

## Article

# Impact of Pure, Co-, and Sequential Fermentations with *Hanseniaspora* sp. and *Saccharomyces cerevisiae* on the Volatile Compounds of Ciders

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**Abstract:** Pure, co-, and sequential fermentations of *Hanseniaspora uvarum*, *H. guilliermondii*, and *Saccharomyces cerevisiae* strains were evaluated to improve the aromatic quality of ciders. In sequential fermentations, *Hanseniaspora* strains were used as starter, followed by *S. cerevisiae* inoculation succeeding one, two, and three days of fermentation. Kinetics, physicochemical parameters, and volatile compounds were assessed during 10 days of fermentation. The headspace technique was used to capture the volatile compounds from the ciders obtained in each experiment and analyzed by gas chromatography. Fermentations with pure strains of *Hanseniaspora* sp. showed a high population ( $>10^{10}$  CFU/mL) but had a low fermentation rate (2.3–3.8 CO<sub>2</sub> g/L/d), low consumption of amino acids (20–40 mg/L) with a high residual content, high sugar consumption (80–90 g/L), and low alcohol content ( $<2.0\%$  v/v). The *H. uvarum* strain produced a notably high ester content (245 mg/L). In the co-fermentations, *H. guilliermondii* with *S. cerevisiae* highlighted a significant production of higher alcohols, similar to that produced by *S. cerevisiae* alone (152–165 mg/L). In general, the maximum fermentation rate of the sequential inoculations was lower than co-fermentations but showed low residual nitrogen content ( $<69$  mg/L) and good conversion of sugars into ethanol (4.3–5.7% v/v). The highest concentrations of volatile compounds were observed in treatments involving the two non-conventional strains: *H. uvarum* with *S. cerevisiae* inoculation after three days (564 mg/L) and *H. guilliermondii* after just one day (531 mg/L) of fermentation. These differences stemmed from the metabolic activity of the strains. *H. uvarum* was influenced by the presence of *Saccharomyces*, whereas *H. guilliermondii* did not exhibit this effect. Thus, a pure *H. uvarum* inoculum has the potential to produce a demi-sec cider with low alcohol content and high content of esters, contributing to a fruity aroma. In addition, ciders with sequential inoculation were the most promising for dry cider processing concerning fermentation parameters and bioaroma enrichment.

**Keywords:** non-*Saccharomyces*; cider fermentation; *Hanseniaspora uvarum*; *Hanseniaspora guilliermondii*; cider quality



**Citation:** Sola, I.M.M.S.; Evers, L.D.; Wojcickowski, J.P.; Assis, T.M.d.; Marinho, M.T.; Demiate, I.M.; Alberti, A.; Nogueira, A. Impact of Pure, Co-, and Sequential Fermentations with *Hanseniaspora* sp. and *Saccharomyces cerevisiae* on the Volatile Compounds of Ciders. *Fermentation* **2024**, *10*, 177. <https://doi.org/10.3390/fermentation10040177>

Academic Editor: Antonio Morata

Received: 25 February 2024

Revised: 12 March 2024

Accepted: 22 March 2024

Published: 23 March 2024



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## 1. Introduction

Cider, or hard cider, consists of sparkling beverages obtained by the total or partial alcoholic fermentation of apple musts from blends of dessert and industrial apples. Apple varieties and cider processing technology vary between countries, regions, and producers worldwide [1,2]. This impacts its diversity of sensory attributes, such as color, turbidity,

acidity, astringency, sweetness, carbonation, and foam. However, there is a consensus that the fruity aroma of ciders, mainly from esters and higher alcohols, is a significant indicator of quality [3].

Traditionally, the alcoholic fermentation of ciders made only with *Saccharomyces* sp. ensures a complete fermentation and avoids sensory deviations. However, pure *S. cerevisiae* inoculum, mainly in the presence of potassium metabisulphite, will result in no notable differences in the aromatic quality of ciders and can provide sensory notes such as a neutral or weak aroma [1,4]. On the other hand, with some non-*Saccharomyces* strains as inoculum, it is possible to increase and improve the flavor of fermented beverages, including cider. Regarding ciders, sensory notes of “fruity, floral, and fruit aromas” may appear [2]. The use of non-*Saccharomyces* in cider processing has been researched since the 1990s [5]. However, recent studies with these strains were assessed, as single cultures or in co-inoculation with *S. cerevisiae*, for their capacity to increase the flavor complexity of cider [2,4,6–8].

Among the non-*Saccharomyces*, *Hanseniaspora uvarum*, frequently isolated from ciders, has been one of the most studied [4,9]. The ability of *Hanseniaspora* species to develop the aroma profile of cider, wine, and beer, even when in mixed-culture fermentations with *S. cerevisiae*, has been demonstrated in several studies [4,9–12]. Recently, *Hanseniaspora guilliermondii* was isolated from apple must [13]. However, *H. guilliermondii* was not yet employed, to the best of our knowledge, in mixed and sequential fermentations with *S. cerevisiae* for cider bioflavoring. The positive impact of this species on ciders’ aromatic characteristics could be promising.

Nevertheless, there are still limited data on flavor-active cider yeasts, and further research is needed to identify novel strains with interesting properties suitable to enrich cider flavor. Therefore, the present study aimed to evaluate the physicochemical properties, kinetics parameters, and aromatic profile of ciders, obtained by pure, co-, and sequential fermentation of *H. uvarum*, *H. guilliermondii*, and *S. cerevisiae*.

## 2. Materials and Methods

### 2.1. Materials

Gala apples (100 kg) samples were obtained in Ponta Grossa, Paraná, Brazil. To check the ripeness of these apples, the starch-iodine test was employed [14]. The iodine value fell between the range of 4–5, which indicates the ripe stage of the apples. The *H. uvarum* and *H. guilliermondii* strains were previously isolated from apple must [13] and identified by molecular profile analysis (PCR-fingerprinting) using the PCR mini/microsatellite (MSP-PCR) technique with synthetic oligonucleotide (GTC) 5 [15], along with DNA extraction [16]. For sequencing, the D1/D2 domains of the larger subunit of the rRNA gene were amplified, and the sequences obtained were compared with those deposited in the GenBank database using the Basic Local Alignment Search Tool. The *Hanseniaspora* strains were stored at  $-80\text{ }^{\circ}\text{C}$  with 20% sterile glycerol. The fermentative yeast used was *S. cerevisiae* Bouquet (Fermol Bouquet, ref. PB 2004, AEB Group, Brescia, Italy), chosen to produce high concentrations of volatile compounds, which contributed to a fruity and floral aroma [17].

