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Sample Availability: Samples of the compounds are not available from the authors.

molecules

MDPI

Article

Volatile Profile of Mead Fermenting Blossom Honey and Honeydew Honey with or without *Ribes nigrum*

Giulia Chitarrini [1], Luca Debiasi [1], Mary Stuffer [1,2], Eva Ueberegger [1], Egon Zehetner [2], Henry Jaeger [2], Peter Robatscher [1] and Lorenza Conterno [1,*]

[1] Laimburg Research Centre, Ora (BZ), 39040 Auer, Italy; giulia.chitarrini@laimburg.it (G.C.); luca.debiasi@laimburg.it (L.D.); mary.stuffer@students.boku.ac.at (M.S.); eva.ueberegger@laimburg.it (E.U.); peter.robatscher@laimburg.it (P.R.)

[2] Institute of Food Technology, University of Natural Resources and Life Sciences (BOKU), 1190 Vienna, Austria; egon.zehetner@boku.ac.at (E.Z.); henry.jaeger@boku.ac.at (H.J.)

* Correspondence: lorenza.conterno@laimburg.it; Tel.: +39-0471969591

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Abstract: Mead is a not very diffused alcoholic beverage and is obtained by fermentation of honey and water. Despite its very long tradition, little information is available on the relation between the ingredient used during fermentation and the aromatic characteristics of the fermented beverage outcome. In order to provide further information, multi-floral blossom honey and a forest honeydew honey with and without the addition of black currant during fermentation were used to prepare four different honey wines to be compared for their volatile organic compound content. Fermentation was monitored, and the total phenolic content (Folin–Ciocalteu), volatile organic compounds (HS-SPME-GC-MS), together with a sensory evaluation on the overall quality (44 nontrained panelists) were measured for all products at the end of fermentation. A higher total phenolic content resulted in honeydew honey meads, as well as the correspondent honey wine prepared with black currant. A total of 46 volatile organic compounds for pre-fermentation samples and 62 for post-fermentation samples were identified belonging to higher alcohols, organic acids, esters, and terpenes. The sensory analysis showed that the difference in meads made from blossom honey and honeydew honey was perceptible by the panelists with a general greater appreciation for the traditional blossom honey mead. These results demonstrated the influences of different components in meads, in particular, the influence of honey quality. However, further studies are needed to establish the relationship between the chemical profile and mead flavor perception.

Keywords: gas chromatography-mass spectrometry; fermentation; honey; black currant

1. Introduction

Mead, also called honey wine, is traditionally an alcoholic beverage obtained through yeast fermentation of diluted honey. Mead is found in the history of many countries all around the world, and it is one of the oldest alcoholic beverages with variable alcohol content (8–18% alcohol *v*/*v*), mostly depending on the honey to water dilution ratio. Besides the traditional mead (the fermented diluted honey), many variations can be found, containing also herbs and spices (metheglin) or fruit (melomel) [1].

Fructose and glucose are generally the most abundant simple sugar found in honey, and fructose is the dominant one (on average from 32% to 42% depending on the honey origin [2]). The mead fermentation process is usually longer than most alcoholic fermentation, where other sugars are present and in higher concentrations. In fact, this fermentation often takes several months to complete, depending on the type of honey, yeast strain, and honey-must composition. Mead contains ethanol and

many other compounds, such as sugars, acids, vitamins, phenolic compounds, and minerals, also in dependence on the added ingredient beside honey (reviewed [3]).

The three main factors mead flavor depends are the honey, the yeast strain carrying out the alcoholic fermentation, and the fermentation conditions. Flavor perception may also be influenced by the final alcohol content, the residual sugars, and the acidic value.

Besides the botanical and the geographical origin, honey can be divided into two main groups: blossom or floral honey and honeydew honey. The former is produced starting from nectar from the flower of blossoming plants, while the latter from the exudates from certain plants (such as *Pinus*, *Abies*, *Castanea*, and *Quercus*, among others), usually with the concourse of insects, mainly from the family Aphididae [4]. Honeydew has a stronger taste than blossom honey and is perceived as less sweet. It has higher antioxidant activity and a higher concentration of oligosaccharides. Many researchers have found out that honeydew honey with a darker color has a higher concentration of total phenolic compounds and a higher antioxidative capacity [5,6]. For honey wine production, wine yeast strains are usually used because the sugar, pH, and nitrogen characteristics in mead are similar to the ones of grape must [1]. Yeast produces during fermentation many metabolites, which have a large impact on the beverage flavor. Most of the unique flavors of mead depend on the type of honey. For every additional ingredient, additional flavor molecules may be developed [7]. The formation of metabolites during fermentation not only depends on the raw materials used but also on the yeast strain. Most by-products are synthesized when there is a high rate of sugar and another nutrient uptake by yeasts, which all join the catabolizing pathway at the level of pyruvate. Many chemically different metabolites in diverse concentrations contribute to the flavor of alcoholic beverages, such as organic acids, fusel alcohols, aromatic alcohols, esters, carbonyls, and various sulfur-containing compounds [8,9]. Yeast activity behavior during fermentation caused by stressful conditions can lead to the production of unwanted flavors, for example, high volatile acidity or undesired esters [10].

The acids in the mead are coming from the honey, the added fruits, and the acids used for acidification of the must [11]. The acids in honey are usually citric, malic, succinic, formic, proglutamic, acetic, gluconic, and lactic [12]. Organic acids have a very important function in alcoholic beverages influencing organoleptic characteristics and product stability. The addition of organic acids is regulated by the European legislation for wine and recommended because low pH helps to minimize the risk of microbial spoilage by preventing bacterial growth. On the other hand, pH values below 3 could make a stressful environment for the yeast, which leads to the production of undesirable by-products [13].

Volatile organic compounds (VOCs) in mead are present due to raw materials or produced by yeast during fermentation. VOCs belong to various chemical classes, such as esters, higher alcohols, acids, aldehydes, ketones, etc. They have an impact on the aroma and odor of mead and especially contribute to the fruity and floral nuances [7]. Alcohols, such as n-propanol, iso-butanol, 2-phenylethanol, amyl alcohol, and others, influence the flavor of alcoholic beverages. In high concentrations, their flavor is usually described as a solvent, and they contribute to the intensification of an alcoholic taste, which creates a warm mouth feeling [8,14]. In lower concentrations, they may have a positive effect, increasing the complexity of fermented beverages [15]. Esters contribute to a floral and fruity flavor of mead. Ethyl acetate, for example, is considered as fruity or solvent (depending on concentration and combination with other volatiles), and isoamyl acetate has a banana or apple flavor. Ethyl esters are the most present in mead because ethanol is the most available substrate [8]. Terpenic compounds are mostly produced by plants and some insects but also from yeast [16] and have been found in remarkable concentrations in mead [7,13,17]. Organic acids have a sour flavor, and additionally, they can have individual characteristic flavors. Short-chain fatty acids have a mostly negative influence on the flavor, depending on the concentration and combination of the product. Furthermore, they affect foam performance [8]. In the mead fermentation process, acetic acid and succinate acid are formed in considerable amounts. They reduce the pH, increase the total acidity, and reduce the dissociation of fatty acids. High amounts of acetic acid, succinic acid, and a high concentration of fatty acids may cause a slowdown of the fermentation process [18].

Despite the long tradition of mead making, a limited scientific background is available in this field, which may be due to the medium and small-scale production. Therefore, systematic information and knowledge are needed in order to be able to define those parameters necessary to understand how to master mead quality, develop adequate formulations, and optimize the fermentation conditions as reviewed by Iglesias et al. [3].

The present work aimed to contribute to filling some of the gaps on the influence of different ingredients used for mead making on the final flavor profile. To reach this aim, multi-floral blossom honey and a forest honeydew honey, with or without black currant (*Ribes nigrum*), added before fermentation, were used to prepare four different honey wines to be compared for their volatile organic compounds content. Due to the high complexity of the *Ribes nigrum*, its addition was decided to originate a mead variant rich in polyphenols, minerals, and vitamins, which might have a positive effect on the fermentation process or the end product quality [19]. Polyphenol content and consumer acceptance were explored, as well.

2. Results and Discussion

Four different meads of honey wine were prepared either with multi-floral blossom honey and forest honeydew honey alone (B and H, respectively) or with black currant added (BC and HC, respectively).

2.1. Fermentation Kinetics

Fermentation kinetics were monitored by measuring the weight loss due to CO_2 effluence. HC was the first product reaching the stationary phase in fermentation after approx. 14 days, whereas H and BC took approx. 20 days. B had the longest fermentation time with approx. 30 days (Figure S1). According to these results, the products prepared with blossom honey had a longer fermentation time, and the addition of black currants accelerated the fermentation. Differences in honey composition [20] and black currant [19] might provide factors influencing the yeast rate of sugar depletion.

Total CO_2 production in g/L was equal to 86.1 (±0.3), 90.6 (±0.7), 75.5 (±0.2), and 79.2 (±0.5) for B, BC, H, and HC, respectively, in theory corresponding to 176.3 (±0.7), 185.5 (±1.4), 154.5 (±0.5), and 162.1 (±0.9) of fermented glucose in g/L. Measurement of sugar content in the original honey showed 690 ±10 g/L for the blossom honey and 650 ±10 g/L for the honeydew honey, expressed as total glucose (sum of glucose, fructose, and sucrose contribution). Glucose and fructose measured in the prepared product before fermentation showed a lower amount of glucose and fructose in H and HC (177.0 and 177.1 g/L, respectively), compared to B and BC (202.5 and 201.5 g/L, respectively). Further measurement of honey sugars besides the one measured might provide a more detailed explanation.

Being the residual sugar content in all honey wines below 5 g/L (Table 1), even if differences in fermentation length were observed, all the fermentation could be considered successfully completed.

Table 1. Physiochemical parameters characterizing the four products prepared with blossom honey (B), blossom honey and blackcurrant (BC), honeydew honey (H), and honeydew honey and black currant (HC). Parameters were measured before fermentation (t0) and/or at the end of the fermentation process (END). END data are reported as the average of the three biological replicates ± standard deviation (in brackets). Apex letter in the same row shows the results of the statistic evaluation, and different letters correspond to significant different parameters ($p \leq 0.05$).

	Analysis Time	B	BC	H	HC
pH	t0	3.17	3.15	3.16	3.22
pH	END	3.13 (±0.03) a	3.33 (±0.01) b	3.27 (±0.01) bc	3.29 (±0.01) c
Brix (%)	t0	21.6	22.9	21.7	21.5
Glu+Fru (g/L)	t0	202.5	201.5	177.0	177.1
Residual sugar (g/L)	END	4.3 (±0.6) a	0.4 (±0.1) bc	1.0 (±0.1) b	0.1 (±0.1) c
Ethanol (% vol/vol)	END	11.32 (±0.44) a	10.63 (±0.67) a	8.60 (±0.13) b	8.66 (±0.18) b
Acetic acid (g/L)	END	0.40 (±0.02) a	0.26 (±0.02) a	0.32 (±0.01) a	0..20 (±0.01) a
Acetaldehyde (mg/L)	END	6.2 (±1.4) a	11.3 (±2.1) ab	10.4 (±3.4) ab	16.8 (±2.7) b
L-lactic acid (g/L)	END	0.14 (±0) a	0.23 (±0.02) a	0.30 (±0) a	0.36 (±0.01) a

2.2. Physiochemical Parameters

The mean values for all parameters measured in the fermented product are listed in Table 1. B, which had a residual sugar value of 4.3 ± 0.6 g/L, significantly differed from the others, although usually meads can be considered dry [11]. Ethanol concentration in the four products was 11.32% *v*/*v*, 10.63% *v*/*v*, 8.60% *v*/*v*, and 8.66% *v*/*v*, respectively, measured in B, BC, H, and HC. Ethanol content was significantly higher in varieties with blossom honey. Even if prepared at a similar honey dilution rate, blossom honey used in B and BC products had a higher fermentable sugar content, explaining both the higher ethanol content and the higher carbon dioxide loss observed in the B and BC products.

The pH did not change to a great extent during the fermentation, and the lower one was recorded for the B mead, and L-lactic acid content was not significantly different in the four products. Acetic acid content at the end of fermentation ranged between 0.20 g/L and 0.40 ±0.02 g/L. Besides being yeast strain-dependent and related to the amount of sugar fermented, acetic acid might be also providing information on the yeast stress status or be the symptoms of microbial spoilage. This latter event seemed not to have occurred in the investigated products, and the acetic acid was detectable in amounts comparable to previous studies [3,13,18]. Acetaldehyde concentration ranged between 6.2 ± 1.2 and 16.8 ± 2.2 mg/L. Acetaldehyde concentration in meads usually ranges between 18.2 and 125.5 mg/L, as reported in the literature [21]. In general, 0.5–286 mg/L is the concentration of acetaldehyde produced by *Saccharomyces cerevisiae* in white wine [22]. A maximum of 0.5 g/L has been indicated in beer [8]. High concentrations of acetaldehyde would lead to a pungent, green, and grassy flavor, and it is associated with microbial spoilage of the fermented beverages. The acetaldehyde concentration found in this study testified the absence of microbial spoilage. Lactic acid was measured in very few quantities of 0.14–0.36 g/L. This was an indication that no lactic fermentation from bacteria took place.

2.3. Total Polyphenolic Content in Honey Wine

Total polyphenolic content measured using Folin–Ciocalteu reagent was 54.91 mg/L (±2.16) in B honey wine, 289.09 mg/L (±14.97) in BC, 101.95 mg/L (±5.78) in H, and 304.44 mg/L (±14.13) in HC, all expressed as mg/L of gallic acid. Results for traditional meads (B and H) were similar to the results reported in the literature [23,24]. Blossom honey varieties had less polyphenolic content than honeydew honey varieties, which corresponded to the result found in the literature [5,6]. Products prepared with currants (BC and HC) had much higher concentrations than the traditional meads as

expected. The black currants added about 200 mg/L of polyphenols to the mead. Together with the red color, black currant imparted to the honey wine a higher capacity to counteract oxidation, due to the higher amount of compound recognized to protect from oxidation [25] and as found in polish mead by Socha [26].

2.4. Volatile Organic Compounds

Meads, fermented from honeydew honey and blossom honey with and without the addition of *Ribes nigrum,* were analyzed to evaluate the influence of the starting honey and blackcurrant addition on the volatile organic compounds profile using HS-SPME-GC-MS method. The flavor and aroma of the final product depend on the type of honey and the floral source, the fermenting yeasts and conditions, and the presence of additives and fruits [27–29].

In this work, a semi quantification of 62 compounds was reported as an average of three biological replicates (Table S1); the table includes the compound name and class, Chemical Abstracts Service (CAS) number, retention index, retention time, and level of identification. Peaks in the chromatograms, acquired in full scan mode, had been integrated and reported as the area ratio of the peak with the internal standard (2-octanol). An ANOVA test was performed with a Turkey posthoc method to identify significant differences between the samples. In total, 4 acids, 13 alcohols, 4 aldehydes, 14 esters, 1 ketone, 16 terpenes and derivatives, 7 others, and 3 unknown compounds were identified. A general view of the results showed a higher compound formation or increase in samples analyzed after the fermentation.

To explore the dataset, a principal component analysis (PCA) was performed using the three replicates for each mead (Figure 1). The first and second components explained 80% of the total variance; the eigenvalues and the correlation results between variables and PCs are reported in Supplementary File 1. The first component allowed the separation between pre-fermentation and post-fermentation samples; the second component allowed to separate meads based on the honey (honeydew honey or blossom honey) and the blackcurrant addition. To better understand which metabolite influenced the diversification of the products, a heatmap was used (Figure 2) with a log10 transformed data (average of three replicates).

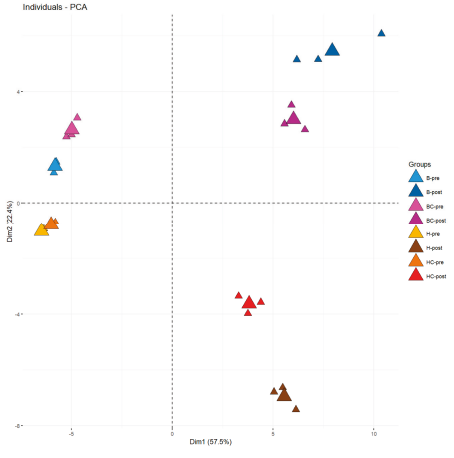

Figure 1. Principal component analysis (PCA) of the volatile compounds found in four honey wines made with blossom honey (B), blossom honey with blackcurrant (BC), honeydew honey (H), and honeydew honey with blackcurrant (HC), analyzed before (pre) and at the end of the fermentation process (post). The first component explained 57.5%, and the second component explained 22.4% of the total variance. Samples replicates are shown by the smaller triangle-shaped dots, while the average is represented by the bigger shaped dots.

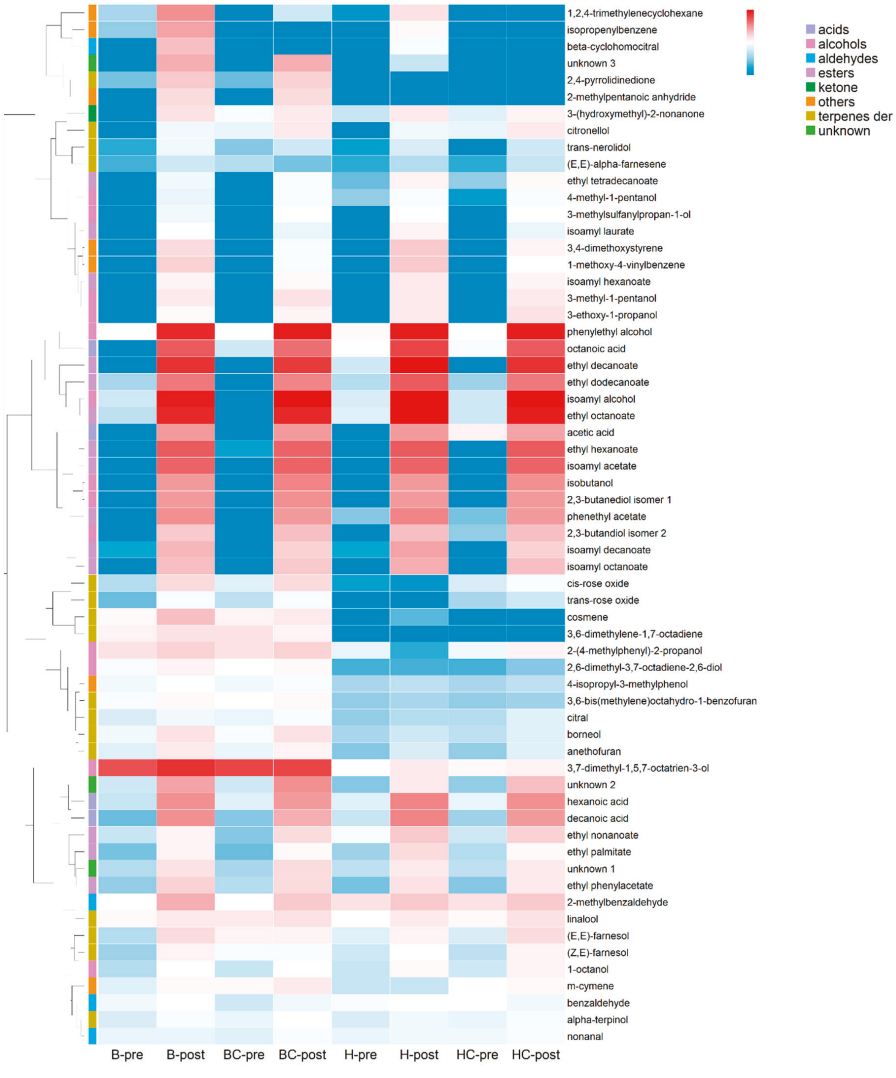

Figure 2. Heatmap and one-dimensional hierarchical dendrogram of the volatile compounds found in the investigated samples. Heatmap represents a log10 transformed data (average of three replicates). The heatmap color represents the magnitude of each compound. Dark red color indicates the higher magnitude, and then the magnitude gradually decreases to light red, white, light blue, up to dark blue (one order each), with the latter, indicating the lower magnitude. The magnitude represents transformed data (log10 of the ratio) to fit them in the same range. On the left: the colored sidebar indicates the class of metabolites. Sample legend: B = blossom honey, BC = blossom honey with blackcurrant, H = honeydew honey, HC = honeydew honey with blackcurrant, pre = pre-fermentation, and post = post-fermentation.

