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Characterisation of *Diospyros kaki* (persimmon) vinegars produced with different microorganisms

Matteo Bordiga [a,*], Raffaele Guzzon [b], Marcello Manfredi [c], Elettra Barberis [c], Simona Grasso [d], Ana M. Benítez-González [e], Antonio J. Meléndez Martínez [e], Fabiano Travaglia [a], Marco Arlorio [a], Jean Daniel Coisson [a]

[a] Department of Pharmaceutical Sciences, Università degli Studi del Piemonte Orientale "A. Avogadro", Largo Donegani 2, 28100, Novara, Italy
[b] Fondazione Edmund Mach, Via Mach 1, 38010, San Michele all'Adige, Italy
[c] Department of Translational Medicine and Center for Translational Research on Autoimmune & Allergic Diseases – CAAD, Università degli Studi del Piemonte Orientale, corso Trieste 15/A, 28100, Novara, Italy
[d] School of Agriculture and Food Science, Science Centre South, University College Dublin, Dublin 4, Ireland
[e] Food Colour and Quality Laboratory, Facultad de Farmacia, Universidad de Sevilla, 41012, Sevilla, Spain

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ABSTRACT

The aim of this work was to evaluate the characteristics of nine kaki vinegars produced using different yeasts and bacteria traditionally involved in wine production, and to evaluate their acidity, density, total phenolic content, and antioxidant activity. Furthermore, the study characterized the volatile fingerprinting by headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS) and by two-dimensional gas chromatography coupled to mass spectrometry (GCxGC-TOF-MS). Finally, individual carotenoids were characterized using high performance liquid chromatography (HPLC). More than a thousand distinguishing molecules were found. It was discovered that vinegars fermented with S. *cerevisiae* produced a larger number of volatile chemicals. Among the three vinegars produced with this strain, the one fermented with *Acetobacter* vino seemed to have a more elaborate flavour profile than all the other samples. The vinegar produced utilizing the mixture of T. *delbruekii* and *Acetobacter* was the only kind to have a high concentration of carotenoids.

1. Introduction

Vinegar is a fermented food made of an aqueous solution of acetic acid and other constituents. Despite the use of different raw materials and productive processes, it is known worldwide as a food condiment and, more importantly, as a natural preservative due to the antimicrobial action of acetic acid (Ho et al., 2017). Vinegar is considered a functional food, for its antibacterial and antioxidant activity, effect on blood pressure, diabetes and cardiovascular diseases (Budak et al., 2014).

Vinegar is the result of two successive fermentations. The first step is the alcoholic fermentation of sugars present in the raw materials, mediated by yeasts, the second step is the oxidation of ethanol into acetic acid by acetic bacteria (Liu et al., 2022). The raw materials (vegetable juice from grapes, apples, apricots, etc.) rich of mono, disaccharide, starch and complex carbohydrates, also need a saccharification step before alcoholic fermentation to release fermentable sugars (Yetiman & Kesmen, 2015).

The persimmon (*Diospyros kaki*) is a perennial plant, widely grown in Asian countries, also present in Europe and north America. In addition to being consumed fresh, the persimmon fruit is used in preparation of various foods and it can also be used in vinegar production (Özdemir et al., 2022; Wang et al., 2022).

A possible way to improve fruit vinegar quality passes through the exploitation of a wide range of microbes, capable to contribute to the fermentations in a different manner, compared to the most known microorganisms, such as *Saccharomyces cerevisiae*. Mixed fermentation using *Lachancea thermotolerans* and *S. cerevisiae* allows the biological acidification of wine lacking in acidity due to clime southernization (Hranilovic et al., 2021). In winemaking of over ripe grapes, the use of *Torulaspora delbrueckii* ensures the moderation of volatile acidity and the disappearance of vegetal notes in favour of sweet and red fruity attributes (Azzolini et al., 2012). *Candida pulcherrima* is another yeast used in commercial application. It is known to produce an extra-cellular

* Corresponding author.
E-mail address: matteo.bordiga@uniupo.it (M. Bordiga).

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α-arabinofuranosidase that increases varietal aromas, terpenes, and volatile thiols. This yeast is also a higher esters producer in mixed fermentations with *S. cerevisiae* (Sadoudi et al., 2012). *Candida zemplinina* is another yeast that showed promising applications in alcoholic fermentation. It increases the accumulation of glycerol, lowering ethanol and increasing wine mouthfeel. Due to its fructophilic character it may help *S. cerevisiae* to accomplish alcoholic fermentation in high concentrated grape must (Prior et al., 2017). Finally, the genus *Saccharomyces*, outward the *cerevisiae* species, offers useful applications in beverages production. *Saccharomyces bayanus* is applied in at the production of fruit wines, sparkling wine, or lager beer with satisfactory results both in singular or in mixed fermentation with *S. cerevisiae*. The most interesting characters are the high volatile production and the capability to ferment at low temperature or high pressure (Rainieri et al., 2006).

Acetic bacteria are able to oxidise ethanol and other minor components of alcoholic beverages to accumulate acetic acid and peculiar organoleptic active compounds. The conversion of ethanol to acetic acid is an ingenious strategy to obtain energy and prevent the development of other microorganisms, considering the high toxicity of acetic acid (Wang et al., 2022). Among the main species of acetic bacteria with technological interest, *Acetobacter aceti* and *Gluconobacter oxidans* are generally involved in vinegar production. *Gluconobacter* can metabolize sugars, which are abundant in raw materials, such as fruits, rich in glucose and fructose and in the first steps of vinegar production. Conversely, *Acetobacter aceti* can oxidise ethanol to acetic acid, surviving at concentration up to 5-6%, with a key role in the production of several fruit vinegars (Oliveira et al., 2010).

Persimmon vinegar is little known in Western countries but is popular in Asia for its health properties. In Italy the consumption of this fruit is constantly reducing, especially among young people who prefer other types of fruit to be eaten fresh, thus causing a surplus in production and consequent waste of raw material, therefore alternative uses for this fruit should be explored.

Different production conditions were evaluated, focusing the attention on two areas: volatile aroma and carotenoids. The *Saccharomyces cerevisiae* strain ATCC 9763 was selected to be used as a fermentation standard, since it is one of the most used yeasts in the food industry. Unlike the previous one, *Saccharomyces uvarum* ATCC 36362, has a greater development capacity at low temperatures and is responsible for the production of different aromatic characters. Finally, *Torulaspora delbruekii* DSM 70483, despite being less resistant to high concentrations of ethanol, was selected because it is more osmotolerant and is currently used in different food fermentations. *Acetobacter aceti* DSM 3508 and *Gluconobacter oxydans* ATCC 621H were selected among the bacteria because they are the main genres used in industrial fermentations. Gluconobacter can degrade sugars unlike Acetobacter which does not metabolize them during the production of acetic acid. Finally, it was also decided to use a spontaneous culture of acetic bacteria isolated from an artisanal vinegar, called Acetobacter_vino, to evaluate the impact in the production of vinegar of a microbial consortium as usually found in the artisanal vinegar factories (Wang et al., 2016).