The standards of volatile compounds were ethanal, ethyl propanoate, ethyl 3-methyl butanoate, propyl ethanoate, 2-methylpropyl ethanoate, ethyl butanoate, hexanone, 2-heptanone, 2-methyl-1-butanol, 2-hexanol, 2-octanone and ethyl hexanoate, purchased from Interchim (Analytical grade, Montluçon, France); ethyl ethanoate, butyl ethanoate, 3-methylbutyl ethanoate, hexyl ethanoate, 2-hydroxyethyl propanoate, 1-hexanol, ethyl octanoate, ethyl decanoate, butanoic acid, diethyl butanedioate, 2-phenylethanol, ethyl dodecanoate, and octanoic acid, all acquired from Sigma–Aldrich ( $\geq 99.7\%$ , Steinheim, Germany); and 3-methyl-1-butanol was purchased from Merck ( $\geq 97\text{--}99\%$ , Darmstadt, Germany). The aqueous solutions were prepared using ultra-pure water (Millipore, São Paulo, Brazil). The culture media and reagents used were of analytical grade.

## 2.2. Methods

### 2.2.1. Processing of Apple Must

The commercial apples were carefully selected, weighed, and sanitized. The fruits were crushed and pressed (AGM Máquinas, Bento Gonçalves, RS, Brazil) at 294 kPa for 5 min, resulting in the extraction of the integral apple must. The physical yield ( $\eta_p$ ) averaged 72.5%. Apple must was blended and depectinized using a pectinolytic enzyme (Pectinex-Batch<sup>®</sup> 1201371L, Novozymes Latin America LTDA, Araucária, Brazil) at 3.0 mL/hL at room temperature ( $25 \pm 2$  °C) for 4 h. The depectinized apple must was racked, filtered in a line filter with a 0.5  $\mu\text{m}$  opening (Hidro Filtros do Brasil<sup>®</sup>, Joinville, Brazil) at 0.5 bar, and stored at  $-18$  °C until cider processing.

### 2.2.2. Processing of Cider

The apple must was transferred to glass fermenters of 1450 mL (sterilized in an autoclave 121 °C/15 min, previously) with a working volume of 1200 mL, and kept in a climate room at a temperature of 20 °C. Antibiotic chloramphenicol was added to the apple must at 0.01% (Henrifarma Supplier) to control bacterial growth. The *S. cerevisiae* was inoculated with an initial population of approximately  $4.0 \times 10^6$  CFU/mL and  $2.0 \times 10^6$  CFU/mL for *Hanseniaspora* sp. To reach this population, one colony of each strain of *Hanseniaspora* sp., isolated in YMA (Merck) in 5 mL of GPBY broth—glucose peptone broth yeast (Merck), was inoculated, and after incubation for 24 h at 25 °C, the culture was transferred to 100 mL of the same broth, time, and temperature, reaching a population of approximately  $10^{12}$  CFU/mL [18]. Then, a sufficient volume was collected to achieve the desired population in fermenters (about  $2.0 \times 10^6$  CFU/mL). On the tenth day, fermentation was stopped (time required to keep residual sugar) and the cider was centrifuged at  $10,200 \times g$  at 5 °C (Hitachi Himac CR21GII centrifuge, Tokyo, Japan) for 20 min, then racked, bottled, and stored at  $-18$  °C before further analysis.

The strains *H. uvarum*, *H. guilliermondii*, and *S. cerevisiae* were inoculated as either pure or monoculture (Hu; Hg, and Sc, respectively), mixed or co-fermentation (Hu + Sc and Hg + Sc), and sequential, with the first inoculum being *Hanseniaspora* sp. The *S. cerevisiae* was inoculated after one (Hu + Sc 1d and Hg + Sc 1d), two (Hu + Sc 2d and Hg + Sc 2d), and three days of fermentation (Hu + Sc 3d and Hg + Sc 3d). The experiments were carried out in triplicate.

### 2.2.3. The Growth Curves of Yeast

The total yeast counting was performed in culture medium potato dextrose agar (PDA, Acumedia<sup>®</sup>, Lansing, MI, USA), then hydrated and sterilized in an autoclave (Phoenix<sup>®</sup> VA 13811, Araraquara, São Paulo, Brazil) for 15 min (121 °C, 1.01 kgf/cm<sup>2</sup>). Ten milliliters of culture medium and 50  $\mu\text{L}$  of 10% tartaric acid (Biotec<sup>®</sup>, Pinhais, Paraná, Brazil) were poured into 90 mm Petri dishes (J. Prolab<sup>®</sup>, São José dos Pinhais, Paraná, Brazil). Subsequently, the solid medium received 100  $\mu\text{L}$  of the sample with scattering and using a sterile Drigalski spatula. The plates were then incubated at 26 °C for 24 h, with subsequent counting of specific colonies. The results were expressed as CFU/mL.

To perform the plate count of *Hanseniaspora* sp., aliquots of 1.0 mL suspension obtained in the recovery were withdrawn. After serial dilutions, the plate surfaces were sown using a Drigalski handle in YMA. The plates were incubated at 25 °C for 48 h [19].

### 2.2.4. Monitoring of Fermentation and Characterization of Ciders

Fermentation was monitored by the loss of mass from the system caused by the release of CO<sub>2</sub>, and the weight was determined every two hours with a sensitivity of 0.01 g (BL3200h, Shimadzu-Brazil, São Paulo, Brazil) for 10 days [20]. The fermentation rate was calculated by the variation in the loss of CO<sub>2</sub> vs time, Equation 1, where V is the fermentation rate (CO<sub>2</sub> g/L/day),  $\Delta\text{CO}_2$  is the variation in production of CO<sub>2</sub> (g/L), and  $\Delta t$  is the variation in time (days):

$$V = \Delta\text{CO}_2 / \Delta t \quad (1)$$

Samples of the apple juice and cider were analyzed for total reducing sugars (TRS) by the traditional method of Somogyi-Nelson [21]. The ethanol content was determined by an ebulliometer (accuracy of 0.1%) and expressed as percentage by volume. Total titratable acidity was expressed as malic acid (g/L), volatile acidity was expressed as acetic acid (g/L), and total nitrogen was determined by the Kjeldhal method [22]. The pH was determined using a pH potentiometer (pH digital micro process Tecnal<sup>®</sup>, model TEC3-MP, Piracicaba, São Paulo, Brazil). All the analyses were performed in triplicate.