2.4.1. Volatile Profile in the Products before and after Fermentation with *S. cerevisiae*

Looking at the PCA, a clear separation between the pre- and post-fermentation samples was noticeable due to the volatile organic compounds produced during the fermentation. The number of

identified VOCs increased in post-fermentation samples since some VOCs are produced by the yeasts during the alcoholic fermentation [30]. At least 18 compounds produced by the yeasts during the fermentation process were found, being mainly alcohols and esters (Figure 2), already described as the products of *S. cerevisiae* EC1118 fermentation [31,32]. As reported in the literature, it is well known that yeasts are VOCs producers; in wine, the main groups of compounds that form the fermentation bouquet are the acids, alcohols, and esters and, to a lesser extent, aldehydes and ketones [33].

Furthermore, it is known that the compounds influencing the aroma of alcoholic beverages are mainly higher alcohols, esters, volatile acids, and aldehydes [34], making yeasts strain the main actor in establishing the sensory characteristics. Looking at our results, B and BC post-fermentation had a higher number of volatile compounds than H and HC post-fermentation. Being that the yeast strains are the same in all fermentation processes, the different numbers are due to the different starter matrix. In the product B-post, 62 VOCs were detected, while BC-post was characterized by 60 different compounds. In H-post and in HC-post, 56 and 54 VOCs were, respectively, identified. The product prepared with blackcurrants, in pre- and post-fermentation, had a lower VOCs content than those produced with the same honey but without fruit addition. The compounds that contributed to this difference were 1,2,4-trimethylenecyclohexane found in B-post and H-post and a very low amount in BC-post, isopropenylbenzene found in B-post and H-post, and beta-cyclohomocitral only found in B-post (Figure 3). These three compounds were not confirmed by the standard injection; for this reason, their identification could be only considered as putative. It is reasonable to assume that *Ribes nigrum* added the nutrients, shaping the yeast metabolism; however, there is no evidence in the literature that which metabolic pathway leads to the synthesis of these compounds that might be regulated during fermentation by yeast.

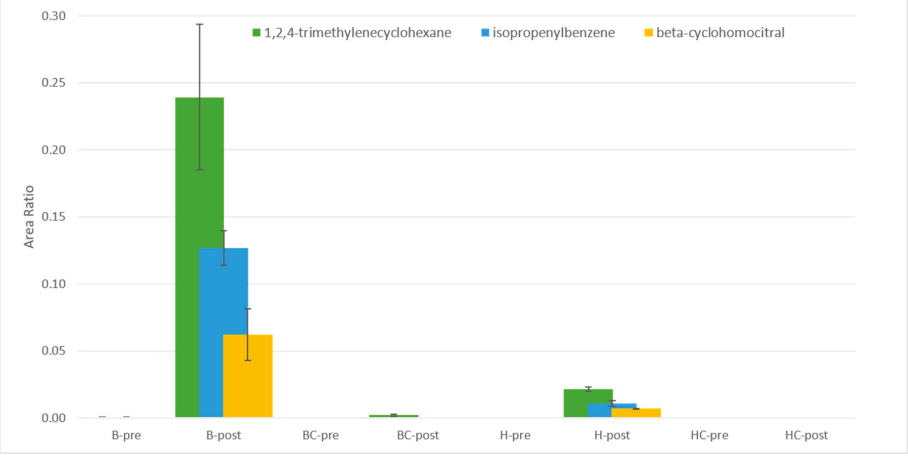

Figure 3. 1,2,4-trimethylencyclohexane, isopropenylbenzene, and beta-ciclohomocitral in honey wine before and after fermentation by *S. cerevisiae* EC1118. Samples were named as follows: B = blossom honey, BC = blossom honey with blackcurrant, H = honeydew honey, HC = honeydew honey with blackcurrant, pre = pre-fermentation, and post = post-fermentation.

The post-fermentation volatile organic compounds had been highlighted in the heatmap (Figure 2). Among them, isoamyl alcohol was found featuring in many fermented alcoholic beverages and already reported in mead [10,17,35], giving a solvent, sweet, and nail polish aroma and also part of fusel oil [36]. Another detected fusel oil representative was isobutanol, only found in post-fermentation samples (Figure 2), which lead to green notes in the flavor of beverages [37]. Esters are contributing to the fruity and floral nuances of meads [38]. In the samples, 14 esters were found; these compounds appearing in different concentrations in the samples, as shown in Figure 4, with a visible magnitude increased

at the end of the fermentation process. H-pre had a higher content of esters and a slightly different profile of this class of compounds compared with the other pre-fermentation samples (Figure 4a). H-pre was, in fact, characterized by the presence of ethyl octanoate end ethyl nonanoate in higher amount and by the presence of ethyl decanoate not revealed in the other worts. In the same product, at the end of the fermentation process, the esters profile seemed to be more similar, although H-post exhibited a higher content in ethyl octanoate and ethyl decanoate compared to the other honey wines (Figure 4b). All found esters are common components in alcoholic beverages and are often found in fruits. In detail, the most abundant compounds determined in our fermented samples were isoamyl acetate, a characteristic compound of banana flavor, ethyl hexanoate and ethyl octanoate that contribute to a sweet and strawberry-like aroma, and ethyl decanoate with a sweet, fruity apple flavor [39]. Ethyl octanoate and ethyl decanoate aroma have also been described as waxy and soapy [40].

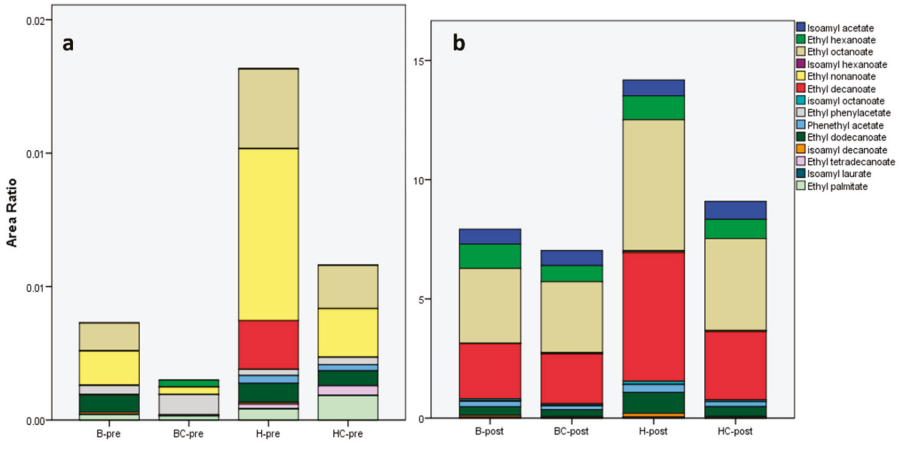

Figure 4. Esters composition in pre (**a**) and post (**b**) fermentation samples. Values represent the area ratio with the internal standard (n = 3). B-pre: blossom honey pre-fermentation; B-post: blossom honey post-fermentation; BC-pre: blossom honey with blackcurrant pre-fermentation; BC-post: blossom honey with blackcurrant post-fermentation; H-pre: honeydew honey pre-fermentation; H-post: honeydew honey post-fermentation; HC-pre: honeydew honey with blackcurrant pre-fermentation; HC-post: honeydew honey with blackcurrant post-fermentation.

2.4.2. Honey Influence on the Aromatic Characteristics of the Product

The honey used for the fermented product represented another important factor influencing the final aroma of the beverages. In our study, two types of honey were used: blossom honey (B) and honeydew honey (H). The PCA showed a separation on the second component, based on the honey used for the fermented beverage (Figure 1); looking at the heatmap, we identified some compounds potentially related to the types of used honey (Figure 2).

Few compounds seemed to be higher in B-pre product compared to the others and were accumulated in B-post products in comparison to the H samples; they were 2-(4-methylphenyl)-2-propanol, 2,6-dimethyl-3,7-octadiene-2,6-diol, 3,6-bis(methylene)octahydro-1-benzofuran, 3,6-dimethylene-1,7-octadiene, 3,7-dimethyl-1,5,7-octatrien-3-ol, 4-isopropyl-3-methylphenol, citral, and m-cymene.

This cluster showed to be more abundant in all B meads (with and without blackcurrant addition) compared with H samples. Other characteristic compounds of B meads were *trans*-nerolidol, *cis* and *trans*-rose oxide, anethofuran, borneol, and ethyl phenylacetate. These compounds produced during the fermentation process (Figure 2) could be responsible for a floral, rose, and balsamic camphor perception in the final product. Ethyl phenylacetate can not only contribute to a positive note, but it is

also considered as off-flavor formed in beer during aging from precursors, which are produced during the fermentation [41].

Regarding the honeydew honey samples, no typical compounds were found in pre-fermentation samples. However, the final beverage seemed to be richer in acids, such as hexanoic acid, octanoic acid, decanoic acid, and esters, such as ethyl octanoate, isoamyl hexanoate, ethyl nonanoate, ethyl decanoate, ethyl dodecanoate. Short-chain fatty acids, such as octanoic acid (caprylic acid) and hexanoic acid (caproic acid), were also reported [17]. These compounds were present in higher concentrations and are associated with negative characteristics of "rancid," "cheese," and "fatty" aroma [7].

Terpene compounds were found in both pre- and post-fermentation samples, and, as expected, these compounds mostly originate from the raw materials. Many of the detected terpenes are known for their positive influences on mead aroma. Citral, for example, has a lemon-like pleasant odor [42], linalool a floral and spicy odor [43]. The stereoisomers *cis*-rose oxide and *trans*-rose oxide are found in flowers, and fruit and essential oils can contribute to fruit and floral notes in fruits and grapes. Mentofuran is a constituent of peppermint oil [44].

2.4.3. Influence of *Ribes nigrum* Addition

Blackcurrant addition, with its peculiar composition, had the capability to modify the fermentation environment for the yeast, leading to different volatiles, influencing, therefore, the aroma profile of the final product. Blackcurrant aroma is characterized by various volatile components, including esters and terpenoids. As reported, cultivars and growing and storage conditions can affect the flavor component [45,46]. Among the most reported compounds for the characteristic of blackcurrant fruit, 2-methylbutyl acetate, methyl butanoate, ethyl butanoate, and ethyl hexanoate, belonging to the esters class, that confer fruity and sweet notes are mentioned. Besides, nonanal, beta-damascenone, and monoterpenes, ketones, and sulfur compounds, such as 4 methoxy-2-methyl-butanethiol (catty note flavor), are reported. In our results, we found few compounds with significantly higher amounts in BC and HC post-fermentation: citronellol, a-terpinol, and nonanal, as shown in Figure 2. It was noted that besides the esters nonanal, an aldehyde C-9 could give the classical aldehyde note of waxy, citrus, floral, and green.

2.5. Sensory Test

The sensory test was carried out in order to find a possible link between the volatile compound profile of the product and their sensory properties (Table S2). Among the 44 nontrained participants to the sensory panel, 77% correctly paired the honey wine prepared with the same honey; the sensory differences linked to the honey used for mead production were, therefore, significantly perceivable.

A nine-point hedonic evaluation scale was structured, point 1 stated for "dislike extremely", while point 9 stated for "like extremely". Using this scale, panelists described the samples as likable as on average; most judged the product as like slightly, like moderately, or like very much. There was no significant difference between the samples in relation to the overall impression and no significant difference between the samples in relation to the olfactory impression. The acceptance level for the honey wine for the sensorial analysis was expressed as a mean value. None of the means presented significant differences. Overall impression and flavor attributes for all beverages variated from 64 to 68% and between 65 and 72%, respectively. According to Dutcosky [47], the acceptance factor (AF) $\geq$ 70% represented good acceptability for the attribute analyzed in a sensorial analysis. Honey wine obtained with blossom honey showed an AF of 72%.

Honey wine B ranked first, followed by BC, HC, and H, for both the overall impression and the odor. According to the Friedman test, only the ranking for the odor impression showed to be significant. This seemed to be in line with the AF. This might be related to the higher amount of hexanoic acid, octanoic acid, decanoic acid, or esters, such as ethyl octanoate, isoamyl hexanoate, ethyl nonanoate, ethyl decanoate, ethyl dodecanoate found in H and HC product compared to B and BC. Short-chain fatty acids, such as octanoic acid (caprylic acid) and hexanoic acid (caproic acid), have been associated

with negative characteristics, such as "rancid," "cheese," and "fatty" aroma [7]. The odor of esters like ethyl octanoate and ethyl decanoate odors have also been described as waxy and soapy [40].

In general, it seems that the VOCs profile imparted by the honey, described above, also has some impact on the sensory perception. However, for a more descriptive sensory evaluation, a trained panel will be able to provide a clearer link between the sensory perception and the specific volatiles or group of volatile as distinguished in the heatmap.

3. Materials and Methods

3.1. Mead Ingredient

Honeydew honey (Bosco, Mieli Thun, Vigo di Ton, Italy) and blossom honey (mixed honey with a prevalence of *Ailanthus altissima*) (Mieli Thun, Vigo di Ton, Italy) were used. Honeydew honey was collected in the northern Italian wood; dark amber color was described by the supplier as spicy (black pepper, juniper berries, and cloves) with a note of fresh vegetables, carob, rhubarb, and liquorice stick. Blossom honey had a golden color and a creamy consistency and was characterized, according to the supplier, by the smell of muscat grapes and peach syrup; lychee was conferred by the prevalence of *Ailanthus altissima*, also known as the tree of heaven. One part of honey (*w*/*w*) was used in all the preparation. Warm tap water was used to dissolve the honey. Black currant (*Ribes nigrum*), common berry fruit in South Tyrol, Italy, had been purchased from a local producer and stored at −80 °C until used. Half part of berry fruit (w/w) was used for the *Ribes nigrum* added recipes. The berries were crushed before the addition of other ingredients. The final mixture had a temperature of about 30 °C before acidification. Acidification was carried out using a citrate buffer in order to reach a pH value between 3–3.2. A preliminary test was carried out to establish the amount of citrate buffer to be added to each mixture.

Saccharomyces cerevisiae yeast strain EC1118 (Lallemand Inc., Montreal, QC, Canada) was used in the dry active form at the ratio of 25 g/hl after rehydration, according to the manufacturer's instructions. As a yeast protectant in the rehydration step (GoFerm Lallemand Inc., Montreal, QC, Canada), it was used at a 1:1 ratio with the weighted yeast. This product contained all essential vitamins, minerals, and amino acids required to create a non-stressful environment for yeast rehydrating in water.

To ensure the necessary amount of nitrogen and avoid a stuck fermentation, "FermaidE" (Lallemand Inc., Montreal, QC, Canada) was added to the must as a vitamin, organic, and inorganic nitrogen source at the ratio of 30 g/hl.

3.2. Honey Wine Wort Preparation and Fermentation Follow Up

Four different recipes for honey wine preparation were used. Blossom honey with and without black currant recipes was compared with honeydew honey with and without black currant (B, BC, H, and HC, respectively). Each recipe was tested in triplicate, carrying out the fermentation in a 5 L glass flask filled up to 3.5 L. All flasks were closed with an air-lock valve. All recipes are described in Table 2. Nitrogen exogen source was added, according to the manufacturer's instructions: two-third at the yeast inoculum and the remaining one-third after one-third of the fermentation was completed. Fermentation was monitored, measuring the weight loss once or twice per day. All the flasks were incubated at 18 °C. Fermentation end was detected when two subsequent weight measurements did not differ for more than approx. one gram: at this stage, flasks were left overnight at 4 °C. After overnight cold storage, berry solids were separated with the aid of a strainer, honey wine was separated by the yeast sediment, and samples were collected for further analysis. The remain was bottled and left at 4 °C until the sensory test. Samples were immediately analyzed for pH and total acidity. For the other analyses, samples were stored at −80 °C until used.

Table 2. Ingredients used for the four recipes tested for honey wine production. Weight is referring to the total amount of 11 kg prepared for each recipe before aliquoting the 3.5 L of each replicate (n = 3). B: blossom honey; BC: blossom honey with black currant; H: honeydew honey; HC: honeydew honey with black currant.

Recipe	Component	Weight (kg)	Ratio
B	Honey	2.750	1.00
	Water	8.075	2.94
	citrate buffer	0.175	0.06
BC	Honey	2.750	1.00
	Water	6.871	2.50
	Buffer	0	0
	black currants	1.379	0.50
H	Honey	2.750	1.00
	Water	7.750	2.82
	citrate buffer	0.500	0.18
HC	Honey	2.763	1.00
	Water	6.682	2.43
	Buffer	0.180	0.07
	Black currants	1.375	0.50

3.3. Honey Wine Analysis

3.3.1. Physicochemical Parameters

The total soluble solids determined as Brix were measured using a digital refractometer (PAL-BX/RI, Atago, WA, USA). For the pH, a pH electrode was used (pH70+ DNH pH meter with Digital pH electrode mod 201 T, XS instruments, Carpi, Italy).

The content was the sum of glucose and fructose after inversion was measured, according to the OIV-MA-AS311-02 R2009 + OIV-MA-AS2-03B R2012. The alcohol content in volume percent (% vol) was measured following the international methods of wine and must analysis (OIV-MA-AS312-01A R2016 par 4.B). Fructose, glucose, and sucrose in honey were expressed as g/L of glucose, acetic, and lactic acid, and residual sugars were measured enzymatically with the CDR BeerLab® Touch (CDR s.r.l., Ginestra Fiorentina, Italy), according to the manufacturer's instructions.