In this study a comprehensive two-dimensional gas chromatography (GCxGC) and mass spectrometry approach in combination with headspace solid phase microextraction (HS-SPME) was used to deeply investigate the volatiles components of persimmon vinegars produced with different microorganisms. Furthermore, the study was focused on the qualitative characterization of volatile fingerprinting by headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS). *Diospyros kaki* is a source of bioavailable carotenoids in humans, including lycopene, lutein and the provitamin A carotenoids α-carotene, β-carotene, β-cryptoxanthin. Important qualitative and quantitative differences have been reported in samples (Bordiga et al., 2019; Dias et al., 2018). Carotenoids are versatile food components of interest due to the accumulating evidence that they can intervene in health-promoting biological actions contributing to reduce the risk of developing diverse diseases (several types of cancers, cardiovascular disease, metabolic, bone, skin, or eye disorders, etc.), and to improve cognition (Meléndez-Martínez et al., 2019; Meléndez-Martínez, 2019). Hence, their interest as components of products intended for health promotion and well-being (functional foods, supplements, nutricosmetics, etc.) (Meléndez-Martínez et al., 2021).

2. Materials and methods

2.1. Samples and microorganisms

For this work, a total of 3 kaki wines and nine kaki vinegars were produced (Table 1). These were then further divided into 3 types, resulting from the different stages of vinegar production. The selection of microorganisms (bacteria and yeasts) used in this study was made based on some of their characteristics. The microorganisms selected for this study were: *Saccharomyces cerevisiae* (strain ATCC 9763); *Saccharomyces uvarum (*ATCC 36362); *Torulaspora delbruekii (*DSM 70483); *Acetobacter aceti (*DSM 3508); *Gluconobacter oxydans (*ATCC 621H) and *Acetobacter_vino*. Microorganisms are collected by official international collection as indicated in the identification code of each of them (DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen; ATCC: American Type Culture Collection). Yeast and bacteria were multiplied prior the utilization in alcoholic and acetic fermentations in the appropriate synthetic medium: Yeast Medium Broth (YPD, Bacteriological peptone, 20 g/L, Glucose, 20 g/L, Yeast extract, 10 g/L) for yeast, ATCC Medium 1 (Yeast Extract, 5.0 g/L, Peptone, 3.0 g/L, Mannitol, 25.0 g/L) for acetic bacteria. The cultures were stored at 30°C and incubated for 3 (yeast) or 5 (bacteria) days. After growth, each culture was centrifuged at 4000 rpm for 10 min, cells were separated from the medium and washed with peptone water (1 g/L of Mycological peptone, Oxoid). The operation was repeated twice. At the end cells culture were resuspended in peptone water to reach a nominal concentration of 8 log CFU/mL and utilized to start the fermentation process.

To produce the samples, 12 kg of kaki were used with an average value of 16.5° Brix. The kakis were ground using a blender, then 0.05 g/L of Rapidase Clear pectolytic enzyme (Oenobrands SaS, F) was added to the blended kaki. After 6 h, the liquid was poured into a new container where 1 g/L of a mixture of ammonium phosphate and thiamine (Enologica Vason, I) was added as nitrogen supplementation. The kaki mixture was transferred into 5 L glass bottles and inoculated with the yeasts (inoculum ratio 1% v/w) to obtain a final nominal concentration of 10^6 cfu/g of yeast. Bottles were placed at 22 ° C for 7 days, once the alcoholic fermentation was completed the liquid was poured out and the various remaining solid substances were eliminated by filtration. Fermented juice yield was about the 65% of initial fruit weight. To obtain the nine vinegar samples, after pressing and fermentation, each kaki juice was subdivided in 3 parts, then each fermented kaki juice was subdivided in 3 aliquots, and the various acetic bacteria were inoculated

Table 1
Microorganisms and types of samples produced.

Microorganisms used	Abbreviation	sample type
Kaki juice	Juice	juice
Torulaspora delbruekii	T	wine
Saccharomyces uvarum	U	wine
Saccharomyces cerevisiae	SC	wine
T. delbrueckii + *A. aceti*	T + A	vinegar
T. delbrueckii + *G. oxidans*	T + G	vinegar
T. delbrueckii + *A. vino*	T + W	vinegar
S. uvarum + *A. aceti*	U + A	vinegar
S. uvarum + *G. oxidans*	U + G	vinegar
S. uvarum + *A. vino*	U + W	vinegar
S. cerevisiae + *A. aceti*	SC + A	vinegar
S. cerevisiae + *G, oxidans*	SC + G	vinegar
S. cerevisiae + *A. vino*	SC + W	vinegar

(nominal concentration in the product equal to 10^6 cfu/g) and then left for 45 days at 30 °C.

2.2. Acidity

Acidity was measured on the vinegar samples only. This parameter was expressed as g of acetic acid per litre of sample. It was calculated using the AOAC official method (AOAC, 2007).

2.3. Density

The samples (100 μL) were weighted using an analytical scale (Precisa XR 205SM-DR). As a reference deionized water was used for the measurement. The determination of the density allows to express the results of the vinegars and wines on g of sample instead of on mL of sample. This way you can compare the data with those of the juice which, following extraction, are expressed on g of sample.

2.4. Antioxidant activity

Total Phenolic Content (TPC). To determine the TPC the Folin-Ciocalteu method was used as per (Locatelli et al., 2016). Results were expressed as mg of catechin equivalent.

DPPH method. The determination of the antioxidant activity was carried out using the radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) assay as per (Locatelli et al., 2009). Results were expressed as mg of Trolox Equivalents per g of sample.