#### 2.2.5. Analysis of Volatile Compounds

The capture of compounds using headspace was performed according to the method of Saerens et al. [23] with modifications. Samples (6 mL) of apple juice and ciders were placed in glass vials with a capacity of 20 mL. A solution of internal standards containing 50 µL of heptanoic acid (Merck<sup>®</sup>) and 20 µL of 4-methyl-1-amyl alcohol (Merck<sup>®</sup>) was added.

Before analysis, the samples remained for 10 min at 60 °C under agitation in the oven of the automatic injector YL 6100 GC (Young Lin Instrument<sup>®</sup> Gas Chromatograph, Anyang, Republic of Korea) equipped with a flame ionization detector (FID) and a 30 m-long capillary column (Phenomenex) with an internal diameter of 25 µm and 0.25 µm-thick ZB-WAX film.

The analysis of aromatic compounds was performed by gas chromatography according to the method of Pietrowski et al. [11]. The injector temperature was 220 °C, the detector temperature was 230 °C, and the carrier gas was nitrogen at a flow rate of 2.5 mL/min. The injection technique was split 1:1.2 and the gas chromatography automatic injection mode was used, with a volume of 1500 µL. The analysis conditions were programmed with an initial temperature of 40 °C for 5 min and an increase of 10 °C/min to 150 °C, before remaining at this temperature for 10 min. This was followed by a rise of 10 °C/min to 200 °C, remaining there for 5 min, and a further rise of 10 °C/min to 220 °C, where it remained for 16 min. The compounds were identified by comparing retention times with those obtained in the reference solution.

#### 2.2.6. Chromatographic Analysis of Amino Acids

A solution containing 3 mL of samples and 2 mL of 0.25 mmol/L norleucine (to monitor the recovery rate) was prepared and centrifuged at 13,000 g, 4 °C for 20 min (Hitachi Himac CR 21 GII, Tóquio, Japan). The supernatant was filtered through a 0.2 µm nylon syringe (Waters, Milford, CT, USA). The amino acids were derivatized using a Waters AccQ Tag<sup>™</sup> reagent kit (flask 1:200 mmol/L borate buffer, pH 8.8; flask 2A: 6-aminoquinolil-N-hydroxysuccinimidyl carbamate, AQC; flask 2B: acetonitrile). Identification and quantification were performed by HPLC Waters 2695 Alliance (Milford, CT, USA) with an FLD 2475 fluorescence detector, according to the AccQ Tag<sup>™</sup> kit methodology (Waters, Milford, CT, USA), with a Pico Tag column (4 µm, 3.9 × 150 mm) [1].

#### 2.2.7. Statistical Analysis

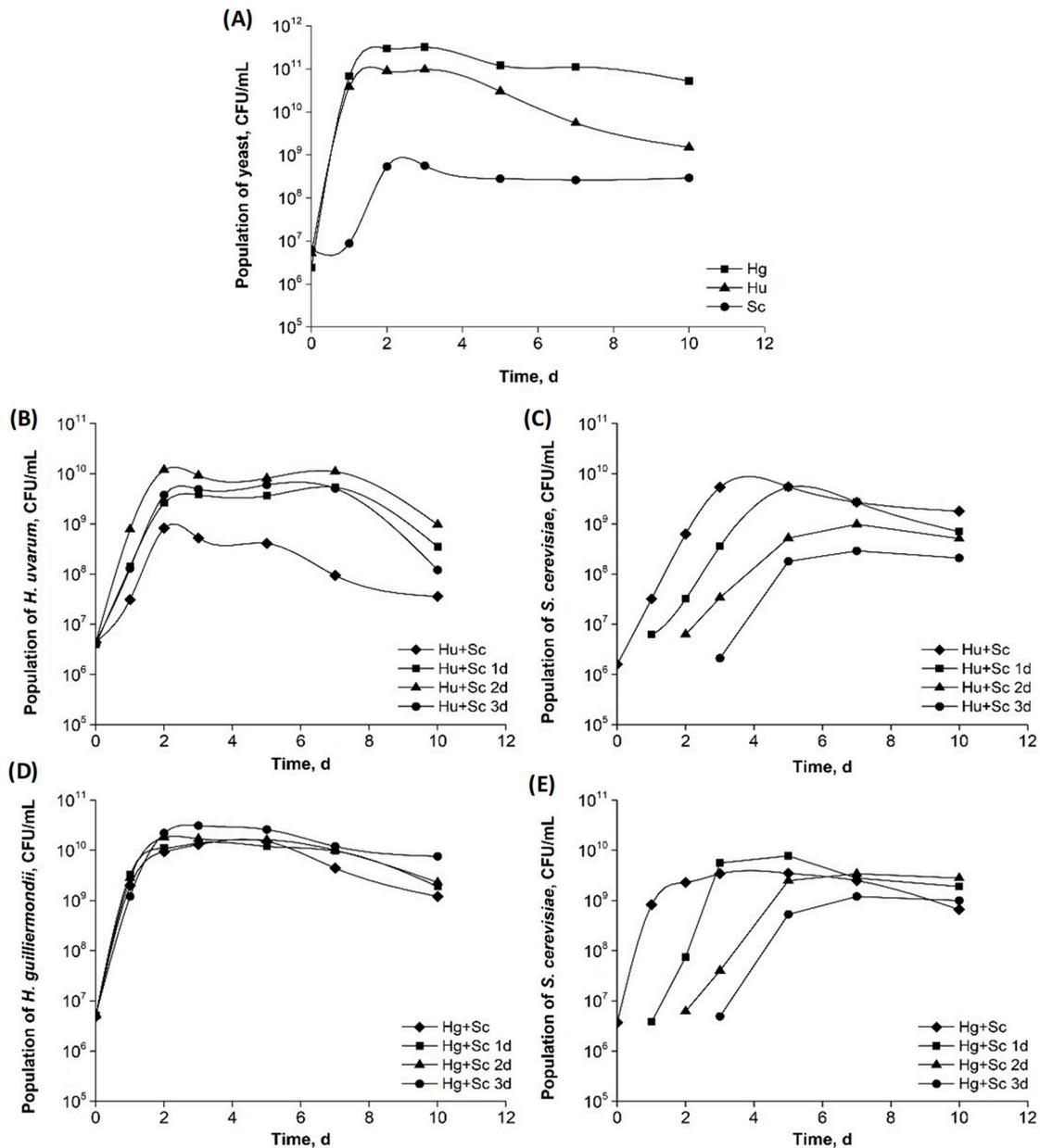
The data were shown as average and standard deviation. The homogeneity of variance was verified by Levene's test or the F-test ( $p \geq 0.05$ ). The differences between the samples were evaluated using one-way ANOVA, followed by Fisher's LSD test. The statistical analysis was performed using Statistica v. 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA) and Origin<sup>®</sup> 9.0 (OriginLab, Northampton, MA, USA).