Acetaldehyde content was measured with the aid of a spectrophotometric-enzymatic-based kit for acetaldehyde, according to the manufacturer's instructions (K-ACHYD 06/18, Megaenzyme International Ireland INC. Bray, Co. Wicklow, Ireland), measuring the absorbance variation at 340 nm with the aid of a microtiter plate reader spectrophotometer (Multiscan Sky Spectrophotometer, Thermo Fisher Scientific Life Technologies Italia, Monza, Italy) where a 96-well microplate was used.

3.3.2. Total Polyphenolic Content

The polyphenolic content of the meads was measured using Folin–Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) in a colorimetric assay. The method was adapted from a published method [48]: Folin-Ciocalteu reagent was added to the sample, and after a reaction time of 3 min at room temperature, 2 M sodium carbonate solution was added. After two hours at 21 °C, the absorbance at 765 nm was read. For the quantification, a standard curve ranging from 50–500 mg/L of gallic acid was prepared. The polyphenol content was determined by linear regression from the standard curve, and the results were expressed as mg/L of gallic acid.

3.4. Volatile Organic Compounds

For the volatile organic compounds (VOCs) analysis, a headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS) was used (QP2010 SE

Shimadzu, Kyoto, Japan). The fermented mead, as well as the unfermented musts, were examined. The method was adapted from Ravasio et al. [49]. Briefly, 2.5 mL of sample was placed in 20 mL glass vials with 0.75 g of NaCl and 10 µL of internal standard (2-octanol, 50 µg/mL). Samples were incubated for 10 min at 40 °C and 250 rpm. Compounds in the headspace were adsorbed for 40 min at 40 °C using 2 cm DVB/CAR/PDMS 50/30 µm fiber from Supelco (Bellefonte, PA, USA). Compounds were desorbed in the GC inlet at 250 °C for 4 min. Chromatographic separation was carried out using a ZB-WAX (30 m × 0.25 mm × 0.25 µm, 40–260 °C) Capillary GC-Column Zebron (Phenomenex, Torrance, CA, USA), using helium as carrier gas at 1.2 mL/min.

The temperature program for the oven was set as follows: 40 °C for 4 min, then up to 250 °C, at 6 °C/min held for 5 min. The total run time was 44 min. The mass spectrometer (quadrupole) was operating in full scan mode, detecting fragments in a mass range of 35 to 350 m/z. Data processing was performed using GC-MS solution Software from Shimadzu. The most intensive ion was used as a quantifier and the ratio of the second and third as a qualifier. The identification of VOCs was carried out by comparing mass spectra using the NIST 2017 database, retention indexes, and with the standard injection when available. The experimental linear temperature retention index of each compound was calculated using a series of n-alkanes (C8-C20) in the same experimental conditions as the samples. Results were expressed as area ratio between compound and internal standard.

3.5. Sensory Analysis

A sensory consumer test was carried out with the aid of 44 nontrained consumer panelists; each participant was asked to evaluate all four types of honey wine. Forty milliliters of samples of each product were served for each panelist. Samples were coded with the number served in different sequences and arrangements randomly. The panelists were asked to rate each sample, with a nine-point hedonistic scale, for the (a) the overall taste impression and (b) for the odor overall impression. The nine-point scale was structured as follows: 9: like extremely, 8: like very much, 7: like moderately, 6: like slightly, 5: like neither nor, 4: dislike slightly, 3: dislike moderately, 2: dislike very much, 1: dislike extremely. The acceptability of the tested beverages was assessed by calculating the acceptability factor (AF) using standardized criteria: AF = A*100/B, where A is the mean value obtained for each attribute, and B is the maximum value used to judge each attribute [47,50]. They were asked also to pair the product made with the same honey. Participants were asked to rank the four samples for both the overall impression and the odor overall, assigning the number one to the best one and number four the worst.

3.6. Statistical Methods

For statistical analyses, SPSS Statistics software Version 26 (IBM Inc., Armonk, NY, USA) and Microsoft Excel 2019 software were used. Means for every data are expressed as arithmetic mean ± standard deviation of the three replicates for every product. To determine if there was a significant difference between results, a one-way analysis of variance (ANOVA) and a Tukey posthoc test were performed. For all analyses, $p < 0.05$ was considered statistically significant. Used test and corresponding *p*-value were indicated together with the result in each specific session. In addition, Microsoft Excel 2019 was used to verify the significance of the pairing test [51]. Friedmann-Test for statistical analysis with n = 44 test subjects and k = 4 samples was carried out using Microsoft Excel 2019 [48]. The R FactoMineR package was used to perform the PCA [52], and the factoextra package for extracting and visualizing the results. The data had been scaled to unit variance before the analysis to avoid some variables to become dominant just because of their large measurement units. The NMF package was used for the heatmap and hierarchical cluster analysis with a Euclidean distance [53].

4. Conclusions

Two different kinds of honey in the presence or absence of black currant were tested for honey wine production. Using these ingredients, no stressful condition seemed to be occurring for the yeast,

leading to fermentation delay or arrest, producing a medium or low alcoholic drink. The fermented products were described by a large number of volatile organic compounds capable of allowing the distinctive metabolic contribution of the yeast, also as a response to the honey and the fruit added in fermentation. In particular, the honey contributed to shaping a specific volatile profile, somehow perceivable during sensory analysis. To a lesser extent, also using berry fruit, such as black currant, provided a way to shape flavor and polyphenols content of the final drink. Further investigation would be necessary to evaluate the specific sensory contribution of every single volatile organic compound alone or in association with others found in this study. More information on volatile metabolites associated with mead had been provided that might help to develop alternative medium to low alcoholic drinks at a reasonable cost, adding value to beehive products.

Supplementary Materials: The following are available online. Figure S1. Fermentation kinetic; Figure S2. Volatile organic compound measured in the honey wines before and after fermentation; Table S1. Volatile organic compound measurement and descriptions; File S1. Eigenvalues and correlation of the PC analysis; Table S2. Sensory evaluation of the honey wine.

Author Contributions: Conceptualization, L.C.; methodology, G.C. and L.C.; validation, G.C., L.C., and P.R.; formal analysis L.D., M.S., and E.U.; investigation, G.C., L.D., and M.S.; resources, L.C. and P.R.; data curation, G.C. and L.C.; writing—original draft preparation, G.C., L.C., and M.S.; writing—review and editing, G.C., L.C., L.D., H.J., P.R., E.U., and E.Z.; visualization, G.C. and L.C.; supervision, L.C., H.J., and P.R.; project administration, L.C.; funding acquisition, L.C. and P.R. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Samples of the compounds are not available from the authors.

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Identification of Aroma Differences in Refined and Whole Grain Extruded Maize Puffs

Kenneth Smith [1] and Devin G. Peterson [2,*]

[1] Department of Food Science and Nutrition, 1334 Eckles Avenue, 145 FScN Building, University of Minnesota, St. Paul, MN 55108, USA; smit4423@umn.edu

[2] Department of Food Science and Technology, 2015 Fyffe Rd., 317 Parker Food Science & Technology Building, The Ohio State University, Columbus, OH 43210, USA

* Correspondence: peterson.892@osu.edu; Tel.: +01-614-688-2723

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Abstract: Differences in the aroma profiles of extruded maize puffs made from refined grain and whole grain flour were investigated. Gas chromatography/mass spectrometry/olfactometry (GC/MS/O) analysis reported 13 aroma compounds with a flavor dilution (FD) value ≥16. Quantitative analysis identified eight compounds as statistically different, of which seven compounds were higher in concentration in the whole grain sample. Sensory recombination and descriptive analysis further supported the analytical data, with higher mean aroma intensities for cooked, corn chip, roasted, and toasted attributes for the whole grain sample. Generally, the compounds responsible for perceived differences in whole grain maize extruded puffs were associated with increased levels of Maillard reaction products, such as 2-ethyl-3,5-dimethylpyrazine and 2-acetyl-2-thiazoline.

Keywords: whole grain; refined grain; GC/O; Maillard reaction; maize; aroma; flavor

1. Introduction

The consumption of whole grain has been associated with a range of health benefits such as body-weight regulation, reduced risk of chronic pathological conditions, and reduced blood glucose levels [1–3]. However, most Americans fail to consume the recommended whole grain intake (48 g/day), which has a direct effect on health and was recently identified as a main contributor to suboptimal diets that are responsible for 1 out of 5 deaths globally [4,5]. In cereal-based foods, refined grains are often preferred in comparison to their whole grain counterparts. The negative flavor attributes associated with whole grain products including bitter taste and vegetative aromas have been reported as one of the most influential factors limiting consumption [6,7].

Breakfast cereals are cooked products introduced in the human diet at a young age and constitute an excellent opportunity for early exposure to whole grain flavor [8]. Maize is a common grain used for cereal production and the impact of extrusion (cooking) parameters on physico-chemical and sensory properties of extruded cereals has been largely studied [9–12]. Flavor generation during the extrusion of cereals involves thermally induced reactions, such as the Maillard reaction and lipid degradation. Extrusion conditions such as heat, water content, and residence time have been shown to exert significant effects on the flavor profiles of extruded products [9,12] with cooking temperature identified as a main influential factor for the formation of flavor compounds. Flavor development in extruded products has been investigated with a focus on processing conditions and has overlooked the impact of whole grain versus refine grain flour formulation. In wheat bread, the utilization of whole versus refined grain flour had a significant impact on flavor generation [13]. Several key compounds that give refined wheat bread its typical aroma attributes were less abundant in whole wheat bread due to the suppression of key Maillard-type flavor formation pathways caused by the elevated levels

of phenolic compounds. In addition to aroma generation, taste compounds are thermally generated by Maillard-type pathways during bread making [14]. Whole grain flour, as compared to refined grain flour, has elevated levels of phenolic compounds, lipids, vitamins and is composed of a unique protein composition, all of which can significantly alter the thermal generation of flavor compounds [13,15–17]. During extrusion processing, high temperature and short time conditions favor Maillard and lipid oxidation flavor-formation pathways [9]; however, the characterization of the flavor differences between whole and refined grain maize products has not been reported.

The objective of this work was to investigate the influence of whole versus refined maize flour on the aroma of extruded puffs and the sensory impact of the identified differences. Aroma-active compounds were identified using gas chromatography/mass spectrometry (GC/MS) and sensory differences were characterized using sensory descriptive analysis.

2. Results and Discussion

To characterize the main differences in the aroma profiles of extruded maize puffs made from whole grain versus refined grain flour, odorants were identified using gas chromatography/mass spectrometry/olfactometry (GC/MS/O) and selected based on cut-off flavor dilution (FD) values ≥16 (see Table 1). Thirteen compounds were selected based on this criterion and all the odor compounds identified have been previously reported in extruded maize products [9,12]. However, the influence of flour type on their generation and their impact on the aroma profile of extruded maize whole grain puffs (WGP) and refined grain puffs (RGP) has not been previously reported. Further quantitative analysis of the 13 compounds was conducted and is reported in Table 2.

Table 1. Identified aroma compounds in extruded maize refined grain puffs (RGP) and whole grain puffs (WGP).

Compound [a]	Odor Descriptor [b]	LRI [c]		Flavor Dilution Value ≥ 16 [d]	
		DB-Wax	DB-5	WGP	RGP
hexanal	Green/Oxidized	1084	801	64	32
2-methylpyrazine	Roasted	1176	827	128	64
2,3-dimethylpyrazine	Roasted	1240	911	128	32
2,5-dimethylpyrazine	Roasted	1253	912	16	32
2-methyl-2-thiazoline	Roasted/Toasted	1436	933	128	64
2-pentylfuran	Earthy/Oxidized	1240	993	128	32
2-ethyl-3,5-dimethylpyrazine	Roasted	1457	1081	64	32
3-hydroxy-2-methyl-4*H*-pyran-4-one	Caramel/Toasted	1955	1087	128	64
2-methoxyphenol	Smokey	1872	1088	128	64
2-acetyl-2-thiazoline	Popcorn/Corn Chip	1772	1103	64	32
(*E*,*E*)-2,4-decadienal	Oxidized	1815	1312	64	32
2-methoxy-4-vinylphenol	Clove	2189	1322	128	64
4-hydroxy-3-methoxybenzaldehyde	Vanilla-like	2589	1410	64	16

[a] Compounds positively identified by linear retention index (LRI), mass spectrometry (MS), and authentic compound; [b] Odor described at sniffing port during gas chromatography/olfactometry (GC/O), [c] LRIs calculated using GC-MS on DB-WAX and DB-5 columns, values relatively to the n-alkane ladder, [d] Flavor dilution based on the average of two panelists.

Eight compounds including six Maillard reaction products and two phenolic compounds were found to be statistically different between WGP and RGP. All the compounds were found in greater amounts in the WGP samples except for 2,5-dimethylpyrazine, which was found in higher concentration in the RGP with 140 μg/kg compared to 100 μg/kg in the WGP. A higher formation of Maillard reaction aroma compounds, in general, in the WGP can be explained by compositional differences between whole and refined grain flours. Milling cereals alters the concentration and composition of proteins and lipids in the flour. The milling process used to produce refined maize flour removes the protein-rich pericarp/germ leaving primarily the starchy endosperm flour [16]. Protein and amino acid content in whole grain maize flour is altered compared to refined maize flour [16,17]. Amino acids are very influential for the progression of Maillard reaction pathways [21,22] and key precursors for the

formation of key odorants such as 2-acetyl-2-thiazoline (cysteine [23]) and 2-ethyl-3,5-dimethylpyrazine (alanine [24]). These two aroma compounds showed concentrations 2.2 and 2.1-fold higher in WGP when compared to RGP, respectively. A lower concentration of amino acids (i.e. cysteine and alanine) in refined maize flour could have resulted in the observed changes in the aroma generation noted or perhaps are due to differences in sugar fragmentation. The ratio of precursors, i.e., reducing sugar to *N*-containing compounds, has been demonstrated to selectively favor formation pathways through the modification of the intermediate reactive chemistry [25,26]. In glucose model mixtures in particular, changes in the glucose to amino acid ratio ultimately modulate the generation of pyrazines; a greater ratio of sugars:amino acids in the RGP could have favored the generation of reactive intermediate species involved in the formation of 2,5-dimethylpyrazine, while suppressing other products, such as 2-acetyl-2-thiazoline or 2-ethyl-3,5-dimethylpyrazine.

Table 2. Concentration of aroma compounds in extruded maize refined grain puffs (RGP) and whole grain puffs (WGP).

Compound	Mean ± CV [2]		Concentration Ratio (WGP/RGP)	Odor Threshold in Water (μg/L) [18–20]
	WGP (μg/kg)	RGP (μg/kg)		
hexanal [1]	470± 8	436 ± 24	1.1	4.5–5.0
2-methylpyrazine	363± 7 [b]	292± 3 [a]	1.2	60,000–105,000
2,3-dimethylpyrazine	653± 7 [b]	280± 2 [a]	2.3	2,500–35,000
2,5-dimethylpyrazine	100± 8 [b]	141± 5 [a]	0.7	800–1800
2-methyl-2-thiazoline [1]	147± 10	137± 13	1.1	2
2-pentylfuran [1]	183± 12	153± 9	1.2	6
2-ethyl-3,5-dimethylpyrazine [1]	260± 16 [b]	124± 18 [a]	2.1	1
3-hydroxy-2-methyl-4*H*-pyran-4-one	370± 9 [b]	321± 10 [a]	1.2	35,000
2-methoxyphenol [1]	317± 5	297± 15	1.1	3–21
2-acetyl-2-thiazoline [1]	377± 5 [b]	168± 10 [a]	2.2	1
(*E*,*E*)-2,4-decadienal [1]	293± 14[b]	243± 3 [a]	1.2	0.07
2-methoxy-4-vinylphenol [1]	3600± 4 [b]	843± 3 [a]	4.3	3
4-hydroxy-3-methoxybenzaldehyde [1]	3517± 7 [b]	1218± 5 [a]	2.9	20–200

[1] Concentration above aqueous odor threshold values; [2] Different letters (a, b) indicate a significant difference between samples using a t-test, $p < 0.05$, $n = 5$.

Two ferulic acid degradation products 2-methoxy-4-vinylphenol and 4-hydroxy-3-methoxybenzaldehyde were quantified in higher amounts in WGP (Table 2) and described in GC/O with clove and vanilla aroma descriptors (Table 1), respectively. In grains, the phenolic material is mainly distributed in the bran layer. For example, in sweet maize the pericarp and germ contain approximately 325 and 702 ug/g of ferulic acid, respectively while the endosperm, the primary component of refined maize flour, contains approximately 13 mg/g [27]. In general, the total phenolic content of the pericarp is approximately 30-fold higher than the endosperm [28]. In bread, the liberation of phenolic compounds (i.e., ferulic acid) from whole wheat flour during baking was reported to suppress the generation of Maillard aroma compounds through carbonyl trapping mechanisms [13]. The noted increase in Maillard aroma compound generation in the WGP versus RGP suggested the phenolic-carbonyl reaction mechanisms that suppressed aroma formation in bread were not relevant in extruded maize products, albeit the heating profile of extrusion cooking is drastically different than baking bread. Others have shown that the addition of the phenolic compound, rutin, during the preparation of baked rye-buckwheat biscuits resulted in higher levels of Maillard-type aroma compounds, such as alkyl-pyrazines [29].

Two lipid oxidation compounds, hexanal and 2-pentylfuran were not found to be statistically different in concentration between RGP and WGP, however (*E*,*E*)-2,4-decadienal was significantly higher (approximately 20%) in the WGP sample. Thus, the higher content of lipid in the whole grain maize did not have a major impact on the formation of these lipid oxidation aroma compounds, perhaps because of the elevated levels of antioxidative components of the pericarp.

To draw further insight regarding the impact of quantitative differences of aroma compounds of the maize extruded puffs (Table 2) on the aroma profile, sensory descriptive analysis (DA) was conducted on both the WGP and RGP samples (Figure 1), as well as aroma recombination models (Figure 2).