2.5. Qualitative volatile analysis (volatile fingerprinting by HS-GC-IMS)

Headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS) (FlavourSpec®, G.A.S., Dortmund, Germany) was used to assess the volatile composition with an untargeted fingerprinting approach. According to the type of sample, a 20 mL glass vial was filled with: a) 100 mg for the juice; b) 50 μL of sample in 450 μL of water for vinegars and wines, respectively. Samples were treated for 5 min at 40 °C. Then, in splitless mode, a 100 μL headspace sample was automatically delivered through a 70 °C heated syringe. Using an MXT-5 column (15 m × 0.53 mm i.d., 1 μm film thickness; Restek Corporation, Bellefonte, PA, USA), the volatile chemicals were separated at 40 °C. As the carrier gas, 99.999% pure nitrogen was employed, and the flow rate program was configured as follows: 2 mL/min for 3 min, followed by a 17-min rise to 25 mL/min and a 5-min hold. A ^3H ionization source ionized the eluted analytes before driving them to a drift tube, which was run at a constant temperature of 45 °C and voltage of 5 kV.

2.6. Quantitative volatile analysis (GCxGC-TOF-MS)

Sample preparation. A volume of 4.0 mL of kaki vinegar and wine samples were placed into a 20-mL glass vials with septa. Following saturation with Na_2CO_3 a small magnetic stirring bar was added, until the complete gas development. A final volume between 1.8 and 1.95 mL was obtained. The sample was equilibrated for 30 min at 40 °C before being extracted for 20 min at the same temperature. The fiber was desorbed at 250 °C for 5 min. A sample of kaki's juice was also prepared diluting 2.39 g of pulp in water (1:1), the equilibration and extraction of the sample was the same used for vinegar.

GCxGC-TOF-MS analysis. A two-dimensional gas chromatography coupled to mass spectrometry was used to characterize volatile molecules using headspace-solid phase microextraction analysis (SPME). A Pegasus BT 4D GCxGC-TOFMS instrument (Leco Corp., St. Josef, MI, USA) equipped with a LECO dual stage quad jet thermal modulator was used. The volatiles were extracted by SPME, using a 50/35 μm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Inc., Bellefonte, PA, USA). The SPME fiber was preconditioned for 30 min at 270 °C and reconditioned between each run to minimize carry over effects. The first-dimension column was a Stabilwax-DA (Restek Corp., Bellefonte, PA) MS capillary column, with an internal diameter of 0.25 μm and a stationary phase film thickness of 0.25 mm, while the second-dimension chromatographic column was a 2 m Rxi-17Sil MS (Restek Corp., Bellefonte, PA) with the same diameter and thickness of the first one. High-purity helium (99,9999%) was used as the carrier gas with a flow rate of 1.4 mL/min. The temperature program of the oven was as follows: the initial temperature was set at 50 °C for 5 min, then ramped at 4 °C/min up to 250 °C for 5 min. The secondary column was maintained at +15 °C relative to the GC oven temperature of the first column. Electron ionization was applied (70 eV). The ion source temperature was set at 250 °C, while the mass range was 35-550 m/z with an extraction frequency of 32 kHz. The acquisition rates were 200 spectra/s and the modulation period 4s for the entire run. Leco Chroma TOF software (ver. 5.54) was used to find all peaks in the raw bi-dimensional chromatogram with a signal-to-noise (S/N) ratio more than 20 and a similarity value larger than 700, in order to select only the higher hits which were correctly assigned with high confidence. The mass spectra assignment was performed by matching with NIST MS Search 2.3 library, implemented with MoNa Fiehn. For all the identified molecules, the respective retention index based on n-alkane scale was calculated in order to remove the potential false-positive identifications generated by mass spectrum matching. Only the identified peaks were taken in consideration and for all molecules the linear retention index was calculated. The peaks resulting from stationary phase bleeding were manually removed. Monovariate and multivariate statistical analysis were performed with Metabonalyst software 5.0 (www.metabo.metaboanalyst.org).

2.7. Carotenoids

The method proposed by (Stinco et al., 2019) was followed for the extraction of carotenoids with slight modifications. Between 5 and 10 ml of the different vinegars were taken, and methanol, water and trichloromethane (1:1:2 v/v/v) were added to each sample. The samples were shaken in an Ohaus VXMTDG automatic vortex (OHAUS Europe GmbH, Nänikon, Switzerland) for 5 min at maximum speed and then centrifuged for 5 min at 4°C and maximum speed. The lipophilic phase was at the bottom and transferred to another tube. Trichloromethane was added to each sample and the process was repeated until the colour of the lipophilic phase was exhausted. All extractions were collected in a single tube and concentrated to dryness in a rotary concentrator under vacuum. Once concentrated, the samples were saponified. The saponification process consisted of adding 2 mL of dichloromethane and 2 mL methanolic potassium hydroxide at 20% (m/v). The samples were left under gentle agitation and protected from light for 1 h. After this time aqueous NaCl at 5% (m/v) was added to remove the alkali. The washing process was repeated until the pH of the washed water was neutral. The extracts were again brought to dryness and kept at -20 °C and in a nitrogen atmosphere to prevent oxidation, until further analysis by HPLC.

Carotenoids analyses were performed by RRLC on an Agilent 1260 system (Agilent, Waldbronn, Germany) equipped with a UV-Vis diode array detector (DAD) according to the method proposed by (Stinco et al., 2019). DAD was set at 472 nm for lycopene and 450 nm for β-cryptoxanthin, zeaxanthin and β-carotene. Separation was carried out on a C30 column (150 × 4,6 mm I.D. 3 μm particle size; YMC Europe, Dinslaken, Germany) maintained at 28 °C with a guard pre-column (10 × 4,0 mm I.D. 3 μm particle size; YMC Europe, Dinslaken, Germany).

The mobile phase was methanol (A), methyl *tert*-butyl ether (B) and water (C). Separation was performed using the following gradient: 0 min 90% A + 5% B + 5% C, 0 - 5 min, 95% A + 5% B; 5 - 10 min, 89% A + 11% B, 10-16, min,75% A + 25% B; 16-20 min, 40% A + 60% B; 22.5–25 min, 15% A + 85% B, 25-28 min, 90% A + 5% B + 5% C. After each analysis a re-equilibration time of 2 min was used to return to the initial composition of the mobile phase. The mobile phase was pumped at 1 ml/min.

2.8. Statistical analysis

The results of the analyses, unless otherwise specified, are expressed as the mean ± standard deviation of at least three independent determinations for each individual sample. The differences between the samples were evaluated through the one-factor analysis of variance (ANOVA), followed by Tukey's test (Honest Significant Difference test). Values of $p < 0.05$ were considered significant. The statistical analysis was conducted using the R software (version 4.1.0). Also, using the VOCal GC-IMS software (version 0.1.3), a principal component analysis (PCA) was performed.

3. Results and discussion

3.1. Acidity, density, TPC (total phenolic content) and antioxidant activity

Table 2 summarizes the acidity, density, TPC (total phenolic content), and antioxidant activity of the samples.