### 3. Results and Discussion

#### 3.1. Yeast Growth Kinetics

The growth curves of the pure strains showed significant differences (Figure 1A). This indicates specific metabolisms during cider fermentation. The *H. uvarum* and *H. guilliermondii* strains reached their maximum population ( $10^{11}$  CFU/mL) on the first day of fermentation. In comparison, *S. cerevisiae* reached a lower population ( $10^8$  CFU/mL)

after three days of alcoholic fermentation and remained stable during the stationary phase (8 days). *H. guilliermondii* had the highest population, and it remained high during the stationary phase. However, *H. uvarum* showed a significant population reduction during this same phase (Figure 1A). The same behavior was observed in co-fermentation with *S. cerevisiae* (Figure 1B).



**Figure 1.** Rate of yeast growth with inoculum of pure (A) (Hg: *Hanseniaspora guilliermondii*; Hu: *Hanseniaspora uvarum*; and Sc: *Saccharomyces cerevisiae*); mixed (B,C) (Hu + Sc) and (D,E) (Hg + Sc); and sequential *H. uvarum* (B,C) and *H. guilliermondii* (D,E). Note: 1d, 2d, and 3d represent days of alcoholic fermentation of *Hanseniaspora* strains in which the *S. cerevisiae* inoculum was performed.

Figure 1B,C correspond to the growth in co- and sequential fermentations of *H. uvarum* and *S. cerevisiae*, respectively. The growth of *H. uvarum* was affected by co-fermentation with *S. cerevisiae* (Figure 1B). On the other hand, the growth of *S. cerevisiae*, in co- and sequential fermentation with one day of inoculum, provided higher growth than that observed in the pure strain (Figure 1A,C). In these two treatments, there was no competition for nutrients, but *H. uvarum* probably released some growth factor into the medium. However, with the

*S. cerevisiae* inoculum after 2 and 3 days, it was possible to observe a smaller population, though it was similar to the pure yeast (Figure 1A,C), indicating a possible depletion of nutrients essential to the growth of *S. cerevisiae*.

The growth of *H. guilliermondii* was not affected by the inoculum in co- and sequential fermentation (Figure 1D), demonstrating better adaptation to competition with *S. cerevisiae*. The *S. cerevisiae* showed the same growth behavior observed with *H. uvarum* (Figure 1E).

### 3.2. Characterization of Ciders

Table 1 shows the composition of apple must. The values correspond to the Gala apple must at the stage of ripeness used [1,3]. This table also shows the composition values of ciders with different yeast inoculums after 10 days of fermentation.

The *H. guilliermondii* strain was able to consume larger quantities of sugar (89.6 g/L) than the *H. uvarum* strains (80.8 g/L). *S. cerevisiae* consumed only 10 to 20 g/L more sugar than the *Hanseniaspora* sp. strains, demonstrating the ability of the non-Saccharomyces strains to ferment apple sugars (glucose, fructose, and sucrose). According to Moreira et al. [24], during fermentation, *H. uvarum* metabolize glucose and fructose similarly, but *H. guilliermondii* has a different behavior (fructophilic), consuming fructose more quickly than glucose.

The ethanol content produced by the *Hanseniaspora* sp. strains was <2.0% vol. after 10 days of fermentation. This shows a low conversion of sugar into ethanol when compared to *S. cerevisiae* (>5.0% vol.). Moreira et al. [24] found higher ethanol concentration with longer fermentations, reaching 5.4% vol. for *H. uvarum* and 7.2% vol. for *H. guilliermondii*. However, these results indicate that pure *Hanseniaspora* sp. inoculum with 10 days of fermentation can be used to develop low-alcohol ciders. There is a growing tendency to reduce the concentration of alcohol in commercialized alcoholic beverages [25].

The ciders made with mixed inoculum showed the highest fermentation rates and high conversion of sugar into ethanol. However, when the inoculum was sequential, the reduction in fermentation rate, and consequently in ethanol production, was proportional to the inoculation time of *S. cerevisiae* (Table 1). In France, slow fermentation by reducing biomass is used to improve aromatic quality and facilitate the logistics of cider processing operations [1].

Total Kjeldahl nitrogen content classifies apple must as a high concentration (>120 mg/L) [17]. Nitrogen is mostly found in apples in the form of amino acids, such as asparagine, glutamine, aspartate, glutamate, and serine, which together account for 86–95% of the nitrogen portion of the juice [17]. In addition, these amino acids are the most relevant precursors and intermediates for the biosynthesis of many volatile compounds [11,26]. In ciders elaborated with pure inoculum, nitrogen consumption by *Hanseniaspora* sp. strains were between 35–45 mg/L. However, *S. cerevisiae* consumed 87 mg/L. This difference in consumption is related to the size of the *S. cerevisiae* cell, which can be twice as large as that of *Hanseniaspora* sp. Nitrogen consumption was similar (around 80 mg/L) in ciders with mixed and sequential inoculum. This consumption can mostly be attributed to *S. cerevisiae*, which used similar amounts when inoculated in pure culture (Table 1). Therefore, this nutrient was not limiting for cell growth (protein synthesis) and fermentation activity during the initial stages of alcoholic fermentation.

**Table 1.** The chemical composition and kinetics parameters of ciders (10 days of fermentation) elaborated with different yeast inoculums (pure, mixed, and sequential).