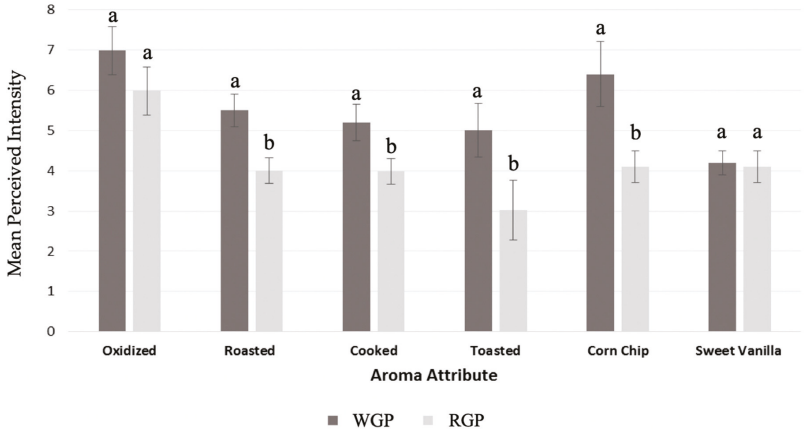

Figure 1. Mean aroma attribute intensity scores of extruded maize refined grain puffs (RGP) and whole grain puffs (WGP); Different letters (a, b) indicate a significant difference between samples for each attribute according to Tukey's HSD, $p < 0.05$

The perceived intensities of the aroma descriptors were significantly higher in whole grain versus refined maize puffs for four out of six attributes (Figure 1). In the whole grain sample, the highest reported mean intensities were for the attributes cooked, corn chip, roasted, and toasted and likely were associated with the increased concentration of the Maillard-derived compounds (Table 2). All the Maillard compounds, with the exception of 2,5-dimethylpyrazine, were statistically higher in concentration in the WGP compared to the RGP. The oxidized attribute was not rated as significantly different in intensity between WGP and RGP. The lipid oxidation compounds typically associated with oxidized sensory properties including 2-pentylfuran and hexanal were indeed found at similar levels in both samples, whereas 2,4-decadienal was approximately 20% higher in the WGP (Table 2). These results indicated that lipid oxidation did not play a major role in aroma differences between WGP and RGP. Finally, the intensity of the vanilla attribute was rated similarly in both samples, and thus was not established as a discriminant sensory trait common to both WGP and RGP despite higher levels of these phenolic degradation compounds in the WGP sample.

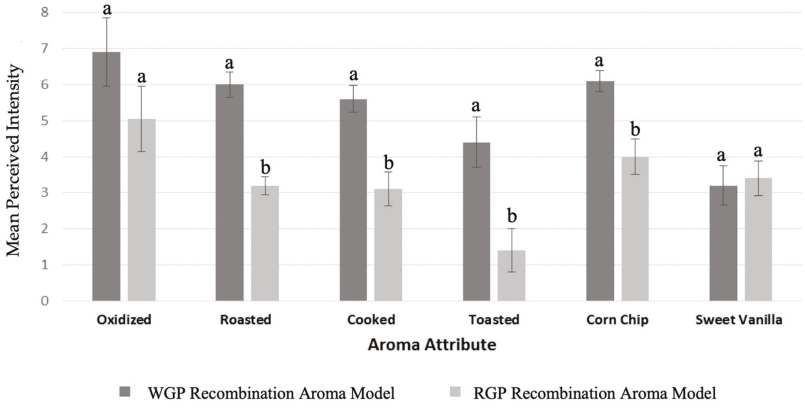

Figure 2. Mean aroma attribute intensity scores of aroma recombination models for extruded maize refined grain puffs (RGP) and whole grain puffs (WGP); Different letters (a, b) indicate a significant difference between samples for each attribute according to Tukey's HSD, $p < 0.05$.

Aroma recombination models were developed to determine the contribution of the aroma composition (Table 2) on the sensory attributes of the WGP and RGP samples (Figure 1). In general, the recombination models of the maize puff samples (Figure 2) agreed with the authentic samples (Figure 1) and showed that toasted, roasted, corn chip, and cooked attributes had significantly higher mean intensities in WGP in comparison to RGP. The odor threshold values are also shown in Table 2, with 10 of the 13 compounds being reported at concentrations above the threshold. Odor thresholds provide a basis to understand sensory relevance; however, some caution would be warranted when extrapolating these threshold values in water to the aroma attributes perceived by the orthonasal evaluation of a low-moisture high-starch puffed cereal product. Nonetheless, when focused on compounds above their aqueous odor threshold values that were also significantly different in concentration (Table 2) and considering the odor properties (Table 1), two Maillard reaction products, 2-ethyl-3,5-dimethylpyrazine and 2-acetyl-2-thiazoline were indicated as the main contributors to the noted sensory differences in the aroma profile of the WGP and RGP samples (Figure 1).

In summary, the Maillard reaction products were established as the main aroma differences between WGP and RGP. This study showed a predominance of Maillard aroma compounds and sensory traits in the WGP likely induced by the heat conditions of the extrusion process. Historically, the aroma attributes of Maillard compounds associated with roasted, toasted, corn chip, and cooked are viewed as positive traits in heat-processed foods. Thus, the aroma attributes of extruded whole grain maize did not appear to negatively alter the flavor profile (compared to refined grain product). However, further work is needed to understand if these changes could contribute to an unbalanced aroma profile when present at higher intensities. Moreover, the impact of whole grain maize on the taste profile (i.e., bitterness) could also play a role in product acceptance.

3. Materials and Methods

3.1. Materials

Hexanal, 2-methylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2-methyl-2-thiazoline, 2-pentylfuran, 2-ethyl-3,5-dimethylpyrazine, 3-hydroxy-2-methyl-4*H*-pyran-4-one, 2-methylphenol, 2-acetyl-2-thiazoline, (*E*,*E*)-2,4-decadienal, 2-methoxy-4-vinylphenol, 4-hydroxy-3-methoxybenzaldehyde, 4-heptanone, 2-methyl-3-heptanone, trisodium phosphate, calcium carbonate, anhydrous sodium sulfate, corn starch and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and methylene chloride, in GC-Resolv® grade, were purchased from Fischer Scientific (Pittsburgh, PA, USA). Maizewise™ whole grain and Innovasure™ refined maize flours were purchased from Cargill (Minneapolis, MN, USA). All the sensory reference materials, dry uncooked Bob's Red Mill Steel Cut Oats, Bergen Unsalted Lightly Roasted Almonds, Organic Valley Whole UHT Milk, Toasted Wonder Bread™, Nilla® Wafers, and Old Dutch Restaurante® Style Yellow Corn Tortillas Chips, were purchased from a local grocery store.

3.2. Twin-Screw Extrusion

Extrusion conditions were designed to yield uniform cell structure throughout each puff [10]. Briefly, extrusion processing was carried out using The Joseph J. Wartheson pilot plant (Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN) with a Buhler DNDL-44 twin-screw extruder (Uzwil, Switerland). Two formulations were produced, a refined maize flour formulation and a whole grain maize flour formulation. The refined maize flour dry formulation consisted of 970 g (97%) refined maize flour with 10 g (1%) trisodium phosphate, 10 g (1%) calcium carbonate, and 10 g (1%) sodium chloride. The whole grain maize formulation consisted of 465 g (48%) refined maize flour and 505 g (52%) whole grain maize flour with 10 g (1%) trisodium phosphate (TSP), 10 g (1%) calcium carbonate, and 10 g (1%) sodium chloride. The ingredients were added to a mixer and mixed for 10 min. The mixture was added with 14% (*w*/*w*) water into the extruder with a low work screw configuration via a feeder and processed per the following extrusion parameters:

computer-controlled shaft speed of 350 rpm, measured die pressure of 10.1 ± 0.5 bar, die temperature of 160 ± 1 °C, material throughput of 50.8 ± 0.1 kg/h with 7 kg/h water, and a cutter speed of 1200 rpm. Due to differences in the physical and chemical characteristics of the refined and the whole grain flour mixes, the refined maize flour formulation showed an increased shaft torque of 224 N·m over the whole grain maize flour formulation, which had a shaft torque of 215 N·m. The specific mechanical energy for refined maize flour formulation was 164 kW/h, while the whole grain maize flour was 159 kW/h. Other parameters were constant across both formulations. The puffed product was collected, dried on a liquid air bed, and stored in high-density polyethylene bags at −40 °C for later analysis.

3.3. Solvent Extraction

Briefly, 300 g of maize puffs were ground and placed in a 1 L Erlenmeyer flask. Next, 600 g of methanol spiked with 0.1 mg/L 4-heptanone were added to the flask, which was then shaken for 24 h on an orbital shake table set at 200 rpm. Methanol was collected and the ground maize puffs were re-extracted for 2 h using 400 g of methanol at 200 rpm. Organic layers were pooled, and 600 g of the methanol collected was subsequently combined with 600 mL of reverse osmosis purified water. The water-methanol mixture was then poured into three 1 L separatory funnels and extracted using 500 g of methylene chloride (DCM) spiked with 0.1 mg/L of 2-methyl-3-heptanone. DCM was added in 100 mL aliquots to each funnel for a total of 5 extractions. The DCM extract was then placed in a –20 °C freezer overnight to separate and remove any residual water-methanol. The DCM extract was collected and then dried using sodium sulfate and subsequently concentrated via distillation to 1.0 g. The concentrated extract was stored at –80 °C until analysis. Additionally, internal standards used were analyzed for reproducibility during extraction. Methanol was spiked with 4-heptanone and DCM was spiked with 2-methyl-3-heptanone to achieve 100 and 150 mg/L, respectively, in the concentrated solvent. This protocol, when compared to DCM extraction, resulted in 30% more aroma actives detected during GC/O (data not shown).

3.4. Gas Chromatography/Olfactometry/Mass Spectrometry (GC/O/MS): Aroma Extraction Dilution Analysis (AEDA)

GC/O analyses were performed on an HP6890 GC (Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-5 column (30 m × 0.25 mm i.d. × 0.25 μm film thickness (Agilent Technologies)) coupled with a 5973 MS (Agilent Technologies) operated in electron impact mode as similarly described by Moskowitz et al. [13]. The system was also equipped with an olfactometry port (Gerstel, Mülheim an der Ruhr, Germany). The effluent was divided 1:1 between the MS and the olfactometry port. The GC conditions were as follows: 0.5 μL sample was injected via air sandwich technique into the inlet which was held at 250 °C set to splitless mode, helium carrier gas was at a constant pressure of 180 kPa. The GC oven temperature program was as follows: initial conditions 40 °C held for 2 min, followed by a 7 °C/min ramp until 250 °C, which was held for 10 min. Each sample was diluted by half-volume in dichloromethane until the dilution had been carried out to a concentration of 128th of the original extraction had been achieved. The largest dilution at which each compound was detected was defined as the FD value. Each dilution was analyzed in triplicate by two panelists. Compound identification was performed using mass spectral data, odor descriptors, and the linear retention index (LRI) of the authentic compound. LRI values were calculated using an *n*-alkane ladder.

3.5. GC/MS Identification and Quantitation

The GC/MS analysis was performed using a 7890 GC (Agilent Technologies) coupled to a time of flight (TOF) MS (LECO Pegasus 4D, St. Joseph, MI, USA). The isolate was analyzed on two alternate column chemistries, namely DB-5 and DB-Wax. For the DB-5 analysis analogous column and oven conditions were as previously described. For the DB-Wax (60 m × 0.25 mm i.d. × 0.25 μm film thickness, Agilent Technologies) the GC conditions were as follows: 0.5 μL was injected into an inlet heated

to 250 °C. The GC oven temperature program was as follows: initial conditions 40 °C followed by a 5 °C/min ramp to 250 °C and then held for 10 min, flow at 1 mL/min.

Quantification was carried out using five-point calibration curves for each of the 18 compounds in the following concentration ranges (μg/kg) listed, hexanal (50–800), 2-methylpyrazine (55–80), 2,3-dimethylpyrazine (51–815), 2,5-dimethylpyrazine (54–860), 2-methyl-2thiazoline (61–975), 2-pentylfuran (43.5–775), 2-ethyl-3,5-dimethylpyrazine (52.5–840), 3-hydroxy-2-methyl-4*H*-pyran-4-one (44–700), 2-methylphenol (60–965), 2-acetyl-2-thiazoline (61–975), (*E*,*E*)-2,4-decadienal (92.5–1480), 2-methoxy-4-vinylphenol (438–7000), 4-hydroxy-3-methoxybenzaldehyde (612–10000), all curves had high linearity ($R^2 > 0.98$ for all compounds) as similarly descripted by Trikusuma et al. [30].

3.6. Sensory Evaluation

The aroma of the maize puffs was evaluated by 12 trained panelists (4 male and 8 female, ages 22–32) from the University of Minnesota Department of Food Science and Nutrition (St. Paul, MN, USA). Training consisted of 10 sessions of 1 h. The first training session was dedicated to lexicon development and selection of references. Panelists generated the six following descriptive terms: oxidized, roasted, cooked, toasted, vanilla, and corn chip. Representative food samples were selected as references for sensory attributes: dry uncooked Bob's Red Mill Steel Cut Oats represented the oxidized aroma, Bergen Unsalted Lightly Roasted Almonds represented the roasted aroma, Organic Valley Whole UHT (ultra-heat treated) Milk represented the cooked aroma, toasted Wonder Bread™ represented the toasted aroma, Nilla® Wafers represented the vanilla aroma, and Old Dutch Restaurante® Style Yellow Corn Tortillas Chip represented the corn chip aroma. The recombination samples were prepared by adding 10 μL of the aroma compound mixture (in ethanol) to 15 g corn starch at the levels quantified in refined and whole grain maize puff samples (Table 2) in sealed 50 mL amber glass containers with Teflon® lined lids. The recombination samples were allowed to equilibrate for 12 h and mixed in a drum tumbler prior to evaluation. Panelists were asked to assess the intensity of the six aroma descriptors orthonasally on a 0–15 pt scale with 0 being not noticeable and 15 being intense. All samples and recombination samples were evaluated in duplicate. For each replicate, a new sample bottle was analyzed. Data were analyzed using analysis of variance and Tukey's HSD test with a probability of $p \leq 0.05$. The effect of replicate and the panelist–sample interaction was not significant, indicating that data collected were reproducible and that panel was aligned toward the sensory attributes. Data were processed using SPSS Statistics (IBM, Armonk, NY, USA).

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Sample Availability: Samples of the compounds are not available from the authors.

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Proteolytic Volatile Profile and Electrophoretic Analysis of Casein Composition in Milk and Cheese Derived from Mironutrient-Fed Cows

Andrea Ianni [1,†], Francesca Bennato [1,†], Camillo Martino [2], Lisa Grotta [1], Nicola Franceschini [3] and Giuseppe Martino [1,*]

1 Faculty of BioScience and Technology for Food, Agriculture and Environment, University of Teramo, Via Renato Balzarini 1, 64100 Teramo, Italy; aianni@unite.it (A.I.); fbennato@unite.it (F.B.); lgrotta@unite.it (L.G.)

2 Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Via Campo Boario 37, 64100 Teramo, Italy; c.martino@izs.it

3 Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, Via Vetoio 1, 67100 L'Aquila, Italy; nicola.franceschini@univaq.it

* Correspondence: gmartino@unite.it; Tel.: +39-0861-266950

† These authors contributed equally to this work.

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Abstract: The aim of the study was to evaluate the proteolytic process in Caciocavallo cheese obtained from Friesian cows fed zinc, selenium, and iodine supplementation. Thirty-six Friesian cows, balanced for parity, milk production, and days in milk, were randomly assigned to four groups. The control group (CG) was fed with a conventional feeding strategy, while the three remaining groups received a diet enriched with three different trace elements, respectively zinc (ZG), selenium (SG), and iodine (IG). At the end of the experimental period, samples of milk were collected and used to produce Caciocavallo cheese from each experimental group. Cheese samples were then analyzed after 7 and 120 days from the cheese making in order to obtain information on chemical composition and extent of the proteolytic process, evaluated through the electrophoretic analysis of caseins and the determination of volatiles profile. Both milk and cheese samples were richer in the amount of the microelement respectively used for the integration of the cattle's diet. The zymographic approach was helpful in evaluating, in milk, the proteolytic function performed by endogenous metalloenzymes specifically able to degrade gelatin and casein; this evaluation did not highlight significant differences among the analyzed samples. In cheese, the electrophoretic analysis in reducing and denaturing condition showed the marked ability of β-casein to resist the proteolytic action during ripening, whereas the dietary selenium supplementation was shown to perform a protective action against the degradation of S1 and S2 isoforms of α-casein. The analysis of the volatile profile evidenced the presence of compounds associated with proteolysis of phenylalanine and leucine. This approach showed that selenium was able to negatively influence the biochemical processes that lead to the formation of 3-methyl butanol, although the identification of the specific mechanism needs further investigation.

Keywords: proteolysis; microelement; dairy cow; caciocavallo cheese; casein; volatile compound

1. Introduction

High-yielding animals require feeding strategies that guarantee the right contribution of all the necessary microelements, such as zinc, manganese, copper, cobalt, iodine, and selenium. Dietary microelements deficiency in livestock commonly leads to a wide range of disorders especially

associated to growth depression, inefficient feed utilization, lower production performance, and depressed immunocompetence that may increase animals' susceptibility to infectious diseases [1].

Zinc is a ubiquitous element in cells and represents an essential component of several metalloenzymes [2] and transcription factors, with relevant roles in the metabolism of essential nutrients in animals. Zinc is not stored in the animal body; therefore, a constant dietary supply is necessary to avoid the onset of a wide range of pathological conditions, such as skin parakeratosis, reduction or cessation of growth, general debility, lethargy, and increased susceptibility to infection [3]. Selenium is involved in numerous biological mechanisms, including cellular response to oxidative stress, redox signaling, cellular differentiation, immune response, and protein folding [4]. Selenium was also reported to improve rumen fermentation, milk yields, and feed digestion in Holstein dairy cows [5]. Iodine is the main component of the thyroid hormones and when its requirement is not satisfied, a reduced functionality of the thyroid gland could occur (hypothyroidism) with consequences for proper mental development, body growth, and decreased fertility. In animal husbandry, iodine supplementation is needed because the native iodine content of plant straight feed-stuffs is low; moreover, the increasing use of rapeseed meal in livestock diets is associated with the intake of glucosinolates, which are known to be iodine antagonists, inhibiting the activity of sodium iodide symporter [6].

Different studies have been carried out in order to evaluate the effect of dietary microelements intake on ruminants' metabolism [7–9] and chemical-nutritional quality of dairy products [10–12], but the topic concerning the microelements' influence on ripened cheese flavor has received less attention. Conversion of lactose and citrate, lipolysis, and proteolysis represent the main chemical processes involved in the development of aroma in dairy products. Among these processes, proteolysis of caseins is an important biochemical pathway responsible for the formation of flavor and texture in hard- and semi hard-type cheeses [13]. Proteolysis in cheese can be divided into the primary and the secondary phase. Primary proteolysis is performed by rennet, native milk enzymes and induces degradation of caseins into large, well-defined polypeptides. Further proteolytic processes operated by starter and nonstarter bacteria during ripening contribute to secondary proteolysis, which cause formation of small polypeptides and free amino acids responsible for cheese aroma and taste [14]. Branched chain amino acids (leucine, isoleucine, and valine), aromatic amino acids (phenylalanine, tyrosine, and tryptophan), and methionine are thought to be the precursors of important volatile compounds in dairy products [15]. Therefore, the aim of the present study was to investigate the effect of dietary microelements' intake on the development of proteolysis in fresh and ripened dairy products obtained from lactating dairy cows. Specifically, the study was conducted on Caciocavallo cheese, a dairy product of bovine origin, which is generally subjected to seasoning for fairly long intervals of time compared to other products, and which is therefore more exposed to both lipolytic and proteolytic processes. The study in any case did not concern only the cheese but was also extended to the milk used for cheesemaking, in order to verify the actual enrichment with the microelements respectively used for dietary supplementation and also to evaluate the presence and the activity of native milk metalloenzymes, which exploit these microelements, especially zinc, as cofactors.