By comparing all the vinegar samples produced with the *Acetobacter bacterium*, the antioxidant activity values are very similar to each other (between 0.36 and 0.40 mg TE/g sample). In terms of TPC, there is a significant difference between the three types. The vinegar produced with the S. *cerevisiae* strain has the highest content, the sample obtained with *Torulaspora delbruekii* has the lowest value, and the vinegar produced with S. *uvarum* has an intermediate concentration (0.35 mg TE/g sample) compared to the previous two samples.

Taking into consideration the antioxidant activity values of the vinegars obtained with fermentation through the use of the *Acetobacter_vino bacterium*, the sample obtained from the fermented *S. uvarum* has a lower activity than those produced using *T. delbruekii* and *S. cerevisiae*. During alcoholic fermentation, the S. *uvarum* strain yielded the highest TPC value. The TPC associated with the samples made from fermented T. *delbruekii* and S. *cerevisiae* were much lower than those associated with the vinegar sample from earlier. In terms of antioxidant activity, while comparing samples made using the *Gluconobacter* bacteria during acetic fermentation, the S. *uvarum* strain used in the initial fermentation had the lowest result.On the other hand, if *T. delbruekii* and *S. cerevisiae* are used as yeasts, significantly higher values of antioxidant activity are obtained; the highest value of all is that of obtained by *T. delbruekii*, but it is very similar to the value reached with *S. cerevisiae*. Instead, for TPC, it can be noted that the three samples are statistically similar, but the sample obtained with the use of *S. cerevisiae* has the greater value unlike that obtained with *T. delbruekii* which presents the lower content.

Table 2
Acidity, density, TPC (total phenolic content) and antioxidant activity of samples.

Sample	Acidity	Density (g/mL)	TPC	Antioxidant activity
juice	-		$0.24^{e} \pm 2x10^{-2}$	$0.34^{d} \pm 1x10^{-2}$
T + A	3.68 ± 0.4^{c}	$1.011^{a} \pm 2x10^{-3}$	$0.30^{d} \pm 2x10^{-2}$	$0.40^{c} \pm 4x10^{-3}$
T + G	3.64 ± 0.2^{c}	$1.014^{a} \pm 5x10^{-3}$	$0.33^{bc} \pm 3x10^{-2}$	$0.42^{b} \pm 2x10^{-2}$
T + W	4.95 ± 0.6^{b}	$1.011^{a} \pm 2x10^{-3}$	$0.32^{c} \pm 2x10^{-2}$	$0.40^{c} \pm 2x10^{-2}$
U + A	1.30 ± 0.2^{e}	$1.003^{b} \pm 3x10^{-3}$	$0.36^{b} \pm 1x10^{-2}$	$0.39^{c} \pm 3x10^{-2}$
U + G	1.15 ± 0.2^{e}	$0.999^{b} \pm 4x10^{-3}$	$0.34^{b} \pm 1x10^{-2}$	$0.32^{e} \pm 1x10^{-2}$
U + W	1.90 ± 0.2^{d}	$1.001^{b} \pm 3x10^{-3}$	$0.37^{b} \pm 1x10^{-2}$	$0.31^{e} \pm 1x10^{-2}$
SC + A	2.36 ± 0.3^{d}	$0.990^{c} \pm 2x10^{-3}$	$0.42^{a} \pm 5x10^{-4}$	$0.36^{d} \pm 3x10^{-2}$
SC + G	1.80 ± 0.1^{d}	$0.991^{c} \pm 3x10^{-3}$	$0.35^{b} \pm 4x10^{-3}$	$0.41^{b} \pm 3x10^{-2}$
SC + W	6.21 ± 0.2^{a}	$1.001^{b} \pm 4x10^{-4}$	$0.32^{c} \pm 1x10^{-2}$	$0.42^{b} \pm 1x10^{-2}$
U	-	$0.995^{b} \pm 6x10^{-3}$	$0.33^{c} \pm 1x10^{-2}$	$0.47^{a} \pm 1x10^{-2}$
T	-	$1.010^{a} \pm 4x10^{-4}$	$0.30^{d} \pm 9x10^{-3}$	$0.39^{c} \pm 9x10^{-3}$
SC	-	$0.982^{d} \pm 5x10^{-3}$	$0.31^{d} \pm 2x10^{-2}$	$0.43^{b} \pm 2x10^{-2}$

T: *T. delbruekii*; SC: *S. cerevisiae*; U: *S. uvarum*; A: *A. aceti; G: G. oxydans*; W: *Acetobacter_vino*; U: *vino S. uvarum*; T: *vino Torulaspora delbruekii*; SC: *vino S. cerevisiae*. Values marked with lower case letters in the same column were significantly different ($p < 0.05$). Acidity: expressed as g acetic acid/litre of sample; TPC: expressed as mg catechin/g sample; Antioxidant activity: expressed as mg of Trolox Equivalents/g sample.

By evaluating the trend of the oxidizing activity results of the samples produced using *S. uvarum*, it can be seen how the value increases in the bioconversion from grape juice to wine (U), but then there is a reduction during the formation of the three vinegars, where two samples (W and G) have a value slightly lower than the juice but similar in terms of significance. The vinegar obtained by fermentation with *Acetobacter* has a higher value than the other two vinegars, but still lower than that recorded in wine. Instead, observing the trend of the TPC, it can be seen that there is an increase during the vinegars production phases. In fact, the three final vinegars have significantly higher values than the starting juice and the intermediate wines. The highest value was obtained by using the *Acetobacter_vino* strain during acetic fermentation.

Taking into consideration the trend of the antioxidant activity values obtained using *T. delbruekii* for alcoholic fermentation, it can be seen that there is an increase during the production phases and that the three vinegar samples recorded comparable values between them and the intermediate wine sample (T); however greater than the starting juice. As for the TPC, it can be noted that, even with this parameter, there is an increase in values during the production process. The trend recorded is in line with that which emerged in relation to antioxidant activity. The vinegars produced with (W) and (G), even if they have higher values than those of wine and the sample fermented with (A), are however similar from a statistical point of view to the latter. For both parameters, the greatest value is developed by the vinegar produced using *Gluconobacter*.

When the antioxidant activity levels of the samples produced by utilizing the S. *cerevisiae* strain are analyzed, there is a rise from juice to wine, but then a little reduction occurs during the vinegar generation. The vinegars obtained with (W) and (G) show significantly homogeneous values to those of wine (SC); while the sample fermented with (A) was similar to the initial juice. Instead, taking into consideration the TPC, there is a different trend. In particular, the vinegar obtained with (A) has the highest TPC compared to the other two vinegars (W and G), but which in any case have higher values than the intermediate wine. According to statistics, the sample generated using (W) has a total polyphenol value comparable to the intermediate SC wine.