Analytical Parameters	Apple Must	Ciders											
		Pure			Mixed		Sequential			Mixed		Sequential	
		Sc	Hu	Hg	Hu + Sc	Hu + Sc 1d	Hu + Sc 2d	Hu + Sc 3d	Hg + Sc	Hg + Sc 1d	Hg + Sc 2d	Hg + Sc 3d	
N mg/L	153.47 ± 0.02	66 <sup>b</sup> ± 7	106 <sup>a</sup> ± 6	118 <sup>a</sup> ± 3	69 <sup>b</sup> ± 6	69 <sup>b</sup> ± 6	67 <sup>b</sup> ± 4	64 <sup>b</sup> ± 4	65 <sup>b</sup> ± 3	74 <sup>b</sup> ± 3	68 <sup>b</sup> ± 3	72 <sup>b</sup> ± 2	
TA g/100 mL	0.34 ± 0.01	0.30 <sup>b</sup> ± 0.01	0.41 <sup>a</sup> ± 0.01	0.36 <sup>a</sup> ± 0.01	0.36 <sup>a</sup> ± 0.00	0.38 <sup>a</sup> ± 0.02	0.39 <sup>a</sup> ± 0.01	0.32 <sup>b</sup> ± 0.01	0.36 <sup>a</sup> ± 0.01	0.38 <sup>a</sup> ± 0.02	0.39 <sup>a</sup> ± 0.01	0.39 <sup>a</sup> ± 0.01	
VA g/100 mL	0.02 ± 0.01	0.05 <sup>a</sup> ± 0.01	0.05 <sup>a</sup> ± 0.01	0.05 <sup>a</sup> ± 0.01	0.03 <sup>b</sup> ± 0.01	0.05 <sup>a</sup> ± 0.01	0.05 <sup>a</sup> ± 0.01	0.05 <sup>a</sup> ± 0.01	0.07 <sup>a</sup> ± 0.01	0.06 <sup>a</sup> ± 0.01	0.05 <sup>a</sup> ± 0.01	0.05 <sup>a</sup> ± 0.01	
RS g/100 mL	9.23 ± 0.09	0.25 <sup>g</sup> ± 0.01	3.86 <sup>a</sup> ± 0.04	2.47 <sup>b</sup> ± 0.11	0.78 <sup>e</sup> ± 0.03	0.79 <sup>e</sup> ± 0.01	1.23 <sup>d</sup> ± 0.06	1.51 <sup>c</sup> ± 0.01	0.18 <sup>g</sup> ± 0.01	0.40 <sup>f</sup> ± 0.01	0.59 <sup>f</sup> ± 0.01	1.12 <sup>d</sup> ± 0.01	
TS g/100 mL	11.78 ± 0.05	1.38 <sup>f</sup> ± 0.04	3.70 <sup>b</sup> ± 0.07	2.82 <sup>d</sup> ± 0.02	2.11 <sup>e</sup> ± 0.08	2.27 <sup>e</sup> ± 0.02	2.90 <sup>a</sup> ± 0.02	4.04 <sup>d</sup> ± 0.02	1.66 <sup>f</sup> ± 0.01	1.57 <sup>f</sup> ± 0.04	2.40 <sup>e</sup> ± 0.03	3.15 <sup>c</sup> ± 0.07	
Ethanol % vol.	-	5.4 <sup>a</sup> ± 0.1	1.9 <sup>f</sup> ± 0.1	1.3 <sup>e</sup> ± 0.1	5.1 <sup>b</sup> ± 0.1	5.0 <sup>b</sup> ± 0.1	4.9 <sup>c</sup> ± 0.1	4.2 <sup>d</sup> ± 0.1	5.5 <sup>a</sup> ± 0.1	5.4 <sup>a</sup> ± 0.1	5.2 <sup>b</sup> ± 0.1	4.9 <sup>c</sup> ± 0.1	
Vmax	-	11.5	3.8	2.3	11.9	9.3	8.0	7.5	14.5	10.1	8.5	6.5	
Time Vmax (d)	-	1.7	4.2	4.7	2.7	3.5	4.2	7.0	1.7	3.0	5.0	5.0	

Note: N: total nitrogen; TA: titratable acidity; VA: volatile acidity; RS: reducing sugars; TS: total sugars; Vmax: Maximum fermentation rate in CO<sub>2</sub> g/L/d; Time Vmax: Time to maximum fermentation rate; Hu: *H. uvarum*; Hg: *H. guilliermondii*; Sc: *S. cerevisiae*. 1d, 2d, and 3d represent days of alcoholic fermentation of *Hanseniaspora* strains days in which the *S. cerevisiae* inoculum was performed; Different letters on the same line indicate that there is a significant difference between the quantities in the samples ( $p \leq 0.05$ ).

### 3.3. Synthesis of Volatile Compounds

Table 2 shows the profile of volatile compounds formed in ciders with different inoculums after 10 days of alcoholic fermentation. Some classes of volatiles, such as esters (78–92% of the total), higher alcohols (6–16% of the total), carboxylic acids, ketones, and aldehydes (2–6%), are formed by yeasts during cider fermentation and contribute to the production of fruity aromas [1]. The ester content of the cider made with the pure culture of *H. uvarum* was four and six times higher than that produced with *S. cerevisiae* and *H. guilliermondii*, respectively. In addition, ciders with sequential fermentation containing *H. uvarum* had a high ester concentration (Table 2); however, the cider made with mixed culture had the lowest ester content. This indicates a possible interference in the biosynthesis of these compounds in the presence of *S. cerevisiae*. Esters are directly related to the formation of fruity aromas in fermented beverages; the concentration of these volatiles after the fermentation process depends on several factors, including the yeast strain and the amount of initial amino acids [17]. In cider fermentation, the amino acids aspartate, asparagine, and glutamate correlate with the production of 3-methyl-1-butanol, 2-phenylethanol, 2-hexanol, ethyl ethanoate, 3-methyl-ethanoate, ethyl octanoate, ethyl butanoate, ethyl decanoate, and hexyl ethanoate [2,17].

The formation of higher alcohols was much higher in the cider with pure *S. cerevisiae*, reaching 14 times more than the cider with *H. guilliermondii*. However, cider with a mixed culture of *H. guilliermondii* and *S. cerevisiae* showed similar results to cider with only *S. cerevisiae*, so there was no interference in the production of these compounds. In the sequential cultures with *H. guilliermondii* and *S. cerevisiae*, the same effect was confirmed, with a higher concentration of higher alcohols compared to the mixed and sequential *H. uvarum* inoculums (Table 2). Studies have demonstrated that more than 90% of acids and higher alcohols (and their acetate esters derivatives) in wine are produced via central carbon metabolism (CCM) [25,27].