2. Results

2.1. Microelements Quantification in Milk and Cheese

At the end of the 56 days of the trial, milk samples obtained from each experimental group (zinc (ZG), selenium (SG), and iodine (IG)) in the feeding strategy were found to be effective in inducing an enrichment of the microelement respectively used for the dietary supplementation (Table 1).

Table 1. Microelements quantification in milk samples obtained from lactating dairy cows fed control diet (CG) and control diet supplemented with zinc (ZG), selenium (SG), and iodine (IG).

Microelement	Milk Samples			
	CG	ZG	SG	IG
Zinc [1]	4.18 [a] ± 0.37	5.76 [b] ± 0.41	3.98 [a] ± 0.33	4.04 [a] ± 0.40
Selenium [1]	0.036 [a] ± 0.004	0.041 [a] ± 0.005	0.049 [b] ± 0.005	0.039 [a] ± 0.004
Iodine [1]	0.12 [a] ± 0.03	0.11 [a] ± 0.02	0.10 [a] ± 0.02	0.17 [b] ± 0.02

[1] Data are reported on a dry matter basis, as mean (mg·kg^{-1}) ± standard deviation (S.D.). [a,b] Different letters in the same row indicate significant differences ($p < 0.05$).

The finding concerning the enrichment with the microelements used for the dietary supplementation was also found in samples of Caciocavallo cheese, both fresh (T_7) and after 120 days of repining (T_{120}). The results concerning the quantification performed on individual samples for the two ripening times is shown in Table 2.

Table 2. Microelements content in cheese samples obtained from lactating dairy cows fed control diet (CG) and control diet supplemented with different trace elements: zinc (ZG), selenium (SG), and iodine (IG).

Trace Element	Ripening Time [1]							
	T_7				T_{120}			
	CG	ZG	SG	IG	CG	ZG	SG	IG
Zinc [2]	41.34 [a] ± 2.03	52.61 [b] ± 2.37	42.77 [a] ± 2.19	40.77 [a] ± 1.98	43.21 [a] ± 2.41	54.74 [b] ± 2.39	41.82 [a] ± 3.09	42.91 [a] ± 2.93
Selenium [2]	0.21 [a] ± 0.03	0.19 [a] ± 0.03	0.32 [b] ± 0.04	0.22 [a] ± 0.03	0.22 [a] ± 0.02	0.18 [a] ± 0.03	0.31 [b] ± 0.04	0.19 [a] ± 0.03
Iodine [2]	0.21 [a] ± 0.03	0.24 [a] ± 0.03	0.19 [a] ± 0.03	0.31 [b] ± 0.04	0.20 [a] ± 0.03	0.22 [a] ± 0.03	0.18 [a] ± 0.02	0.29 [b] ± 0.04

[1] 7 and 120 days of ripening (T_7 and T_{120} respectively); [2] Data are reported in mg·kg^{-1} on a dry matter basis. [a,b] Different letters in the same row indicate significant differences ($p < 0.05$).

2.2. Zymographic Evaluation of Gelatinolytic and Caseinolytic Activity in Milk

Enzymatic activities able to induce the degradation of gelatin and casein in milk samples have been evaluated using a zymographic approach.

The gelatin-zymography (Figure 1) was helpful in highlighting the enzymatic activity closely associated with the two major gelatinases: matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9).

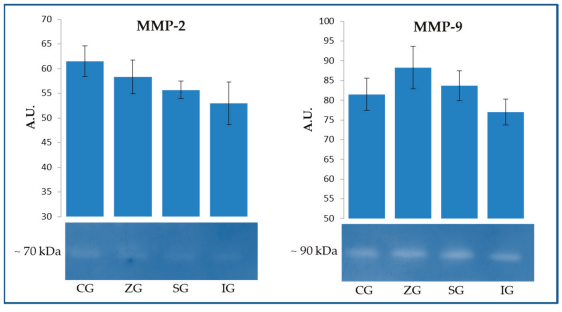

Figure 1. Gelatin-zymography on milk samples obtained from lactating dairy cows fed control diet (CG) and control diet supplemented with zinc (ZG), selenium (SG), and iodine (IG). Analysis was performed in order to obtain information on the enzymatic activities associated to matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9). The ImageJ software was used to perform the quantitative analysis of visualized spots. Data are reported as mean values expressed in arbitrary unit (A.U.) ± standard deviation.

With specific regard to MMP-2, no significant variations were detected ($p > 0.05$), although the quantitative analysis of the spots highlighted a tendency in the samples from the experimental groups (ZG, SG, and IG) to degrade gelatin with less efficacy. In the case of MMP-9, all samples showed greater activity than that found for MMP-2. However, similarly to what was observed for MMP-2, no significant differences between the various samples were identified ($p > 0.05$). The only noteworthy phenomenon concerns the slight tendency of the ZG sample to degrade the substrate more effectively.

Total caseinolytic activity was assessed through casein-zymography. The data shown in Figure 2 showed a picture quite similar to that observed for MMP-9 with the ZG milk sample, which seemed to have a greater ability to degrade the substrate, although this difference compared to the control (CG) and to the other experimental samples (SG and IG) was not significant ($p > 0.05$).

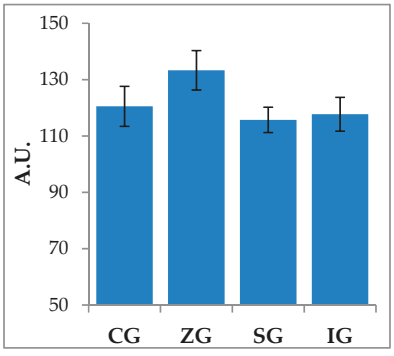

Figure 2. Casein-zymography on milk samples obtained from lactating dairy cows fed control diet (CG) and control diet supplemented with zinc (ZG), selenium (SG), and iodine (IG). The ImageJ software was used to perform the quantitative analysis. Data are reported as mean values expressed in arbitrary unit (A.U.) ± standard deviation.

2.3. Caseins Separation by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the aim of monitoring the degradation of caseins by the rennet and indigenous milk enzymes. As showed in Figure 3, cheese proteins have been separated into three major casein components (αS1-CN, αS2-CN, and β-CN).

Under the applied experimental conditions, the protein profile of both fresh (T_7) and ripened (T_{120}) cheese showed a major β-CN band and less intensive bands corresponding to αS1-CN and αS2-CN. In all samples, five low molecular weight peptides (from 25 kDa to 10 kDa) were also identified as proteolysis products (Table 3). Dietary supplementation with zinc and iodine did not generate significant changes compared to the CG samples both at T_7 and at T_{120}, while selenium influenced the proteolytic process, partly protecting αS2-CN, as evidenced by the lack of significant differences in the proteolysis products corresponding to bands 2, 3, and 4 in SG samples ($p > 0.05$).

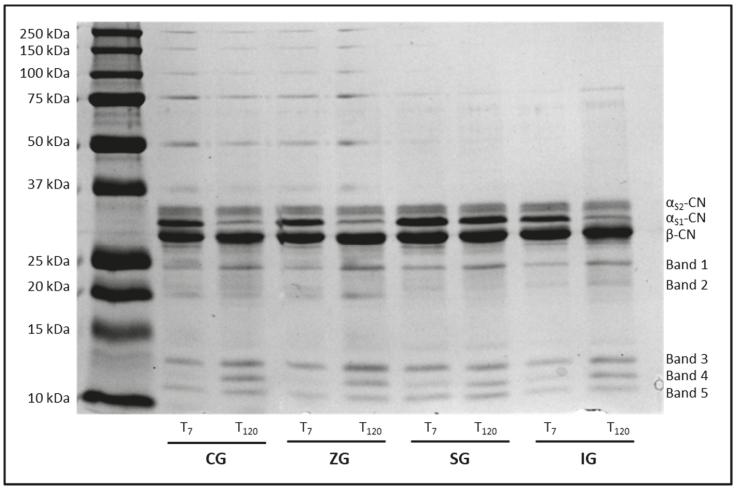

Figure 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) pattern of caseins and peptides resulting from protein degradation in fresh (T_7) and 120-days ripened (T_{120}) cheese samples obtained from lactating dairy cows fed control diet (CG) and control diet supplemented with zinc (ZG), selenium (SG), and iodine (IG) deviation.

2.4. Identification of Volatile Compounds in Fresh and Ripened Cheese

Volatile compounds resulting from secondary proteolysis during ripening were identified in all the analyzed samples (Table 4). Two volatile compounds, phenylacetaldehyde and 2-phenylethyl alcohol, derived from phenylalanine catabolism were identified, while only the 3-methyl-1-butanol was identified as a derivative of leucine degradation.

In both T_7 and T_{120} samples no significant variations were found in the phenylacetaldehyde content ($p > 0.05$). In the case of 2-phenylethyl alcohol, dietary supplementation with zinc, selenium, and iodine, seems to have led to a significant reduction of this compound in T_7 samples (6499 AU in CG vs. 3125, 1364, and 3037 AU in ZG, SG, and IG, respectively; $p < 0.01$). In ripened cheese, the phenomenon was confirmed only in ZG samples, while an increase of 2-phenylethyl alcohol was detected in IG. The only identified compound deriving from the leucine degradation was 3-methyl butanol, which tends to be synthesized in the various cheese samples with a comparable pattern both after 7 and 120 days of ripening. Specifically, no significant differences were observed between CG, ZG, and IG samples, while both at T_7 and T_{120}, the SG samples were characterized by a lower concentration of this compound ($p < 0.01$ at T_7 and $p < 0.05$ at T_{120}).

Table 3. Densitometric analysis of SDS-PAGE protein bands (Figure 3) in fresh (T_7) and 120-days ripened (T_{120}) cheese samples obtained from lactating dairy cows fed control diet (CG) and control diet supplemented with zinc (ZG), selenium (SG), and iodine (IG).

Protein	CG			ZG			SG			IG		
	T_7	T_{120}	*p*	T_7	T_{120}	*p*	T_7	T_{120}	*p*	T_7	T_{120}	*p*
α_{S2}-CN	22.93 ± 2.07	26.59 ± 2.78	ns	20.98 ± 1.87	19.60 ± 1.79	ns	22.30 ± 2.13	21.02 ± 2.04	ns	23.80 ± 2.21	25.10 ± 2.33	ns
α_{S1}-CN	33.16 ± 2.88	10.82 ± 0.85	**	29.66 ± 2.83	8.30 ± 0.78	**	31.82 ± 2.97	21.14 ± 1.99	**	29.98 ± 2.83	14.66 ± 1.42	**
β-CN	26.92 ± 2.54	27.94 ± 2.41	ns	30.57 ± 2.92	30.93 ± 2.82	ns	23.39 ± 2.18	27.21 ± 2.51	ns	31.33 ± 2.86	33.60 ± 3.11	ns
Band 1	5.04 ± 0.56	8.41 ± 0.86	*	5.19 ± 0.53	10.70 ± 0.96	**	5.48 ± 0.55	8.09 ± 0.78	*	4.10 ± 0.42	6.36 ± 0.61	*
Band 2	3.86 ± 0.44	6.36 ± 0.67	**	5.18 ± 0.52	8.76 ± 0.83	**	4.68 ± 0.47	5.96 ± 0.61	ns	3.09 ± 0.32	6.27 ± 0.62	**
Band 3	3.51 ± 0.39	6.77 ± 0.69	**	3.64 ± 0.38	8.20 ± 0.77	**	4.39 ± 0.45	4.27 ± 0.43	ns	2.76 ± 0.29	4.30 ± 0.42	*
Band 4	1.72 ± 0.23	6.70 ± 0.68	**	0.96 ± 0.11	6.77 ± 0.66	**	3.11 ± 0.33	3.99 ± 0.40	ns	2.10 ± 0.22	4.50 ± 0.44	**
Band 5	1.87 ± 0.21	6.41 ± 0.65	**	3.82 ± 0.39	6.74 ± 0.65	**	4.84 ± 0.50	8.32 ± 0.81	**	2.84 ± 0.28	5.21 ± 0.51	**

Data are reported as mean (%) ± S.D. of the total proteins found in the electrophoretic profile of each sample. α_S-CN = α_S-casein and β-CN = β-casein. Bands 1, 2, 3, 4, and 5 = fragments of protein degradation. * $p < 0.05$; ** $p < 0.01$; and ns = not significant.

Table 4. Proteolytic volatile compounds in fresh (T_7) and 120-days ripened (T_{120}) cheese obtained from lactating cows fed control diet (CG) and control diet supplemented with zinc (ZG), selenium (SG), and iodine (IG).

Volatile Compounds	T.I. [2]	Ripening Time [1]									
		T_7					T_{120}				
		CG	ZG	SG	IG	SEM	CG	ZG	SG	IG	SEM
Phenylalanine											
Phenylacetaldehyde	91	750	862	1083	480	227	3015	3658	5748	4334	1222
2-phenylethyl alcohol	91	6499 [a]	3125 [b]	1364 [c]	3037 [b]	399	9596 [a]	6833 [b]	7564 [a]	14815 [c]	2643
Leucine											
3-methyl-1-butanol	55	7481 [a]	6526 [a]	2697 [b]	6091 [a]	987	7648 [a]	6327 [a]	5921 [b]	7597 [a]	1179

[a,b,c] Different letters in the same row indicate significant differences between groups ($p < 0.05$). [1] 7 and 120 days of ripening (T_7 and T_{120} respectively); [2] Target ion. Data are reported as arbitrary units.

3. Discussion

Preventive analysis performed to determine the chemical composition of cheese samples obtained from the various experimental groups did not show significant changes both in relation to the feeding strategy (CG, ZG, SG, and IG) and in relation to the ripening time (T_7 and T_{120}). In particular, there were no differences in the protein content (Supplementary Table S1), testifying that the proteolytic process took place in the presence of equal substrate concentrations among the analyzed cheese samples. This finding is in complete agreement with what has been observed in other studies, which tested the dietary supplementation with essential trace elements in dairy cows [10,16]. With regards to the dosage of zinc, selenium, and iodine in milk and cheese samples, a significant increase of the micronutrient respectively used for the integration of the animal diet was highlighted. Limited to selenium, this result is in agreement with several studies [17], while in the case of zinc and iodine, there are discrepancies with what was previously reported. Pechová et al. [18] evidenced the inability of dietary zinc supplementation to influence its amount in bovine milk and cheese; these authors discussed such phenomenon advancing the hypothesis of an impaired incidence of rumen acidosis in the herd before the start of the experiment. With regard to iodine, Moschini et al. [19] indicated this microelement suitable for milk fortification through feed supplementation but its poor ability to interact with protein structures seems to compromise its transfer to cheese. Given the relevance of zinc, selenium, and iodine in human biochemical mechanisms, their general enrichment in milk and dairy products at the concentrations detected in this study should represent a positive aspect. However, it must be taken into account that an excess of these trace elements can constitute a technological risk factor for dairy products, both for the commercial image of the products and, above all, for consumers' health. This consideration assumes particular value especially in the case of nonessential or toxic metals, such as lead and cadmium, that even in low concentrations are responsible for metabolic disorders with extremely serious consequences [20].

The milk enrichment with the microelements respectively used for dietary supplementation made it necessary to verify the possibility that this event could influence the function of endogenous enzymes. In particular, attention was focused on the activity of matrix metalloproteinases (MMPs), which, following cheesemaking, can be incorporated into the dairy matrix, actively participating in the proteolytic events that characterize the ripening process, especially in the initial stages. MMPs represent a family of calcium-dependent endopeptidases, with a catalytic domain containing a zinc ion coordinated by three histidines. These enzymes are involved in the physiological degradation of the extracellular matrix (ECM) in mammals, a fundamental process for tissue development, morphogenesis, remodeling, and repair. Based on their specificity for the substrate, MMPs are divided into four main subgroups: gelatinases, collagenases, stromelysins, and membrane type MMPs (MT-MMPs) [21]. In this study, the activity of gelatinases MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) have been specifically evaluated; furthermore, the caseinolytic potential of milk samples was also assayed, since casein can be hydrolyzed by different metalloenzymes, such as MMP-1 (Collagenase-1) and MMP-3 (Stromelysin-1) [22]. Although MMPs are constitutively present in all tissues, their release in milk can be partly influenced by the health status of the mammary gland. As previously reported, the inflammatory process during mastitis results in the release of a wide range of proteolytic enzymes, which are mainly secreted by polymorphonuclear cells recruited into the mammary gland from blood circulation [23].

The dairy cows involved in this study maintained good health conditions for the entire duration of the trial, and the preliminary evaluations carried out on milk used for cheesemaking showed particularly low values associated with somatic cells count (SCC) and without significant changes in the data associated with the total bacterial count (data not shown). The zymographic approach did not evidence significant variations both for gelatinolytic (MMP-2 and MMP-9) and caseinolytic activities. Because these enzymes depend on the presence of zinc, an increase in the substrate hydrolysis capacity in ZG samples would have been expected. The justification for this evidence could be sought in the fact that dietary supplementation was performed by using zinc oxide (ZnO), which showed, in alternative

research fields, the ability to even block the activity of MMP-9 [24]. It is therefore presumable that this organic form of zinc is difficult to use by this family of enzymes, which therefore do not undergo an improvement in the hydrolysis kinetics of the respective substrates.

Regarding the analysis of primary proteolysis in cheese, the SDS-PAGE has proved to be particularly useful in monitoring the degradation of caseins by the rennet and indigenous milk enzymes. Cheese proteins have been separated into three major casein components (αS1-CN, αS2-CN, and β-CN) in all the analyzed samples, with molecular weights and electrophoretic mobility that were consistent with those reported in the literature [25]. Under the applied experimental conditions, the protein profile of both fresh (T_7) and ripened (T_{120}) cheese showed a major β-CN band and less intensive bands corresponding to αS1-CN and αS2-CN. In all samples, five low molecular weight peptides (from 25 kDa to 10 kDa) were all identified as proteolysis products. Dietary supplementation with zinc and iodine did not generate significant changes compared to the CG samples both at T_7 and at T_{120}, while selenium influenced the proteolytic process, partly protecting αS2-CN, as evidenced by the lack of significant differences in the proteolysis products corresponding to bands 2, 3, and 4 in SG samples. According to other studies, most of these peptides with molecular weight in the range 10–20 kDa were generated by rennet and plasmin following the degradation of αS-CN and β-CN [26,27]. Selenium has been reported to inhibit the expression of urokinase-type plasminogen activator (uPA), a serine protease, which can convert plasminogen to plasmin, which is capable of degrading extracellular matrix proteins and activating latent forms of MMPs [28]. The αS1 and β caseins did not show significant changes both in relation to the feeding strategy and in relation to the maturing time; this finding is consistent with what was previously reported in other studies and represents an added value if we consider the growing interest on β-casein micelles as a nano vehicle for solubility enhancement of natural compounds in the food industry [29].