Overall, if the values of both parameters of all the vinegar samples are compared, it can be observed that the different starting yeasts affect the result. In fact, we can distinguish three different subgroups in which the values are substantially similar, except for vinegar produced using *S. cerevisiae* associated with *Acetobacter* which has a lower value of antioxidant activity and a higher value of total polyphenols than the other two vinegars fermented with the same yeast.

3.2. Quantitative volatile analysis (GCxGC-TOF-MS)

The different kaki vinegars were manufactured with complex processing and multiple microorganisms, thus the volatile components were rather complex. Based on the analysis of mass spectrum match factors, structured chromatograms, and linear retention indices, more of 1000 characteristic molecules were identified (Table S1). Esters were the most abundant class in terms of number. There were also several ketones, furans and derivatives, aldehydes, alcohols, lactones, phenols, acids, and pyrazines (Table S2).

First, an unsupervised analysis was performed taking into consideration the entire dataset of the nine different kaki vinegars; this analysis was grouped by the three starting types (Fig. 1A), resulting from the different stages of vinegar production (T, U and SC). Principal component analysis (PCA) clearly shows the presence of metabolomic profiles associated with kaki's vinegars: the samples resulted well separated based on the three starting microorganisms. A further supervised Partial Least Square Discriminant Analysis (PLS-DA) was performed in order to discriminate the classes of the kaki vinegars (TA, TG, TW, UA, UG, UW, SCA, SCG, SCW) from the starting wines (T, U, SC) and the kaki juice (JUICE). The analysis showed a clear separation (Fig. 1B) of the different vinegar products (red, blue and yellow circles). A further distinction between the initial products and the result at the ending of fermentation (grey circle) was also observed.

Several ketones (39) and aldehydes (57) were identified among all samples, including cyclic and acyclic ketones and aromatic and hydrogen aldehydes. As reported in the distribution of volatile compounds (Table 3), the number of aldehydes appears to be substantially higher in vinegars than in wines or juice, probably as a result of the final process of fermentation that occurred throughout the vinegar's production. Molecules as Benzaldehyde and its derivatives contribute to give the vinegars the typical almond smell, while the ketones 2-Heptanone, 3-Butanedione and furfural have been to contribute to the yogurt and sweet, fruity and orange peel odor and caramel-like notes respectively (see Table 4).

Organic acids are considered the most essential flavour constituents in vinegars (Zhou et al., 2017) and they were mostly produced by alcohol oxidation from bacteria (Wang et al. 2016). A total of 42 relevant organic acids were identified in the vinegars, the majority of which were saturated monocarboxylic fatty acids, although many saturated fatty acids such as dodecanoic, palmitic and stearic acid were detected, as reported in the Table S2. The kaki vinegar with the highest number of organic acids was the UG: in this sample, *cis*-vaccenic acid was also identified. This compound was previously identified at the end of the fermentation process in some wine and cider vinegars (Fujimori et al., 2009; Trček et al., 2007). In our case, its exclusive presence in kaki vinegar composed by microorganisms *S. uvarum* + *Gluconobacter* could indicate that acetic acid fermentation can change the fatty acid composition during the vinegar manufacture with these microorganisms. Some of these organic acids, as propanoic, acetic and butanoic acid are described as the most pungent powerful odorants (Zhu et al., 2016) and can have big influence on sensory quality of kaki vinegar.

Furans and derivatives, pyrazines and lactones might be significant contributors because they are in part responsible of the quality of notes in vinegar (van Boekel, 2006). Our analysis reported a huge amount of furan compounds, many of which were already present in the kaki pulp, even if their levels increased in all vinegars fermented with *Saccharomyces uvarum* yeast (Table 3). The higher presence of furans in vinegar produced using *Gluconobacter* could provide a wide range of sensory properties in this fermented product. Furans are in fact mainly associated with a caramel-like, sweet, fruity, nutty, meaty, and burnt odor impression. Lactones are predominant in the starting wine U and pulp, the typical coconut and fruity/creamy and apricot-like smell distinguishes them from other vinegars. In addition, a total of 12 pyrazines were identified: most of them were saturated alkylpyrazines, which are known to give taste and aroma to many food products, beverages, and seasoning, including a particular type of Chinese vinegar produced from rice, koji and wheat bran (Xu et al., 2011; Yu et al., 2012). Tetramethyl-pyrazine, known also as ligustrazine, is the most abundant compound in terms of percentage area. A study attributed the ability to synthesize tetramethyl-pyrazines to bacteria from genera *Bacillus*, *Lactococcus* and *Corynebacterium* (Xu et al., 2011). This molecule, identified only in vinegars produced from *Torulaspora delbruekii*, (SC + W and SC + G), has been recently considered as alkaloid with powerful bioactivities and health functions in the treatment of cardiovascular and cerebrovascular diseases, as well as hypertension (Valença et al., 2021).

Furthermore, 10 significant lactones were found in the vinegar, differently distributed depending on the starting microorganisms. The presence of lactones in vinegar has been extensively studied (Kašpar & Česla, 2022; Kašpar et al., 2022; Perestrelo et al., 2018) as these compounds confer a typical sweet flavour. In our vinegars, the aromatic profiles indicate that the selection of the starting microorganism can improve the sensory quality of the final vinegar, positively contributing to the aroma. The exclusively presence of pyrazines in *T. delbruekii* starting vinegar contribute to give the characteristic roasted and nutty odor.