The compounds hexyl ethanoate, ethyl lactate, and 2-octanone showed lower values than the threshold odor, playing little or no role in the aroma of the ciders. Regardless of this, more than 99% of the volatile compounds identified were above the threshold value (Table 2), demonstrating the importance of these compounds in the formation of cider aroma. Consequently, the *H. uvarum* strain showed a greater propensity to produce esters, and the *S. cerevisiae* strain was greater in the formation of higher alcohols. Both the mixed culture and the sequential culture of *H. guilliermondii* with *S. cerevisiae* maintained a high formation of higher alcohols.

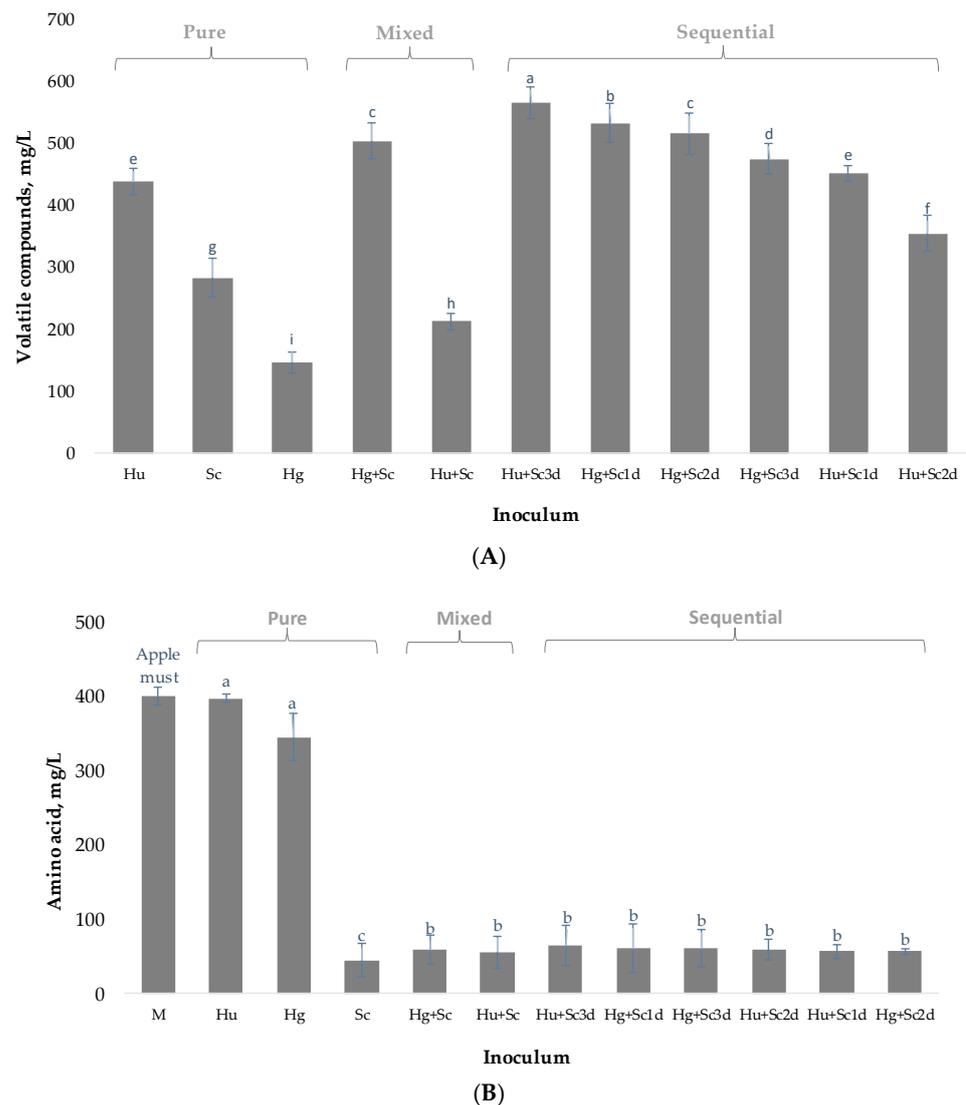
Figure 2A shows the sum of the volatile compounds, giving an overview of the production of each treatment. Considering that the production of volatiles in cider inoculated only with *S. cerevisiae* corresponds to the usual content, only pure *H. guilliermondii* and mixed *H. uvarum* and *S. cerevisiae* fermentations had lower contents. The other treatments showed higher values, with between 1.25 and 2.00 times more volatiles (Figure 2A).

The residual amino acids after 10 days of alcoholic fermentation can be observed in Figure 2B. Consumption by the *Hanseniaspora* sp. strains was between 20 and 50 mg/L and still resulted in high populations and production of volatiles in the pure cultures. High levels of residual nitrogen, usually in the form of amino acids, can cause microbiological instability in cider [17]. Nitrogen consumption was similar in all other treatments with the presence of *S. cerevisiae* at about 88%. Therefore, the use of pure *H. uvarum* fermentations requires further studies to maintain stability in the final product.

**Table 2.** Values of volatile compounds (mg/L) in ciders elaborated with different yeast inoculums (pure, mixed, and sequential).