Several studies have shown how changes in the diet of ruminants can be effective in inducing significant changes in the volatile profile of milk and dairy products [30]. Volatile compounds (VOCs) resulting from secondary proteolysis during ripening were identified in all the analyzed samples. Two volatile compounds, phenylacetaldehyde and 2-phenylethyl alcohol, derived from phenylalanine catabolism, while only the 3-methyl-1-butanol was identified as a derivative of leucine degradation. In both T_7 and T_{120} samples, no significant variations were found in the phenylacetaldehyde content. As reported by McSweeney and Sousa [14], the phenylacetaldehyde metabolism in dairy products may occur by the nonenzymatic Strecker synthesis, through the degradation of phenylalanine, or by enzymatic transamination of phenylalanine as imide that is subsequently degraded to aldehyde. The presence of 2-phenylethyl alcohol is instead due to the phenylacetaldehyde reduction. In this case, the dietary supplementation with zinc, selenium, and iodine seems to have led to a significant reduction of this compound in fresh cheese samples, while in ripened samples, this finding was confirmed only for ZG samples. Aromatic compounds from phenylalanine have a relevant impact on the aroma of cheese. In particular, phenylethyl alcohol is reported to be one of the most odorous aromatic compounds, associated with a rose flower note [31]. With regard to leucine, this amino acid can undergo several biochemical processes in cheese during ripening. After an extracellular enzymatic degradation of casein by proteases, released from starter bacteria, leucine is converted to the corresponding α-keto acid (α-ketoisocaproic acid) by an intracellular transamination; then, such compound can be converted to α-hydroxy acids, aldehydes, or CoA-esters [32]. These leucine metabolites are not considered to be important for the development of cheese flavor, while the 3-methyl-1-butanol, derived from the hydrogenation of the corresponding aldehyde (3-methylbutanal), was reported to be responsible for alcoholic and fruity odors in Swiss-type cheese [33]. In this study, the only identified compound deriving from the leucine degradation was 3-methyl butanol, which tends to be synthesized in the various cheese samples with a comparable pattern both after 7 and 120 days of ripening. Specifically, no significant differences were observed between CG, ZG, and IG samples, while both at T_7 and T_{120} of the SG, samples were characterized by a lower concentration of this compound. A similar behavior was also observed in a 30-days ripened caciotta cheese obtained from dairy cows fed organic

selenium [34], as well as in a 90-days ripened Pecorino cheese deriving from ewes that received a dietary supplementation with organic zinc [35]. In addition to this, the dietary intake of organic zinc by dairy cows was even effective in inducing the reduction of 3-methyl butanol in a typical Italian soft cheese, the Giuncata cheese, after only 7 days storage at 4 °C [36]. Probably, the microelements administered in organic forms negatively influence the biochemical processes that lead to the formation of 3-methyl butanol, although the identification of the specific biochemical mechanisms needs further and more targeted investigations.

4. Materials and Methods

4.1. Experimental Design, Feeding Strategies, Cheesemaking, and Sampling

The experimental plan was performed according to Directive 2010/63/EU of the European Parliament (European Union, 2010) and Directive 86/609/EEC (European Economic Community, 1986), which deal with the protection of animals used for scientific purposes [37,38].

Thirty six healthy Friesian cows, homogeneous for age (41 ± 1.5 months) and lactation days (74 ± 12 days) have been used in this study. Animals, belonging to the same commercial farm, have been randomly divided into four groups of nine cows each. The control group (CG) was fed with a standard diet formulated taking into account the nutritional needs of cows in midlactation and guaranteeing each animal the daily requirement of each microelement, while the three experimental groups received the dietary supplementation with zinc (ZG), selenium (SG), and iodine (IG), respectively. The ZG received an additional total intake of about 100 mg of Zn. For the preparation of the rations, ZnO as a powder was used, and the dose management was performed according to Regulation (EC) No. 1831/2003 of the European Parliament and of the Council of 22 September 2003 (European Commission, 2003) on additives for use in animal nutrition [39]. With regards to SG, animals received a total daily supplementation of 0.47 mg of Se; current EU regulations have limited the use of Se supplementation to the overall content not exceeding 0.5 mg in complete feed daily administered. For the preparation of the ration organic Se as a crystalline powder was used that was incorporated into feed in the form of a premixture according to the recommendation reported in the Regulation No. 121/2014 of the European Commission concerning the authorization of L-selenomethionine as a feed additive for all animal species [40]. Finally, the IG animals were fed with a daily total iodine content of 4.5 mg/cow. For the dietary supplementation, potassium iodide (KI) as a powder was used and the total iodine was set not to exceed the maximum daily amount of 5 mg allowed by law (Reg. 1459/2005) [41].

The study had an overall duration of 70 days, characterized by 14 initial days of adaptation in which all the involved animals received the standard diet, followed by 56 days of dietary supplementation, in which animals of each group were housed in separate areas of free housing with an access to an identical feeding area in which each animal had an individual feeding bin with water freely available all throughout the study. All animals received about 23 kg/head/day of dry matter of total mixed rations (TMR) whose composition (Supplementary Table S2) was defined taking into account the parameters reported on the seventh edition of Nutrient Requirements of Dairy Cattle (2001) [42]. Samples of TMR were analyzed, according to AOAC methods (1990), for crude protein (CD; method 930.15), ether extract (EE; method 920.39), crude fiber (CF; method 962.09), and ash (method 942.05) [43]; detergent procedures reported by Van Soest et al. [44] were used for the determination of neutral detergent fiber (NDF) and acid detergent fiber (ADF). On the 70th day of the trial, individual raw milk samples were collected separately from each group and immediately analyzed for chemical composition. The remaining milk from each group was pooled and manipulated in the same way during the cheese-making process to obtain the Caciocavallo cheese, according to the protocol previously described [45]. In order to evaluate changes in the chemical composition and quality attributes due to ripening, sampling and analyses on Caciocavallo cheese were carried out after 7 days (T_7) and 120 days (T_{120}) from the cheese-making batches. Caciocavallo cheese is a dairy product that lends itself to being aged for quite long periods and on average it is consumed even after 6 months from cheesemaking.

The analysis of the samples at the indicated times (T_7 and T_{120}) therefore allows us to compare the fresh product with a ripened product ready to be consumed. Samples, collected in triplicate from three different cheese-makings, were partly immediately analyzed and partly packed under vacuum and frozen at −20 °C until analysis.

4.2. Microelements Determination in Milk and Cheese

For the determination of the total amount of zinc in milk and cheese, samples were first mineralized by dry incineration, then subjected to atomic absorption spectrophotometry using an air/acetylene flame [46]. Selenium and iodine content was determined instead by inductively coupled plasma mass spectrometry (ICP-MS) by using an Agilent 7500ce (Agilent Technologies, Santa Clara, CA, USA) and following the procedures respectively reported by Gerber et al. and Fecher et al. [47,48].

4.3. Gelatin and Casein Zymography of Milk Samples

The evaluation of the activity of the zinc-dependent proteases in raw milk was performed through a zymographic approach. A total of 10 mL of each sample were placed in 15 mL tubes and centrifuged at 10,000 rpm for 20 min at 4 °C. This allowed us to obtain a separation in three distinct phases: an upper layer consisting of fat, a central serum fraction containing proteins, and a lower layer characterized by the cellular component and any interfering residues. The central phase was then carefully recovered and filtered through with 0.22 μm syringe filters before the total protein dosage that was calculated by the Bradford protein concentration assay [49].

Volumes of each sample corresponding to 10 μg of total proteins were diluted in a nonreducing sample buffer without heating, and resolved by 8% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.2 mg/mL type B gelatin (Sigma Aldrich, Milan, Italy) [50]. The gels were then incubated for 45 min in a renaturation buffer (50 mM Tris-HCl pH 8.0, containing 2.5% Triton X-100) to remove SDS. Subsequently, incubation of 24 h in a developer buffer (50 mM Tris-HCl pH 8.0, containing 5 mM $CaCl_2$, 200 mM NaCl, and 0.02% Brij 35) was performed to allow enzymes renaturation and activity. The gels were then stained in a 0.1% solution of Coomassie Blue R250 in 40% (*v*/*v*) methanol and 10% (*v*/*v*) acetic acid to allow the spot visualization.

The evaluation of caseinolytic activity was instead carried out by 10% SDS-PAGE with the addition of 0.3% of bovine casein (Sigma Aldrich, Milan, Italy). Additionally, in this case, volumes of each sample corresponding to 10 μg of total proteins were diluted in a nonreducing sample buffer without heating, before the electrophoretic resolving. After electrophoresis, gels were subjected to the same protocol reported for gelatin zymography, with the only variations regarding the final incubation period that was prolonged to 48 h.

The ImageJ software (version 1.44, National Institutes of Health, Bethesda, MD, USA) [51] was used to perform the quantitative analysis of visualized spots for both gelatin and casein zymography.

4.4. Caseins Extraction and Separation by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Casein degradation in T_7 and T_{120} cheeses was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Each cheese sample (1 g) was dissolved in 20 mL 0.01 M Tris-Glicina pH 8.3 and 6 M Urea and homogenized for 2 min. The cheese extract was incubated for 2 h at 37 °C to induce the solubilization of casein fraction. The solution was then centrifugated for 15 min at 10,000 g (4 °C), and the supernatant was recovered and filtered through Whatman filter paper to remove fat and other insoluble solids.

Volumes of each sample, containing 10 μg of total extracted proteins, were diluted in a 2X sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and traces of bromophenol blue), boiled for 3 min, and loaded onto a 15% polyacrylamide gel. The electrophoresis was performed in a mini-protean III dual slab cell (Bio-Rad Laboratories, Wartford, UK) at 30 mA constant amperage. Immediately after ending of the run, gels were placed at room temperature for

1 hr in a staining solution containing 40% methanol, 10% acetic acid, and 0.1% Coomassie Brilliant Blue G-250. Finally, the gels were destained by several washings in distilled water containing 40% methanol and 7% acetic acid. Densytometric analysis of displayed bands was performed by using ImageJ software [51].

4.5. Volatile Compounds Extraction and GC-MS Analysis

The extraction of volatile compounds from milk and cheese was performed by a solid-phase microextraction (SPME) and then analyzed using a gas-chromatograph coupled with a mass spectrophotometry (GC-MS) following the procedure previously described by Bennato et al. [52] with slight modifications. Grated cheese (4.5 g) was mixed with a saturated NaCl solution containing the internal standard, the 4-methyl-2-heptanone. The SPME was performed by exposing a 50/30 μm of divinylbenzene/carboxen/polydimethylsiloxane fiber (DVB/CAR/PDMS Supelco, Bellefonte, PA, USA) into the headspace of capped vials with a PTFE septum for 40 min at 50 °C in stirring conditions. Desorption of volatile compounds was obtained into the splitless injector of the GC system set at 250 °C for 1 min. The gas-chromatograph (Clarus 580; Perkin Elmer, Waltham, MA, USA) was coupled with a mass spectrometer (SQ8S; Perkin Elmer, Waltham, MA, USA) and equipped with a PE-ELITE-5MS 30 × 0.25 mm, 0.25 μm column (Perkin Elmer, Waltham, MA, USA). The oven temperature was set at 50 °C for 1 min, then was increased to 200 °C at 3 °C/min for 1 min, and to 250 °C at 15 °C/min, held for 15 min. The carrier gas was helium at 1 mL/min. Source and interface temperature were held at 250 °C. The mass detector operated in electronic impact mode (70 eV) and data were acquired in full scan mode (range 35–350 *m*/*z*, dwell time 0.2 s/scan). The volatile compounds were identified by comparing their mass spectra with those of the National Institute of Standards and Technology library (NIST, Gaithersburg, MD, USA) and comparing the eluting order with Kovats retention indexes. The isoamyl butyrate and isoamyl isobutyrate were identified comparing the mass spectra and the retention time of authentic standard compounds (Sigma Aldrich, St. Louis, MO, USA). Samples were analyzed in triplicate and quantification was carried out considering the relative peak area expressed as arbitrary unit (AU, target ion area × 10^{-3}).

4.6. Statistical Analysis

The statistical analysis of data was carried out by using SAS software, version 9.2 (SAS Institute Inc., Cary, NY, USA). Data on volatile compounds have been processed with two-way ANOVA, considering diet, ripening time, and their interaction as fixed effects, according to the following statistical model: $y_{ijk} = \mu + D_i + T_j + D_i \times T_j + e_{ijk}$, in which y_{ijk} = volatile compound, μ = population average, D_i = effect of dietary supplementation (CG, ZG, SG and IG), T_j = effect of ripening time (T_7 vs. T_{120}), $D_i \cdot T_j$ = interaction between dietary supplementation and ripening time, and e_{ijk} = error. Means separation was assessed by Tukey's test and differences were declared significant at $p < 0.05$.

5. Conclusions

The results highlighted the possibility of fortification with zinc, selenium, and iodine in cheese through animal feeding. The increase in concentration of essential trace elements in dairy products, in addition to representing an advantage for the consumers health, undoubtedly influences the biochemical mechanisms that characterize cheese during aging, also contributing to the development of flavor. No variations were evidenced in the caseinolytic activity in raw milk, whereas in cheese, the electrophoretic analysis in denaturing and reducing conditions of cheese showed the ability of selenium to preserve αS1-CN from primary proteolysis during ripening. With regard to the evaluation of the proteolytic volatile profile, only three compounds, resulting from the degradation of phenylalanine and leucine, have been identified. In this case, further studies are needed to clearly understand the relationship between the dietary microelements supplementation and the characterization of the free amino acids pattern in fresh and ripened cheese, as well as the mechanisms involved in the production of proteolytic compounds.

Supplementary Materials: The following are available online at http://www.mdpi.com/1420-3049/25/9/2249/s1, Table S1: Chemical composition of fresh (T_7) and 120-days ripened (T_{120}) Caciocavallo cheese obtained from lactating dairy cows fed control diet (CG) and control diet supplemented with zinc (ZG), selenium (SG), and iodine (IG). Table S2: Ingredients and chemical composition of complete diets administered to lactating dairy cows belonging to control group (CG), zinc group (ZG), selenium group (SG), and iodine group (IG).

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Sample Availability: Samples analysed during the current study are available from the corresponding author on reasonable request.

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Volatile Profile in Yogurt Obtained from Saanen Goats Fed with Olive Leaves

Francesca Bennato [1,†], Denise Innosa [1,†], Andrea Ianni [1], Camillo Martino [2], Lisa Grotta [1] and Giuseppe Martino [1,*]

[1] Faculty of BioScience and Technology for Food, Agriculture and Environment, University of Teramo, Via Renato Balzarini 1, 64100 Teramo, Italy; fbennato@unite.it (F.B.); dinnosa@unite.it (D.I.); aianni@unite.it (A.I.); lgrotta@unite.it (L.G.)

[2] Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Via Campo Boario 37, 64100 Teramo, Italy; c.martino@izs.it

* Correspondence: gmartino@unite.it

† These authors contributed equally to this work.

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Abstract: The aim of this study was to evaluate the development of volatile compounds in yogurt samples obtained from goats fed a dietary supplementation with olive leaves (OL). For this purpose, thirty Saanen goats were divided into two homogeneous groups of 15 goats each: a control group that received a standard diet (CG) and an experimental group whose diet was supplemented with olive leaves (OLG). The trial lasted 28 days, at the end of which the milk of each group was collected and used for yogurt production. Immediately after production, and after 7 days of storage at 4 °C in the absence of light, the yogurt samples were characterized in terms of fatty acid profile, oxidative stability and volatile compounds by the solid-phase microextraction (SPME)–GC/MS technique. Dietary OL supplementation positively affected the fatty acid composition, inducing a significant increase in the relative proportion of unsaturated fatty acids, mainly oleic acid (C18:1 *cis*9) and linolenic acid (C18:3). With regard to the volatile profile, both in fresh and yogurt samples stored for 7 days, the OL supplementation induced an increase in free fatty acids, probably due to an increase in lipolysis carried out by microbial and endogenous milk enzymes. Specifically, the largest variations were found for C6, C7, C8 and C10 free fatty acids. In the same samples, a significant decrease in aldehydes, mainly heptanal and nonanal, was also detected, supporting—at least in part—an improvement in the oxidative stability. Moreover, alcohols, esters and ketones appeared lower in OLG samples, while no significant variations were observed for lactones. These findings suggest the positive role of dietary OL supplementation in the production of goats' milk yogurt, with characteristics potentially indicative of an improvement in nutritional properties and flavor.

Keywords: goats' milk yogurt; Saanen goat; olive leaves; unsaturated fatty acid; volatile compound; free fatty acid; aldehyde

1. Introduction

The flavor development in dairy products is an important factor determining its acceptability and preference and is strongly affected by the combination of a wide range of compounds, mostly produced by a series of biochemical events involving the metabolism of residual lactose, lactate, and citrate, lipolysis and proteolysis. Among the mentioned catabolic processes, lipolysis is undoubtedly the mechanism that, more than others, contributes to the release of flavor-affecting compounds, mainly free fatty acids (FFAs), aldehydes, alcohols, esters, methyl ketones, γ- and δ-lactones. Milk and cheese are, in fact, characterized by relevant levels of short- and medium-chain fatty acids,

whose specific composition is strongly influenced by the animal species and the administered dietary treatment [1].

In recent years, an increase in consumer demand for goats' milk and its derivatives, due to the high nutritional features and health-promoting benefits of these products, has been observed. Goat dairy products were, in fact, reported to be characterized by proteins with lower allergenicity in comparison to bovine products, and higher concentrations of bioactive compounds, attributed to their greater digestibility [2,3]. Specifically, a growing interest has been developed towards yogurt, commonly considered a safe and nutritious food and not only being rich in vitamins and minerals, but also in calcium and proteins [4]. Furthermore, it was demonstrated that yogurt consumption is helpful for consumers affected by specific gastrointestinal conditions, mainly those associated with lactose intolerance and constipation [5].

In the past, several studies were conducted on the inclusion of different natural sources of bioactive compounds in goats' milk yogurt in order both to improve nutritional value and reduce the unpleasant "goaty" aroma and aftertaste, commonly associated with decreased acceptance by consumers [6,7]. In time, studies performed in the sector of ruminant husbandry led to the development of feeding strategies able to influence the chemical composition of animal products. In this regard, several experiments have been performed by supplementing animals' diets with plant matrices, especially agro-industrial byproducts rich in bioactive compounds with interesting functions from a biochemical point of view. These strategies have shown positive effects on several fronts, preserving, in many cases, animal welfare [8], improving the nutritional quality of animal products [9,10], and inducing, especially in dairy products, the development of volatile compounds capable of influencing the aroma and taste [11].