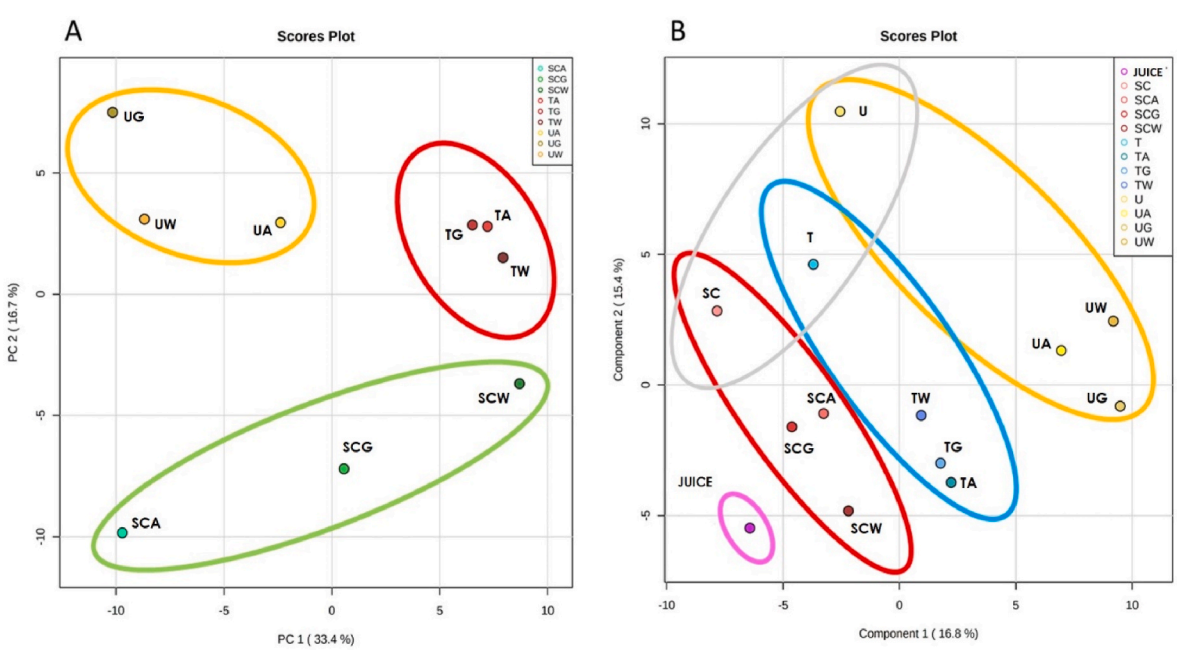

Fig. 1. A) Principal Component Analysis (PCA) of the 9 kaki's vinegars grouped on the three starting microorganisms (yellow: *S. uvarum;* red: *T. delbruekii;* green: *S. cerevisiae*). B) Partial Least Square Discriminant Analysis (PLS-DA) of the nine vinegars, the three wines and the kaki's juice. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
The most abundant class in number calculated for all the samples.

Volatile compounds identified	U + A	U + G	U + W	T + A	T + G	T + W	SC + A	SC + G	SC + W	T	U	SC	Juice
Alcohols	23	40	34	33	19	23	23	30	27	27	18	22	11
Esters	71	112	104	62	43	53	79	76	70	77	68	80	29
Lactones	4	6	5	3	4	3	2	1	2	1	1	1	5
Furans and derivatives	9	21	11	10	12	9	1	7	0	3	2	3	15
Nitrogen containing compounds	4	8	2	7	7	7	2	3	9	4	3	3	0
Ketones	10	17	11	9	14	9	5	7	9	6	6	6	6
Acids	11	31	17	13	12	11	10	14	13	10	12	11	18
Aldehydes	12	23	12	14	12	10	6	12	9	11	10	14	27
Pyrazines	0	0	0	5	5	8	0	1	5	1	0	0	0
Phenols	15	20	18	19	16	15	14	12	17	8	9	7	5
Sulphur- containing compounds	4	8	7	6	7	4	2	5	4	2	1	1	1
Total classes	163	286	221	181	151	152	144	168	165	150	130	148	117

Table 4
Individual carotenoids contents expressed as ug carotenoid/g sample.

Sample	Zeaxanthin	*β*-Cryptoxanthin	*β*-Carotene	(all-*E*)-Lycopene	*Zs*-Lycopene	*Tot*
Juice	$36.8^{a} \pm 4$	$157^{a} \pm 10$	$13.3^{a} \pm 0.2$	$5.11^{a} \pm 1$	$12.1^{a} \pm 4$	224
T	$9.63^{b} \pm 1$	$40.5^{b} \pm 6$	$3.59^{b} \pm 1$	$1.77^{b} \pm 1$	$1.34^{c} \pm 0.3$	56.8
U	$8.89^{b} \pm 1$	$34.0^{c} \pm 2$	$2.64^{c} \pm 0.1$	$1.39^{c} \pm 0.2$	$0.88^{d} \pm 0.1$	47.8
SC	$10.2^{b} \pm 0.3$	$41.0^{b} \pm 1$	$3.12^{b} \pm 0.1$	$2.91^{b} \pm 1$	$4.14^{b} \pm 1$	61.4
U + A	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
U + W	$0.092^{e} \pm 2x10^{-3}$	$0.28^{f} \pm 0.01$	$0.032^{f} \pm 1x10^{-3}$	$0.06^{d} \pm 0.02$	$0.0134^{e} \pm 1x10^{-4}$	0.47
U + G	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
T + A	$9.35^{b} \pm 3$	$29.2^{c} \pm 7$	$2.05^{c} \pm 1$	$0.97^{c} \pm 0.22$	$0.75^{d} \pm 0.2$	42.3
T + W	$1.38^{c} \pm 0.2$	$4.72^{d} \pm 1$	$0.37^{d} \pm 0.1$	$0.064^{d} \pm 5x10^{-3}$	$0.032^{e} \pm 4x10^{-3}$	6.56
T + G	$0.68^{d} \pm 0.1$	$1.76^{e} \pm 0.4$	$0.14^{e} \pm 0.03$	$0.06^{d} \pm 0.05$	$0.0142^{e} \pm 1x10^{-4}$	2.65
SC + A	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
SC + G	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
SC + W	$0.103^{e} \pm 5x10^{-3}$	$0.30^{f} \pm 0.1$	$0.021^{f} \pm 7x10^{-3}$	$0.01^{e} \pm 0.01$	< LOQ	0.43

<LOQ: value below the quantification limit. T: Torulaspora delbruekii; SC: S. cerevisiae; U: S. Uvarum; A: Acetobacter; G: Gluconobacter; W: Acetobacter_vino; Test U: S. Uvarum wine; Test T: Torulaspora delbruekii wine; Test SC: S. cerevisiae wine. Values marked with lower case letters in the same column were significantly different ($p < 0.05$). Total carotenoid content expressed as the sum of the previous individual carotenoids.

Kaki vinegar samples contained also a significant level of sulphur compounds (n = 13), which are mostly produced from metabolism of sulphur sources and precursors. As suggested by the literature (Swiegers & Pretorius, 2007), we hypothesize that this process could be initially acted by different yeasts used, and then implemented by the addition of bacteria.

The high presence of phenols (n = 34) is nearly consistent in all the vinegars and shows a considerably increase during the fermentation (Table 3).