Volatile Compounds mg/L	Different Inoculum and Volatile Composition after 10 Days											Odor Threshold		
	Pure			Mixed			Sequential			Mixed			Sequential	
	Sc	Hu	Hg	Hu + Sc	Hu + Sc 1d	Hu + Sc 2d	Hu + Sc 3d	Hg + Sc	Hg + Sc 1d	Hg + Sc 2d	Hg + Sc 3d			
Ester														
Ethyl ethanoate	26.9 <sup>gh</sup> ± 0.14	147 <sup>a</sup> ± 0.8	9.9 <sup>i</sup> ± 0.6	18 <sup>hi</sup> ± 2	78 <sup>d</sup> ± 7	91 <sup>c</sup> ± 9	133 <sup>b</sup> ± 13	32 <sup>g</sup> ± 3	43 <sup>f</sup> ± 4	48 <sup>ef</sup> ± 4	55 <sup>e</sup> ± 5	7.5 <sup>(1,2,3,6)</sup>		
Ethyl butanoate	0.50 <sup>b</sup> ± 0.01	<LOD	0.05 <sup>g</sup> ± 0.01	0.18 <sup>f</sup> ± 0.01	0.37 <sup>c</sup> ± 0.03	0.17 <sup>f</sup> ± 0.01	1.10 <sup>a</sup> ± 0.08	0.25 <sup>e</sup> ± 0.01	0.26 <sup>e</sup> ± 0.01	0.31 <sup>d</sup> ± 0.01	0.32 <sup>d</sup> ± 0.01	0.02 <sup>(1,2,3)</sup>		
Isopentyl acetate	2.88 ± 0.15	0.63 ± 0.03	<LOD	1.8 ± 0.2	2.1 ± 0.2	1.6 ± 0.2	1.7 ± 0.2	3.9 ± 0.3	3.1 ± 0.2	3.1 ± 0.2	2.4 ± 0.2	0.03 <sup>(1,2,3)</sup>		
Hexyl ethanoate	1.11 <sup>a</sup> ± 0.09	0.18 <sup>g</sup> ± 0.01	<LOD	0.13 <sup>g</sup> ± 0.01	0.25 <sup>f</sup> ± 0.02	0.35 <sup>e</sup> ± 0.03	0.26 <sup>f</sup> ± 0.03	0.43 <sup>d</sup> ± 0.01	0.56 <sup>cd</sup> ± 0.01	0.61 <sup>bc</sup> ± 0.01	0.61 <sup>bc</sup> ± 0.01	0.67 <sup>(2,3,6)</sup>		
2-hydroxyethyl propanoate	26 <sup>c</sup> ± 2	48 <sup>a</sup> ± 4	45 <sup>a</sup> ± 4	3.8 <sup>g</sup> ± 0.1	10.5 <sup>de</sup> ± 0.4	7.5 <sup>ef</sup> ± 0.3	14.5 <sup>d</sup> ± 0.6	8.91 <sup>e</sup> ± 0.03	8.41 <sup>e</sup> ± 0.03	13.1 <sup>de</sup> ± 0.1	15.3 <sup>d</sup> ± 0.1	1.8 <sup>(3)</sup>		
Ethyl octanoate	2.1 <sup>b</sup> ± 0.2	0.10 <sup>g</sup> ± 0.01	0.02 <sup>g</sup> ± 0.01	0.5 <sup>e</sup> ± 0.03	0.54 <sup>b</sup> ± 0.04	0.54 <sup>e</sup> ± 0.04	0.28 <sup>f</sup> ± 0.02	2.3 <sup>a</sup> ± 0.2	1.12 <sup>d</sup> ± 0.09	1.3 <sup>c</sup> ± 0.1	0.96 <sup>d</sup> ± 0.08	0.002 <sup>(1,6)</sup>		
Ethyl lactate	0.85 <sup>a</sup> ± 0.08	0.29 <sup>cd</sup> ± 0.03	0.09 <sup>f</sup> ± 0.01	0.09 <sup>f</sup> ± 0.01	0.10 <sup>f</sup> ± 0.01	0.17 <sup>e</sup> ± 0.01	0.05 <sup>f</sup> ± 0.01	0.69 <sup>b</sup> ± 0.05	0.27 <sup>d</sup> ± 0.02	0.34 <sup>c</sup> ± 0.03	0.21 <sup>e</sup> ± 0.02	0.5 <sup>(1,6)</sup>		
Diethyl succinate	8.0 <sup>a</sup> ± 0.5	6.2 <sup>b</sup> ± 0.4	4.3 <sup>cd</sup> ± 0.2	1.72 <sup>h</sup> ± 0.02	4.51 <sup>c</sup> ± 0.05	2.12 <sup>g</sup> ± 0.02	3.45 <sup>e</sup> ± 0.04	4.12 <sup>d</sup> ± 0.01	3.14 <sup>f</sup> ± 0.01	3.33 <sup>ef</sup> ± 0.01	2.20 <sup>g</sup> ± 0.01	0.02 <sup>(1,2,3)</sup>		
Ethyl dodecanoate	4.7 ± 0.4	4.3 ± 0.4	2.9 ± 0.2	1.79 ± 0.01	3.01 ± 0.01	2.10 ± 0.01	3.50 ± 0.01	3.65 ± 0.08	2.90 ± 0.06	3.59 ± 0.08	4.7 ± 0.1	0.5 <sup>(1)</sup>		
Aldehyde														
Ethanal	28.1 <sup>b</sup> ± 0.4	38.0 <sup>a</sup> ± 0.6	21.5 <sup>c</sup> ± 0.6	9.9 <sup>i</sup> ± 0.9	19 <sup>d</sup> ± 2	12 <sup>egh</sup> ± 1	27 <sup>b</sup> ± 2	11.2 <sup>ehi</sup> ± 0.8	12.5 <sup>fg</sup> ± 0.9	14 <sup>f</sup> ± 1	18 <sup>d</sup> ± 1	0.5 <sup>(3)</sup>		
Acid														
Butanoic acid	10.7 <sup>a</sup> ± 0.6	4.8 <sup>b</sup> ± 0.2	3.3 <sup>c</sup> ± 0.3	<LOD	<LOD	2.86 <sup>d</sup> ± 0.04	<LOD	11.0 <sup>a</sup> ± 0.4	<LOD	<LOD	<LOD	0.24 <sup>(5)</sup>		
Octanoic acid	5.0 <sup>a</sup> ± 0.1	<LOD	<LOD	1.62 <sup>h</sup> ± 0.03	1.75 <sup>g</sup> ± 0.03	1.96 <sup>f</sup> ± 0.03	1.74 <sup>g</sup> ± 0.03	3.7 <sup>b</sup> ± 0.1	3.2 <sup>c</sup> ± 0.1	2.47 <sup>e</sup> ± 0.07	2.67 <sup>d</sup> ± 0.08	10 <sup>(1,6)</sup>		
Higher alcohol														
3-methyl-1-butanol	116.1 <sup>a</sup> ± 0.8	22.0 <sup>h</sup> ± 0.1	12.4 <sup>h</sup> ± 0.4	60 <sup>f</sup> ± 5	64 <sup>ce</sup> ± 6	39 <sup>g</sup> ± 3	59 <sup>ef</sup> ± 5	112 <sup>a</sup> ± 10	76 <sup>bd</sup> ± 7	83 <sup>b</sup> ± 8	70 <sup>cd</sup> ± 6	30 <sup>(1,2,3)</sup>		
2-hexanol	10.1 <sup>a</sup> ± 0.6	0.28 <sup>i</sup> ± 0.02	0.13 <sup>i</sup> ± 0.01	2.4 <sup>fg</sup> ± 0.2	2.6 <sup>f</sup> ± 0.2	2.3 <sup>gh</sup> ± 0.2	1.8 <sup>h</sup> ± 0.2	8.0 <sup>c</sup> ± 0.5	9.0 <sup>b</sup> ± 0.5	6.0 <sup>d</sup> ± 0.3	5.2 <sup>e</sup> ± 0.3	15 <sup>(3)</sup>		
1-hexanol	0.78 <sup>f</sup> ± 0.01	<LOD	<LOD	0.38 <sup>g</sup> ± 0.01	1.39 <sup>c</sup> ± 0.03	0.85 <sup>e</sup> ± 0.02	1.40 <sup>c</sup> ± 0.03	1.88 <sup>a</sup> ± 0.06	1.10 <sup>d</sup> ± 0.04	1.56 <sup>b</sup> ± 0.05	1.58 <sup>b</sup> ± 0.05	0.11 <sup>(6)</sup>		
2-phenylethanol	39 <sup>a</sup> ± 2	<LOD	<LOD	11.1 <sup>g</sup> ± 0.02	15.74 <sup>e</sup> ± 0.02	7.86 <sup>h</sup> ± 0.01	12.78 <sup>f</sup> ± 0.02	31.0 <sup>b</sup> ± 0.5	19.5 <sup>d</sup> ± 0.3	23.0 <sup>c</sup> ± 0.4	18.5 <sup>d</sup> ± 0.3	10 <sup>(1,2,3)</sup>		
Ketone														
2-heptanone	<LOD	<LOD	0.04 <sup>e</sup> ± 0.01	<LOD	<LOD	<LOD	<LOD	1.38 <sup>b</sup> ± 0.04	5.4 <sup>a</sup> ± 0.2	0.31 <sup>d</sup> ± 0.01	0.44 <sup>c</sup> ± 0.01	0.0082 <sup>(4)</sup>		
2-octanone	<LOD	0.06 ± 0.01	<LOD	0.02 ± 0.01	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.15 <sup>(7)</sup>		