Byproducts derived from olive oil production are accumulated yearly in high amounts in the Mediterranean area, and their disposal represents an issue of great importance both from an environmental and economic point of view. Several studies focused their attention on the valorization of these byproducts as feeding supplements for farm animals, both ruminant and monogastric [12,13]. Specifically, olive leaves (OL) have been reported to be a cheap raw material that can be used as a source of phenolic bioactive compounds with antioxidant, antihypertensive and anti-inflammatory functions, such as oleuropein, hydroxytyrosol, verbascoside, apigenin-7-glucoside and luteolin-7-glucoside [14].

Considering the biological relevance of this byproduct, the purpose of this work is to evaluate the effects of dietary OL supplementation of lactating dairy goats on the development of volatile compounds in fresh and stored yogurt samples, with specific attention paid to compounds derived from lipolytic events.

2. Results

2.1. Chemical Properties of Yogurt Samples

The evaluation of chemical properties in yogurt samples did not evidence significant variations between the control group that received a standard diet (CG) and the experimental group whose diet was supplemented with olive leaves (OLG) in relation to moisture (85.74 ± 0.26% vs. 85.17 ± 0.49% in CG and OLG respectively, $p > 0.05$) and the amount of total lipids (19.81 ± 1.22% vs. 19.68 ± 1.35% in CG and OLG respectively, $p > 0.05$). The only significant difference was observed for pH values that decreased in yogurt obtained from goats fed the dietary OL supplementation (4.69 ± 0.02 vs. 4.43 ± 0.04 in CG and OLG samples respectively, $p < 0.05$).

2.2. Fatty Acid Composition

The dietary OL supplementation in the goats' diet induced several modifications in the fatty acid composition of yogurt samples. As reported in Table 1, in OLG samples, we observed a significant increase in the relative proportion of total monounsaturated fatty acids (MUFA; 22.25 ± 0.92% vs. 24.80 ± 0.77% for CG and OLG respectively, $p < 0.05$) and total polyunsaturated fatty acids (PUFA; 4.62 ±

0.14% and 4.84 ± 0.07% for CG and OLG respectively, $p < 0.05$). With specific regard to the sum of the saturated fatty acids (SFA), a slight decrease was instead observed in OLG samples, although this variation was not significant ($p > 0.05$).

Table 1. Fatty acid composition of yogurt samples obtained from goats fed a standard diet (CG) and goats fed a dietary supplementation of olive leaves (OLG).

Fatty Acids [1]	CG	OLG	*p*-Value
C4:0	0.54 ± 0.22	0.83 ± 0.39	ns
C6:0	1.03 ± 0.26	1.31 ± 0.39	ns
C8:0	1.73 ± 0.29	1.92 ± 0.34	ns
C10:0	7.65 ± 0.69	7.33 ± 0.58	ns
C12:0	4.31 ± 0.21	3.48 ± 0.12	*
C14:0	11.72 ± 0.44	10.75 ± 0.33	*
C15:0	0.93 ± 0.07	0.92 ± 0.03	ns
C16:0	29.58 ± 0.80	26.67 ± 0.66	*
C17:0	0.67 ± 0.02	0.66 ± 0.01	ns
C18:0	11.04 ± 0.42	13.10 ± 0.74	**
C20:0	0.27 ± 0.03	0.29 ± 0.01	ns
C22:0	0.09 ± 0.03	0.09 ± 0.01	ns
total SFA	69.54 ± 5.80	67.35 ± 5.61	ns
C14:1	0.42 ± 0.02	0.43 ± 0.01	ns
C16:1	0.33 ± 0.01	0.34 ± 0.01	ns
C18:1 *trans*11	0.43 ± 0.01	0.52 ± 0.15	*
C18:1 *cis*9	20.89 ± 0.59	23.22 ± 0.61	**
C18:1 *cis*11	0.38 ± 0.02	0.30 ± 0.01	ns
total MUFA	22.25 ± 0.92	24.80 ± 0.77	*
C18:2	2.92 ± 0.18	2.60 ± 0.14	*
CLA	0.89 ± 0.07	1.12 ± 0.09	*
C18:3	0.78 ± 0.02	1.13 ± 0.05	**
total PUFA	4.62 ± 0.14	4.84 ± 0.07	*
other FAs	3.20 ± 0.31	3.01 ± 0.30	ns
MUFA/SFA	0.32 ± 0.02	0.37 ± 0.03	*
PUFA/SFA	0.07 ± 0.01	0.07 ± 0.02	ns
UFA/SFA	0.39 ± 0.02	0.44 ± 0.04	*
DI C14:1 *cis*-9/(C14:0+C14:1*cis*-9)	0.04 ± 0.01	0.04 ± 0.01	ns
DI C16:1*cis*-9/(C16:0+C16:1*cis*-9)	0.01 ± 0.01	0.01 ± 0.01	ns
DI C18:1*cis*-9/(C18:0+C18:1*cis*-9)	0.65 ± 0.01	0.64 ± 0.01	ns
DI CLA/(C18:1*trans*-11+CLA)	0.62 ± 0.09	0.69 ± 0.02	ns

[1] Data are reported as mean percentage of total fatty acid methyl esters ± S.D. Saturated fatty acid (SFA); monounsaturated fatty acid (MUFA); polyunsaturated fatty acid (PUFA); conjugated linoleic acids (CLA); desaturation index (DI); not significant (ns); * $p < 0.05$; ** $p < 0.01$.

With reference to individual fatty acids, the OLG yogurt samples were characterized by a decrease in the relative proportion of lauric acid (C12:0, $p < 0.05$), myristic acid (C14:0, $p < 0.05$), palmitic acid (C16:0, $p < 0.05$), and linoleic acid (C18:2, $p < 0.05$), while a significant increase in the relative proportion was observed for stearic acid (C18:0, $p < 0.01$), oleic acid (C18:1 *cis*-9, $p < 0.01$), linolenic acid (C18:3, $p < 0.01$), and conjugated linoleic acids (CLA, $p < 0.05$). In addition to this, in OLG samples, the ratio MUFA/SFA and UFA/SFA was significantly higher ($p < 0.05$), whereas no variations were evidenced in the individual desaturation indices calculated for C14:1, C16:1, C18:1 and CLA.

2.3. Evaluation of the Oxidative Stability in Fresh and Stored Yogurt

The evaluation of the overall antioxidant potential in fresh yogurt highlighted a higher antioxidant capacity in OLG samples (79.69 ± 4.76 μmol TEAC/g and 89.77 ± 5.03 μmol TEAC/g for CG and OLG respectively, $p < 0.05$).

The oxidative process on the lipid component was instead determined by a Thiobarbituric Acid Reactive Species (TBARS) test, both after 1 day (T1) and 7 days (T7) of storage at +4 °C and the results are shown in Figure 1. In T1 samples, the amount of malondialdehyde (MDA) was lower in OLG samples compared with CG samples ($p < 0.01$). In T7 samples, the same trend was observed, although a marked increase in the difference between CG and OLG samples was found ($p < 0.01$). By comparing the obtained results on the basis of the storage time (T1 vs. T7), a significant increase in oxidation was evidenced in CG yogurt ($p < 0.01$), while no variations were induced by the OL dietary enrichment.

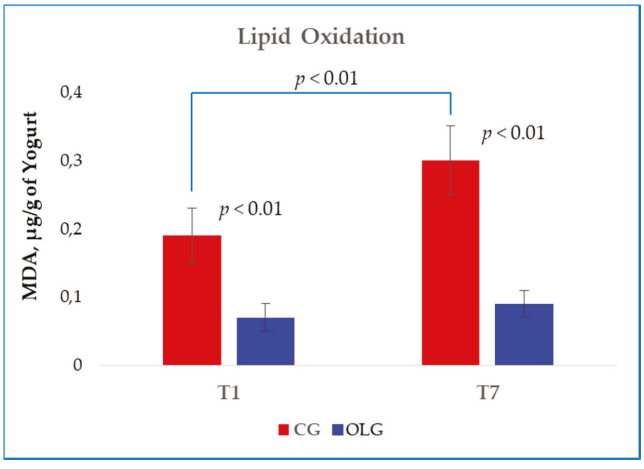

Figure 1. Lipid oxidation evaluated in yogurt samples obtained from goats fed the standard diet (CG) and goats that received the dietary supplementation with olive leaves (OLG). Samples were analyzed after 1 day (T1) and 7 days of storage (T7) at 4 °C. Malondialdehyde (MDA).

2.4. Identification of Volatile Compounds

In all samples, we found 26 volatile compounds (VOCs): seven free fatty acids (FFAs), five alcohols, five aldehydes, one ester, six ketones and two lactones. As schematized in Figure 2, both after 1 and 7 days of storage, the dietary OL supplementation was effective in inducing an increase in the relative proportion of FFAs ($p < 0.05$ and $p < 0.01$, for T1 and T7 respectively) and a significant decrease in the relative proportion of alcohols ($p < 0.05$ and $p < 0.01$, for T1 and T7 respectively), aldehydes ($p < 0.01$), esters ($p < 0.05$), and ketones ($p < 0.05$).

Table 2 reports the specific compositions of the listed VOC families. After 1 day after yogurt production, all the alcohols found were lower in OLG samples in comparison with CG samples: 1-hexanol ($p < 0.01$), 2-ethyl-hexan-1-ol, ($p < 0.05$), 1-heptanol ($p < 0.001$), 1-octanol ($p < 0.01$), and 1-nonanol ($p < 0.001$). After 7 days of storage, such a condition persisted, with the only exception being 1-hexanol, 2-ethyl, for which no significant variations were evidenced. With regard to the aldehydes, only nonanal was significantly lower in T1 samples derived from the dietary OL supplementation ($p < 0.01$), while, at T7, this behavior was also observed for heptanal ($p < 0.05$). Among the FFAs, the OLG samples stored for 1 day after the yogurt production were characterized by higher relative proportions of octanoic acid ($p < 0.05$) and decanoic acid ($p < 0.05$). The finding concerning the octanoic acid was also confirmed after 7 days of yogurt storage ($p < 0.01$) and, in addition to this, the same samples also showed a decrease in the relative proportions of hexanoic acid ($p < 0.05$) and heptanoic acid ($p < 0.01$). Concerning ketones, both after 1 and 7 days of storage, we observed lower relative proportions for 2-heptanone ($p < 0.05$) and 2-nonanone ($p < 0.01$). Butyl heptanoate was the only ester to be detected and was less represented in OLG yogurt compared to CG, both at T1

and T7 ($p < 0.01$). Finally, no significant variations were evidenced for the two identified lactones: δ-decalactone and δ-dodecalactone ($p > 0.05$).

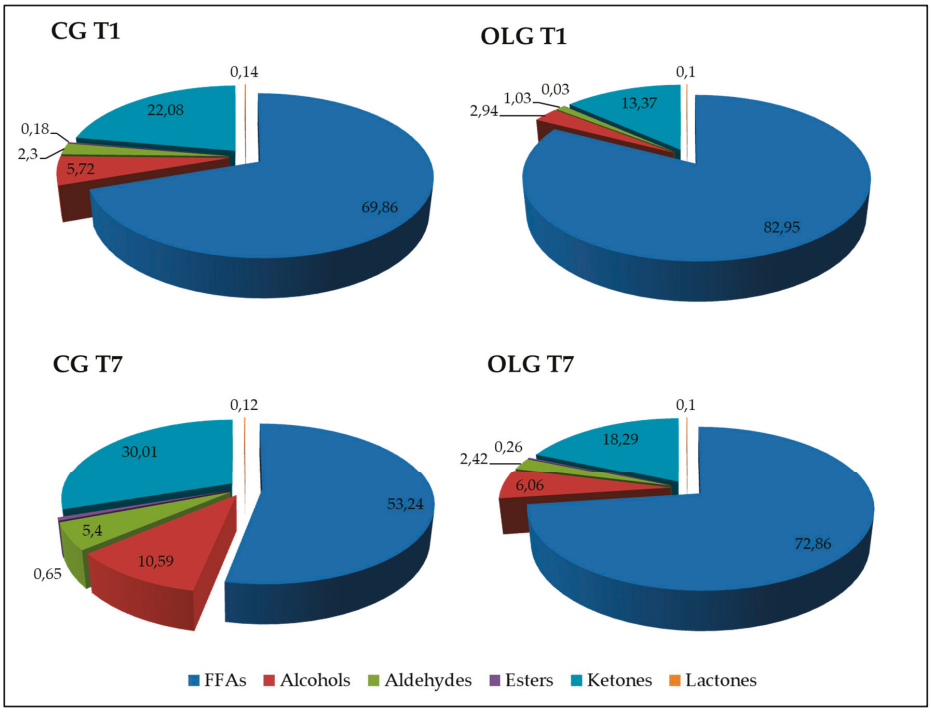

Figure 2. Schematic representation of relative proportions (%) of volatile compound families detected in yogurt samples obtained from goats fed the standard diet (CG) and goat that received the dietary supplementation with olive leaves (OLG). Samples were analyzed after 1 day (T1) and 7 days of storage (T7) at 4 °C. Free fatty acids (FFAs).

Table 2. Volatile compounds (VOC) identified in yogurt samples obtained from goats fed a standard diet (CG) and goats fed a dietary supplementation of olive leaves (OLG). Samples were analyzed after 1 day (T1) and 7 days of storage (T7) at 4 °C.

VOC [1]	T1			T7		
	CG	OLG	*p*	CG	OLG	*p*
FFAs						
Acetic acid	0.26 ± 0.14	0.40 ± 0.15	ns	nd	0.48 ± 0.20	ns
Butanoic acid	0.27 ± 0.23	0.16 ± 0.06	ns	0.10 ± 0.03	0.11 ± 0.03	ns
Hexanoic acid	18.03 ± 1.69	20.80 ± 2.30	ns	15.39 ± 0.99	13.94 ± 0.74	*
Heptanoic acid	0.56 ± 0.05	0.60 ± 0.06	ns	0.62 ± 0.02	0.43 ± 0.01	**
Octanoic acid	38.91 ± 3.31	45.70 ± 4.28	*	22.99 ± 2.61	43.79 ± 4.46	**
Nonanoic acid	0.29 ± 0.14	0.18 ± 0.03	ns	0.11 ± 0.03	0.14 ± 0.04	ns
Decanoic acid	11.54 ± 0.64	15.11 ± 1.50	*	14.03 ± 2.77	13.97 ± 2.26	ns

Table 2. *Cont.*

VOC [1]	T1			T7		
	CG	OLG	*p*	CG	OLG	*p*
Alcohols						
1-Hexanol	1.80 ± 0.13	0.88 ± 0.06	**	3.88 ± 0.71	1.72 ± 0.01	*
2-Ethyl-hexan-1-ol	1.76 ± 0.14	1.13 ± 0.11	*	3.41 ± 0.25	2.45 ± 0.19	ns
1-Heptanol	0.95 ± 0.13	0.24 ± 0.03	**	1.06 ± 0.18	0.67 ± 0.09	*
1-Octanol	0.39 ± 0.04	0.17 ± 0.02	**	0.92 ± 0.03	0.39 ± 0.01	**
1-Nonanol	0.81 ± 0.05	0.52 ± 0.02	**	1.32 ± 0.11	0.83 ± 0.10	**
Aldehydes						
Heptanal	0.05 ± 0.03	0.02 ± 0.01	ns	0.20 ± 0.10	0.04 ± 0.02	*
Nonanal	1.84 ± 0.17	0.64 ± 0.05	**	4.53 ± 0.25	2.06 ± 0.10	*
2-Heptenal	0.09 ± 0.01	0.08 ± 0.04	ns	0.09 ± 0.01	0.02 ± 0.01	ns
2-Octenal	0.12 ± 0.02	0.07 ± 0.02	ns	0.28 ± 0.01	0.11 ± 0.03	ns
2-Decenal	0.20 ± 0.01	0.22 ± 0.02	ns	0.30 ± 0.04	0.19 ± 0.03	ns
Esters						
Butyl heptanoate	0.18 ± 0.01	0.03 ± 0.01	**	0.65 ± 0.05	0.26 ± 0.03	**
Ketones						
Acetoin	1.99 ± 0.08	3.20 ± 0.31	**	3.22 ± 1.59	2.10 ± 0.39	ns
2-Pentanone	0.10 ± 0.01	0.04 ± 0.01	ns	0.13 ± 0.02	0.59 ± 0.02	ns
2,3-Pentanedione	0.06 ± 0.02	0.04 ± 0.01	ns	0.67 ± 0.01	0.03 ± 0.05	ns
2-Heptanone	9.30 ± 3.11	4.43 ± 1.02	*	11.32 ± 2.40	6.81 ± 1.11	*
2-Nonanone	9.25 ± 0.74	4.68 ± 0.41	**	12.73 ± 0.99	7.38 ± 0.53	**
2-Undecanone	1.38 ± 0.25	0.98 ± 0.17	ns	1.94 ± 0.46	1.38 ± 0.20	ns
Lactones						
δ-Decalactone	0.09 ± 0.01	0.03 ± 0.02	ns	0.05 ± 0.03	0.07 ±0.05	ns
δ-Dodecalactone	0.05 ± 0.01	0.07 ± 0.01	ns	0.07 ± 0.01	0.03 ± 0.01	ns

[1] Data are reported as mean percentage of total volatile compounds (VOCs) ± S.D; Free fatty acids (FFAs); not detectable (nd); not significant (ns); * $p < 0.05$; ** $p < 0.01$.

3. Discussion

Flavor is an important factor determining the acceptability of food products. With specific regard to dairy products, the sensory properties are largely dependent on the development of volatile flavor compounds mainly derived from biochemical processes that degrade fat and proteins present in milk. Considerable knowledge has been accumulated on the wide range of aromatic compounds contributing to the development of flavor in yogurt. Most of these compounds include VOCs already present in milk and compounds derived from fermentation processes [15]. Dietary OL supplementation was able to influence VOC development in goats' milk yogurt, with variations particularly evident in the production of lipolytic catabolites.