In agreements with the results obtained from HPLC analysis

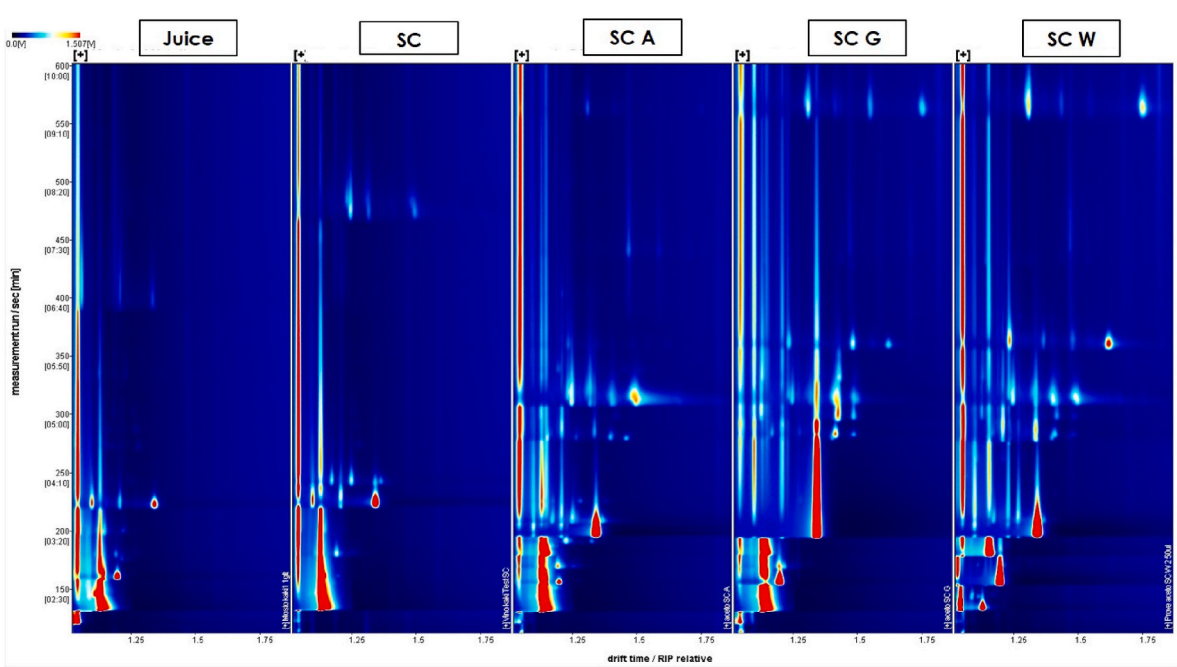

Fig. 2. Comparison among the 2D chromatograms obtained by analyzing the samples obtained by using *S. cerevisiae*.

performed on the carotenoids content (listed in the next section), the identification of three ionone compounds, also known for their woody-violet aroma (López et al., 2020), shows that these derived products significantly decrease throughout the vinegar production process (Fig. S1).

3.3. Volatile fingerprinting by HS-GC-IMS

This study also employed gas chromatography-ion mobility spectrometry, which combines gas chromatography and ion mobility spectrometry. This technique is a quick gas assay with good resolution, sensitivity, and efficiency as well as straightforward operation. The samples do not require any pre-treatment. Headspace sampling swiftly gathers the information on the trace volatile organic compounds in the sample to create a fingerprint that may be used to determine the sample's authenticity, freshness, shelf life, and variety quality (Cavanna et al., 2019; Gu et al., 2020). This method can be used to profile volatile organic compounds in samples using non-targeted screening.

As an example, Fig. 2 shows the top view of a GC-IMS analysis of volatile compounds from samples obtained using *S. cerevisiae* (juice, and corresponding wine and vinegars). The total headspace compounds in the samples are represented by the entire spectrum. Each point in the diagram indicates a volatile chemical isolated from the samples. The number symbolizes the selected areas of various compounds, while blue denotes a lower intensity and red a higher intensity (reported in Fig. S2). According to the gallery plot, the differences between samples during the different stages of fermentation (juice, wine and vinegar) were significant. Vinegars showed higher numbers of volatile compounds than those detected in wines and juice. The compounds detected in all the samples generally showed a higher intensity in the samples obtained using *S. cerevisiae*.

Due to the complexity of the volatile profiles analyzed, a principal component analysis was performed on the data obtained. The visual distribution map for the samples is reported in Fig. 3, describing 47% and 16% of the cumulative variance contribution rate. It is clear that, due to the different profiles, the wine types are located on the left side of the PCA together with the juice, while the vinegars are located on the right side. Within the same typology, two different behaviours can be highlighted. The vinegars produced using *S. cerevisiae* were more clustered together; demonstrating a more peculiar volatile profile. On the contrary, vinegars produced by using *S. uvarum* and *T. delbruekii* showed a more homogeneous profile between them, thus both resulting in negative values on PC2.

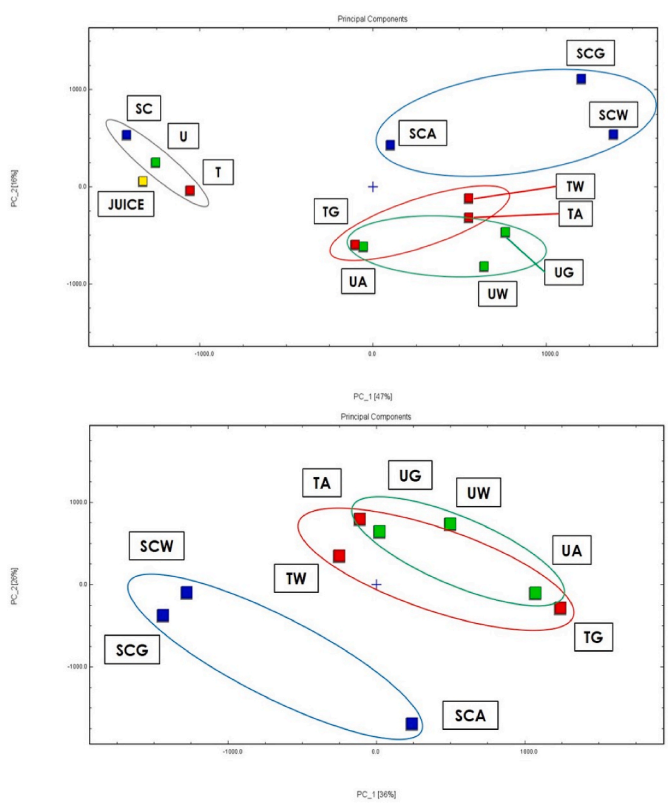

Fig. 3. PCA score plot; all samples (upper side) and vinegars only (lower side).