Note: Concentration of volatile compounds in mg/L; data shows the average of three replicates for headspace; Different letters in the same row indicate significant differences existing between the quantities in headspace samples ( $p < 0.05$ ); nd = not detected; LOD: limit of detection. Pure inoculum (Hu: *H. uvarum*; Hg: *H. guilliermondii*; Sc: *S. cerevisiae*), mixed inoculum (Hu + Sc and Hg + Sc), and sequential inoculum (Hu + Sc 1d; 2d, and 3d; and Hg + Sc 1d, 2d and 3d), 1d, 2d, and 3d represent days of alcoholic fermentation of *Hanseniaspora* strains in which the *S. cerevisiae* inoculum was performed. <sup>(1)</sup> Yu et al. [28]; <sup>(2)</sup> Li et al. [29]; <sup>(3)</sup> Wei et al. [8]; <sup>(4)</sup> Karl et al. [30]; <sup>(5)</sup> Arcari et al. [31]; <sup>(6)</sup> Baiano et al. [32]; <sup>(7)</sup> Wang et al. [33]. It is clear that the *H. uvarum* strain, as a pure and sequential inoculum, showed higher volatile compound content. The inoculum in a mixed culture of *H. uvarum* and *S. cerevisiae* had a low volatile content, making it an uninteresting inoculum. The same was observed with *H. guilliermondii* as a pure inoculum, which also showed low production of volatile compounds. However, in the fermentation with the mixed and sequential cultures of *H. guilliermondii*, the values were interesting and cannot be neglected.



**Figure 2.** Sum of volatile compounds (A) and residual amino acids (B) in ciders elaborated with different yeast inoculum (pure, mixed, and sequential) after 10 days of alcoholic fermentation. Note: M: apple must pure inoculum (Hu: *H. uvarum*; Hg: *H. guilliermondii*; Sc: *S. cerevisiae*), mixed inoculum (Hu + Sc and Hg + Sc), and sequential inoculum (Hu + Sc 1d; 2d, and 3d; and Hg + Sc 1d, 2d and 3d), 1d, 2d, and 3d represent days of alcoholic fermentation of *Hanseniaspora* strains in which the *S. cerevisiae* inoculum was performed.

#### 4. Conclusions

This research evaluated the physicochemical properties, kinetic parameters, and aromatic profile of ciders obtained by pure, co-fermentative, and sequential fermentation by *H. uvarum*, *H. guilliermondii*, and *S. cerevisiae*. In yeast growth, it was possible to observe a different metabolic behavior of volatile production in *H. uvarum* compared to *H. guilliermondii* in the presence of *S. cerevisiae* in co- or sequential fermentations. The ciders produced with pure *Hanseniaspora* sp. inoculum showed a high population but low fermentation rate, low amino acid consumption with high residual content, high sugar consumption, and low alcohol content (<2.0% v/v). However, the *H. uvarum* strain was the one that produced a high ester content (245 mg/L). In the co-fermentations, the highlight was *H. guilliermondii* with *S. cerevisiae* with a high production of higher alcohol, though similar to that produced by *S. cerevisiae* alone (152–165 mg/L). The presence of *S. cerevisiae* in both co- and sequential fermentations improved the fermentation parameters of the *Hanseniaspora* sp. strains.

In general, the maximum fermentation rate of the sequential inoculations was lower than that of the co-fermentations. In addition, they showed low residual nitrogen content and good conversion of sugars to ethanol. The highest concentrations of volatile compounds in these treatments were obtained for the two non-conventional strains, *H. uvarum* with *S. cerevisiae* inoculum, after three days, and *H. guilliermondii* after only one day. These differences were due to the metabolic activity of the strains. *H. uvarum* was influenced by the presence of *Saccharomyces*; however, *H. guilliermondii* did not show this effect.

Thus, pure *H. uvarum* inoculum has the potential to develop a demi-sec cider with a low alcohol content and a high content of esters responsible for a fruity aroma. In addition, the ciders with sequential inoculum were the most promising for industrial cider processing, in terms of fermentation parameters and bioaroma enrichment.

**Author Contributions:** I.M.M.S.S.: formal analysis, writing (original draft); L.D.E.: formal analysis, writing (original draft: formal analysis); T.M.d.A.: statistic and editing; J.P.W.: writing (original draft); M.T.M.: writing (review and editing); I.M.D.: methodology, writing (review and editing); A.A.: supervision, methodology, writing (review and editing); A.N.: resources, project administration, funding acquisition, writing (review and editing). All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Council for Scientific and Technological Development (CNPq) [Research project n. 406799/2023-7], the Araucária Foundation (FA), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES) [grant finance code 001].

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Data are unavailable due to privacy restrictions. Researchers can provide if necessary.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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