related to the analysis of apple juice and cider before and after fermentation and maturation processes.

The innovative approach of the present study consists of both the design and the methods applied in the research. Apple juices obtained from 3 locally grown varieties were used and the variability of yeasts, LAB, presence or absence of nutrients led to a complex study. Comparison of cider obtained by both single strain fermentation, co-fermentation, impact of malolactic fermentation, and secondary fermentation led to a better understanding of biochemical processes and traceability of chemical compounds.

The compounds that have the highest influence on cider quality, according to the reviewed literature, have been investigated (sugars, organic acids, polyphenols, volatile compounds and amino acids) and the results may be of interest to both the scientific community and to engineers and technologists in the alcoholic beverage industry considering the impact of these compounds on the quality of the finished product.

The proposal of the cider production technology in terms of the variability of the microorganisms used is an original aspect that has not been observed in previous studies.