A further PCA was carried out considering only the vinegar samples. The visual distribution map is reported in Fig. 3, describing 36% and 26% of the cumulative variance contribution rate. Also, in this case there was homogeneity in the volatile profiles of the samples obtained with *S. uvarum* and *T. delbruekii*. It should be pointed out that the TG and UA samples, as in the previous PCA, are located very close to each other, demonstrating similar characteristics even if produced with different strains. Finally, the vinegars produced by using *S. cerevisiae* are clustered, thus showing a peculiar volatile profile (as seen above).

3.4. Determination of carotenoid

Among the three wines, the one obtained with *S. cerevisiae* was the one with the highest content of carotenoids, followed by the one fermented with *T. delbruekii* and the one with *S. uvarum*. Finally, after the second fermentation, a further decrease was noted in all vinegars except for the T + A sample in which the content decreases less markedly. Decreases of carotenoid levels from wines to vinegars were foreseeable and could be attributed to a longer exposure to degrading conditions, including increased acidity (Davies et al., 2017). The various parameters considered had different trends during the production process: the different carotenoid contents in the samples, largely due to extraction dynamics and degradation reactions, had a deep impact on the colour of the products, ranging from the colourful orange of the kaki juice to the transparency of some vinegars. The only vinegars that had a significant level of carotenoids were those deriving from the *T. delbruekii* fermentation; while two vinegars from the yeast *S. cerevisiae* (SC + A and SC + G) and two from *S. uvarum* (U + A and U + G) were below the detection limit. The U + W and SC + W samples showed a small content of carotenoids (<0.50 μg/g) (Table 3).

Carotenoids levels were lower (3.64, or 4.68-fold) in the wines than in the juice. These clear differences could be owed to different reasons. On one hand, carotenoids within the fruit cells and cell debris are usually found in subcellular structures collectively named plastids (Meléndez-Martínez et al., 2021). Carotenoids are lipophilic, and their lipophilicity increases when they are esterified with fatty acids, which is the case of *Diospyros kaki* (Cano et al., 2019), so it seems reasonable to think that the ethanol produced as a result of fermentation could only extract a small part of the carotenoids present in the juice. The rest may have remained in the cell debris or was degraded either during winemaking or storage, as carotenoids are usually stable in their biological locations but very labile when extracted. Indeed, carotenoids can be degraded due to oxidants, heat, acids or bases, with differences depending on their structure and the intensity of the processes. It is known that important wine aroma compounds derive from carotenoid breakdown (Mendes-Pinto, 2009). The presence of small amounts of carotenoids in grape wines is well-known. Mendes-Pinto et al. (2005) reported the presence of carotenoids (both typical grape carotenoids such as neoxanthin, violaxanthin, lutein and β-carotene, the derivative of neoxanthin neochrome as well as several unidentified carotenoids) in Port wines and their degradation over storage. More recently, carotenoids (neoxanthin, violaxanthin, lutein and β-carotene) have also been reported in mango wine (Varakumar et al., 2011).

β-cryptoxanthin, zeaxanthin, β-carotene, (all-*E*)-lycopene and Z isomers of lycopene, in decreasing order, were the predominant carotenoids in the juice. Overall, they all were also detected in samples where carotenoids were detectable; β-cryptoxanthin was always the

predominant carotenoid. Indeed *Diospyros kaki* (kaki, persimmon) is one of the best known sources of this provitamin A carotenoid (Bordiga et al., 2019; Meléndez-Martínez, 2019). The second predominant carotenoid was consistently zeaxanthin, and then either β-carotene or lycopene (considered as the sum of isomers), depending on the sample.

4. Conclusions

This work was aimed at evaluating the quality, during the production process, of a kaki vinegar varying different strains (both yeast and bacteria). In fact, the analyses were carried out both on the starting juice, on the intermediate fermentation and on the final acetic fermentation. It can be noted that the anti-radical activity and the polyphenol index tend to increase due to the formation of new bioactive compounds during fermentation processes. The only type of vinegar characterized by a high concentration of carotenoids was the one obtained using the combination *T. delbruekii* + *Acetobacter*.

The types of vinegar produced through the *T. delbruekii* yeast strain, despite having the highest levels of carotenoids and antioxidant activity, were found to be those with the lowest polyphenol index. Finally, from the comparison of the aromatic profile, it emerged that vinegars fermented with *S. cerevisiae* develop a greater quantity of volatile compounds. Among the three vinegars produced with this strain, the one fermented with *Acetobacter_vino* seems to have a more complex flavour profile than all the other samples. GC × GC-TOF-MS was shown to result in more VOCs in the kaki vinegars, especially those compounds at trace levels. HS-GC-IMS identified a limited number of compounds when compared with GC × GC-TOF-MS. However, HS-GC-IMS could present similar clustering on the PCA plots, indicating that also this fast method is suitable to discriminate kaki vinegars (but also kaki juice and wines) on VOCs.

In conclusion, it can be said that kaki vinegar is a product with good properties such as, for example, the antioxidant ones. The results obtained will certainly increase the knowledge of this matrix. The positive characteristics of kaki vinegar will clearly favour its spread also in Western countries in the coming years.

Future studies could be focused on identifying the best fermentation process through some modifications; by integrating the substrate with different substances (for example enzymes) or by trying to decrease the reaction time, with the aim of increasing the polyphenol index value. The purpose of diversifying the use of persimmon (as in the case of producing vinegars) is to arrive shortly at a selection of products whose characteristics will be subjected to the process of consumer acceptability, thus assessing the aroma, taste, and mouthfeel.

Author statement

Matteo Bordiga: Conceptualization, Visualization, Writing–original draft, Methodology, Validation, Formal analysis, Investigation. Raffaele Guzzon: Methodology, Formal analysis, Writing–original draft. Marcello Manfredi: Methodology, Formal analysis, Writing–original draft, Investigation. Elettra Barberis: Methodology, Formal analysis, Writing–original draft, Data curation. Simona Grasso: Methodology, Writing–original draft, Writing–review & editing. Ana M. Benítez-González: Methodology, Formal analysis, Writing–original draft, Investigation. Antonio J. Meléndez Martínez: Methodology, Formal analysis, Visualization, Data curation. Fabiano Travaglia: Methodology, Formal analysis, Investigation. Marco Arlorio: Methodology, Visualization, Writing–review & editing. Jean Daniel Coisson: Conceptualization, Writing–review, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Matteo Bordiga reports financial support was provided by Regione Piemonte.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102987.

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