The experimental feeding strategy was, first of all, able to induce significant changes in the fatty acid profile of yogurt samples. The relationship established between fat and flavor development in dairy products is complex; however, it should be considered that fat is a rich reservoir of flavors, as a consequence of the tendency of many aromatic compounds to be soluble in fat rather than in water [16]. Furthermore, the proportion and structure of fat may affect the rheology of the product, and thus its dispersion in the mouth. It is also well known that lipids present in food represent precursors of several flavor compounds, since may undergo spontaneous oxidation or enzymatic hydrolysis, which are essential for flavor development [17]. It is therefore plausible that variations at the substrate level could have influenced the extent of these biochemical mechanisms, thus influencing VOC production. The most relevant variation was associated to the increase in the relative proportion of MUFAs and PUFAs in OLG samples. Since the calculated desaturation indices (for C14:1, C16:1, C18:1 *cis*-9 and CLA) did not show significant differences, it is presumable that the changes observed in the fatty acid composition are totally ascribable to the different diets administered to goats. Variations found in the calculation of these desaturation indices are, in fact, generally attributed to a greater

expression or activity of stearoyl-CoA desaturase (SCD), also known as Δ^9-desaturase [18]. This enzyme plays a leading role in the lipid metabolism of the mammary gland, due to its ability to catalyze the addition of a double bond in the cis-Δ^9-position in a large spectrum of medium- and long-chain fatty acids [19]. The substrates with which this enzyme interacts with greater affinity are precisely the acyl-CoA of C14, C16, C18 and trans-11 C18:1, which are respectively converted into C14:1, C16:1, C18:1 cis-9, and C18:2 *cis*-9 *trans*-11 [20].

The presence in the OLG samples of lower relative proportions of SFA lends itself to be discussed from different points of view. First of all, the dietary intake of SFA for humans is notoriously associated with an increased risk of developing cardiovascular diseases [21], and the importance of limiting the concentration of these compounds in foods acquires particular relevance in ruminant products because of the biohydrogenation mechanisms that are responsible for dietary PUFA conversion into SFA or MUFA [22]. Additionally, it must be specified that the improvement in the health indices of a food product is more specifically associated with the increase in omega-3 fatty acids and, over time, different feeding strategies have been tested in the zootechnical field, with the aim of obtaining the enrichment of such compounds in goats' milk and its derived dairy products [23]. Therefore, in this study, the increase in the relative proportion of linolenic acid (C18:3) observed in yogurt samples obtained from goat fed the OL supplementation acquires particular value. This finding is consistent with what was recently evidenced in a similar study focused on the evaluation of nutritional quality of Ricotta cheese made from goats' milk [24], and can be fully justified by the fact that C18:3 has been reported to be the major fatty acid in OL [25].

In addition to what has been reported, it must be considered that the increase in the relative proportion of unsaturated forms exposes these food products to greater susceptibility towards oxidative processes, which take place in most cases as an effect of the action of reactive species that are able to interact with C=C double bonds, producing peroxides [26]. The PUFA tendency to undergo oxidation is an aspect of remarkable importance for the food industry, since high concentrations of these compounds can induce detrimental effects on food nutritional quality and may represent a cause of concern for food safety [27]. Despite what has been reported, it should be said that, in the present study, the antioxidant potential in OLG yogurt samples was higher than in the CG samples—a finding also consistent with the results obtained by the TBARS test, which showed a greater resistance to lipid peroxidation in yogurt obtained from goats fed the dietary OL supplementation. The enrichment of ruminants' diets with plant matrices rich in compounds of high value from a biological point of view is generally associated with an improvement in the oxidative stability of animal productions [28,29]. Benavente-Garcia et al. [30] performed an accurate qualitative and quantitative characterization of the phenolic compounds present in OL extracts, assigning each compound its own antioxidant potential. Oleuropein was indicated as the most represented phenolic compound in the extracts. However, the greatest antioxidant function was attributed to hydroxytyrosol which is derived from the oleuropein hydrolysis. There is not much information on the bioavailability of these compounds in ruminants and, above all, there is a lack of information on their possible transfer to milk in the form of secondary metabolites able to act as antioxidants. However, even other studies conducted in the past on dairy cows and goats have highlighted the better oxidative stability of dairy products obtained by feeding animals with olive oil byproducts [13,24].

With regard to the volatile profile, numerous studies have been conducted in recent years, which highlight the diet's role in influencing the production of volatile flavor compounds in ruminant products [11]. Most of the compounds identified in all samples belong to the FFA group, which is testament to the prevalence of lipolytic processes compared to other catabolic mechanisms. Both in the freshly produced yogurt (T1) and in the one kept at 4 °C for 7 days (T7), carboxylic acids are present in higher relative proportions than in the OLG samples. In T1, this finding is mainly due to the greater presence of octanoic and decanoic acids. Both of these compounds have been reported to contribute to the characteristic animal-like, rancid and "soapy" flavor notes [31]. At T7, the data regarding octanoic acid is confirmed, but it is nevertheless interesting to note the slight but significant

reduction in the relative proportion of hexanoic and heptanoic acids. These compounds are associated with pungent, rancid and flowery notes [32]; however, their relative concentrations place them in second place with respect to octanoic acid as contributors to the overall flavor. Apart from the question concerning the flavor development, the greater presence of octanoic acid in OLG samples also lends to be discussed from the perspective of food safety. In fact, the study conducted by Kinderlerer and Lund [33] reported the ability of this compound to inhibit the growth of 10 strains of *Listeria monocytogenes* and two strains of *L. innocua*, with a minimum inhibitory concentration (MIC), which was comparable with that determined in several cheeses. In particular, in T7 samples, the dietary OL supplementation induced significant differences between volatile short-chain fatty acids, while no significant changes were found for total long-chain fatty acids (nonanoic and decanoic). Since no variations were detected in the total lipid content between CG and OLG, it is plausible that this data is the result of the differential expression or activity of the bacterial lipases, whose reaction kinetics could have been influenced by secondary OL metabolites, which reached the milk through the mammary gland. However, in this study, we did not characterize of the OL metabolites in milk; therefore, further and more specific assessments are needed to clarify this aspect.

FFAs also contribute to the formation of cheese flavor indirectly, since they represent the precursors of other chemical families: methyl ketones, secondary alcohols, esters, aldehydes and lactones [34]. In this study, the second group of VOCs in order of importance was that of methyl ketones, which are formed following the oxidation of FFAs to β-ketoacids and subsequent decarboxylation to corresponding methyl ketones [16]. These compounds are reported to be the major determinants for the characteristic flavor of blue-veined and surface-mold-ripened cheeses, as a consequence of their typical odors and low perception thresholds [35]. However, in yogurt, many of these compounds have also been associated with well-defined aromatic notes. In this study, a specific pattern has been observed with regard to 2-heptanone and 2-nonanone, to which the ability to confer floral and green-fruity notes is attributed [15]. These compounds were found to be predominant in several dairy products, and their concentrations are reported to increase during the early stage of ripening, after which levels undergo reduction [36]. This behavior is generally observed over a period of a few weeks, and since the yogurt samples considered in the present study have been stored for few days, presumably only the initial part of the phenomenon, characterized by the increase in the concentrations of these compounds, has been highlighted. The peculiarity concerns the fact that both in T1 and T7 samples, the dietary OL supplementation seems to negatively influence the production of methyl ketones. The most plausible explanation could be precisely the greater antioxidant potential that should characterize the OLG samples and which would have protected the FFAs from oxidation to β-ketoacids.

In the presence of free radicals, the unsaturated fatty acids characterizing the matrix of dairy products can undergo non-enzymatic intrachain oxidation, giving origin to hydroperoxides, which rapidly decompose to form compounds such as propanal, hexanal, heptanal, octanal, nonanal or unsaturated aldehydes, whose presence is commonly associated with a "green grass-like" aroma [16,37]. The accumulation of aldehydes as a consequence of lipid oxidation is responsible for the off-flavor development during yogurt storage. For this reason, Carrillo-Carrion et al. [38] proposed to use the total concentration of volatile aldehydes (mainly from C5 to C9) as a marker of yogurt deterioration during storage. The dietary OLG supplementation was effective in curbing such mechanisms, especially after 7 days of storage, and this evidence also correlates with a presumed improvement in the yogurt's oxidative stability. This finding is consistent with what has been already observed in other dairy products obtained from ruminants fed diets supplemented with vegetable matrices rich in bioactive compounds, mainly polyphenols [11]. In addition to this, it should be mentioned that the oxidation of unsaturated fatty acids can even be mediated by microbial lypoxygenases. These enzymes are described as non-heme iron enzymes able to catalyze the dioxygenation of PUFAs to hydroperoxy fatty acids. Although limited information is available on the bacterial isoforms of these enzymes, it is, however, known that the preferred substrate is represented by linoleic acid [39]. Therefore, the higher

concentration of linoleic acid found in the CG yogurt samples could, in part, have favored a greater accumulation of aldehydes; however, it should also be considered that the extent of this enzymatic phenomenon should have been limited in any case, given the relative percentage of linoleic acid compared to other fatty acids.

Aldehydes that accumulate in dairy products during storage or ripening may be reduced into the corresponding primary alcohols, which are considered transitory compounds [16]. Both in T1 and T7 samples, the dietary OL intake led to a limitation of this biochemical process, resulting in lower relative proportions of all the identified alcohols, precisely 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol and 2-ethyl-hexan-1-ol. Contrary to what was expected, in all samples, the presence of ethanol, which is considered one of the most characteristically volatile compounds participating in the formation of yogurt flavor, was not evidenced. Ethanol can be alternatively released during yogurt manufacture by the starter lactic acid bacteria (LAB), through a mechanism that involves the conversion of lactose into lactate with consequent pH lowering, and lactate metabolization into formate, acetaldehyde and ethanol [15,34]. Therefore, in this case, any secondary metabolites derived from bioactive compounds consumed by the goats through the OL intake could have already influenced the metabolism of the microbial forms involved in the yogurt production in the early stages of the procedure.

Alcohols that accumulate in yogurt can also react with free acids to produce esters such as ethyl-acetate and butyl-acetate. In all the analyzed samples, the only identified ester was butyl heptanoate, which showed significantly lower concentrations in the OLG yogurt. This is a compound not commonly found in yogurt, whose synthesis mechanisms and specific contributions to the development of flavor require further and more specific evaluations.

4. Materials and Methods

4.1. Experimental Design, Yogurt Manufacturing Protocol and Sampling

Thirty Saanen goats, homogeneous in age, lactation period, and number of births were used for the experiment. The trial lasted 28 days, in which the nutritional needs of goats in lactation were satisfied. Animals were randomly divided into two groups, a control group (CG) and an experimental group (EG) that received the dietary enrichment of 350 g/die/goat of olive leaves.

Regarding the yogurt production, whole raw goats' milk was pasteurized at 92 °C. Then, the milk was cooled at 40 °C, inoculated with a lactic starter mixture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus*; Santamaria Srl, Burago di Molgora (MB), Italy), portioned into 300-mL glass containers and incubated at 45 °C for 12 h. For each group of goats, 12 yogurts were produced; six yogurts for each treatment were sampled one day after production (T1) and the remaining six for each group were sampled after seven days (T7) of storage at 4 °C. All the samples were collected and maintained at −20 °C until the analysis.

4.2. Chemical Analysis

The evaluation of the chemical properties of yogurt samples were performed according to AOAC methods [40]. Briefly, pH was measured at 25 °C by using a pH-meter HD 8705 (Delta OHM, Caselle di Selvazzano (PD), Italy) and moisture content (method 933.05) was determined on 5 g of sample left in the stove for 6 h at 105 °C.

With regard to the evaluation of total lipids, 3 g of yogurt was treated with 5 mL of ethanol (Sigma Aldrich, Milan, Italy) and 750 µL of ammonium hydroxide 25% in water to obtain protein precipitation. Then, three consecutive extractions with diethyl ether and petroleum ether (Sigma Aldrich, Milan, Italy) were performed and, every time, the supernatant was recovered in a previously calibrated flask. At the end of the extraction, a rotary evaporator was used to remove the solvent, and the flask containing the lipids was then moved into the stove at 40 °C for 20 min to eliminate humidity.

After cooling at room temperature in a dryer, all flasks were weighed to calculate the total fat percentage for each sample. Results were expressed on a dry matter (DM) basis.

4.3. Fatty Acid Composition

The evaluation of the fatty acids (FA) profile was performed as previously described with slight modifications [41]. Briefly, 60 mg of total fat was solubilized in 1 mL of hexane and methylated with 500 µL of sodium methoxide 2 N in water to obtain the fatty acid methyl esters (FAMEs). Then, 1 µL of methylated extract was injected in the gas chromatograph (GC; ThermoScientific, Waltham, MA, USA) coupled with a flame ionization detector (FID) and equipped with a column Restek Rt-2560 (100 m, 0.25 mm ID, 0.20 µm df). The injector and detector temperature were both set at 280 °C and hydrogen was used as carrier gas at a flow rate of 1 mL/min. The chromatographic run lasted 56 min and the oven temperature was first held at 80 °C for 10 min, then increased from 80 °C to 172 °C at 4 °C/min and held for 30 min, and finally increased from 172 °C to 190 °C at 4 °C/min and held for 10 min. The identification of each FAME was performed by comparing the peak retention time with those obtained from a mix of analytical standards (F.A.M.E. Mix C8-C24, Supelco). The peak area was quantified using ChromeCard Software and the results were expressed as mean percentages of total FA.

4.4. Total Antioxidant Capacity and Lipid Peroxidation

Antioxidant compounds were extracted by mixing 5 g of yogurt with 15 mL of methanol. The solution was gently shaken for 40 min in the dark and then centrifuged for 15 min at 4000 rpm. Then, the supernatants were filtered and used for analysis. The antioxidant capacity was evaluated through the 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method according to the protocol described by Chen et al. [42]. Initially, 100 µL of extract was mixed with 1 mL of opportunely diluted ABTS solution, and the colorimetric evaluations were performed at 734 nm after 4 min of incubation at room temperature. An external seven-point calibration curve for Trolox (ranging from 1 to 50 µmol·g^{-1}; R^2 = 0.9961) was prepared for the quantification and the results were reported on a dry matter basis as µmol·g^{-1} Trolox equivalent antioxidant capacity (TEAC).

The TBARs test was used to evaluate the lipid peroxidation through the identification of acid-reactive substances. The analysis was performed in accordance with the procedure previously adopted by Ianni et al. [43], with slight modifications. Five grams of yogurt were mixed with 500 µL of butylated hydroxytoluene (BHT) 0.1% in methanol to stop the oxidation process. At this point, the sample was distilled, and 2 mL of distillate were mixed with an equal volume of thiobarbituric acid (TBA) solution (0.02 M). Finally, the sample was heated for 1 h at 80 °C in a thermostatic bath; after cooling at room temperature, the absorbance at 534 nm was determined for each sample. Data were expressed in µg of malondialdehyde (MDA) equivalent per gram of yogurt.

4.5. Determination of Volatile Compounds

The volatile compounds (VOCs) were extracted through a solid-phase microextraction (SPME), and then separated and identificated as previously described [44] with the use of a gas chromatograph (Clarus 580; Perkin Elmer, Walthman, MA, USA) coupled with a mass spectrometry (SQ8S; Perkin Elmer) and equipped with an Elite-5MS column (length: 30 m; internal diameter: 0.25 mm; film thickness: 0.25 µm; Perkin Elmer, Waltham, MA, USA). Ten grams of yogurt were transferred in vials and mixed with 5 mL of NaCl water solution (360 g/L), and 10 µL of 4-methyl-2-heptanone, which was used as internal standard with the aim of evaluating the extraction efficiency downstream of the analysis. After sealing the vial, the sample was stirred at 60 °C in a thermostatic bath, and the adsorption of VOCs was performed with a divinybenzene–carboxen–polydimethylsiloxane SPME fiber exposed for 45 min in the headspace. At this point, the extracted VOCs were thermally desorbed into the GC injector for 1 min in a splitless mode at 250 °C. Helium was used as carrier gas with a flow rate of 1 mL/min and the oven temperature program was started at 50 °C, held for 1 min, then increased up to 200 °C with a ratio of 3 °C/min, then held for 1 min and finally increased up to 250 °C with a ratio

of 15 °C/min, then held for 15 min. The mass spectrometer operates in electronic impact ionization mode at 70 eV. Volatile compounds were identified by a comparison with the mass spectra included in the library database (NIST Mass Spectral library (2014), Search Program version 2.0, National Institute of Standards and Technology, US Department of Commerce, Gaithersburg, MD, USA) and by comparing the eluting order with modified Kovats indices, according to the method of Van Den Dool and Kratz [45]. The data were expressed as a percentage of the relative abundance of each compound in relation to the sum of total VOCs.

4.6. Statystical Analysis

All the listed evaluations were performed on 12 samples per group (six samples for T1 and six samples for T7), and the analyses on individual samples were performed in triplicate. Results were reported as mean values with corresponding standard deviations (SD). The analysis of statistically significant differences between the two groups of data were performed by using the SigmaPlot 12.0 Software (Systat software, Inc., San Jose, CA, US) for the Windows operating system (ANOVA, Student's t-test); p values lower than 0.05 were considered statistically significant.

5. Conclusions

The results shown in this study suggest the positive role of dietary OL supplementation on the nutritional characteristics and volatile profile of goats' milk yogurt. Dietary OL intake was effective in inducing an increase in the concentration of unsaturated fatty acids, in addition to the general improvement in oxidative stability. This finding could justify an improvement in the shelf-life of the product and was also confirmed by a reduction in the concentration of volatile aldehydes. The characterization of the volatile profile was also useful in highlighting the accumulation of compounds that could justify an improvement in the yogurt flavor, although further sensorial analysis is necessary to evaluate any variations in consumer acceptability.

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Influence of Different Modalities of Grape Withering on Volatile Compounds of Young and Aged Corvina Wines

Davide Slaghenaufi [1], Anita Boscaini [2], Alessandro Prandi [1], Andrea Dal Cin [2], Vittorio Zandonà [2], Giovanni Luzzini [1] and Maurizio Ugliano [1,*]

[1] Department of Biotechnology, University of Verona, Villa Lebrecht, via della Pieve 70, 37029 San Pietro, Cariano, Italy; davide.slaghenaufi@univr.it (D.S.); aleprandi96@libero.it (A.P.); giovanni.luzzini@univr.it (G.L.)

[2] Masi Agricola, Via Monteleone, 26, Sant'Ambrogio di Valpolicella, 37015 Verona VR, Italy; anita.boscaini@masi.it (A.B.); andrea.dalcin@masi.it (A.D.C.); vittorio.zandona@masi.it (V.Z.)

* Correspondence: maurizio.ugliano@univr.it

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Abstract: Withering is a practice traditionally used in various regions to produce sweet or dry wines. During withering there is an increase in sugar content but also a modification in volatile compound profiles. Controlling metabolic changes through the dehydration process to obtain wines with desired characteristics is therefore a challenging opportunity. The effects of two different withering technologies, post-harvest or on-vine with blocked sap vessel flow, on the volatile profile of young and aged Corvina red wines was investigated. The results showed that modulation of wine aroma due to the withering process is associated with fermentative metabolites, such as esters, higher alcohols, and acids, as well as grape-related compounds such as C6 alcohols, terpenes and norisoprenoids. Significant differences were also found by comparing the two withering techniques. Post-harvest in a traditional "fruttaio" warehouse wines showed higher content of ethyl acetate, ethyl butanoate, β-citronellol and 3-oxo-α-ionol, whereas post-harvest withering on-vine increased β-damascenone in wines. The type of withering technique has an influence on the evolution of some aroma compounds during the aging of wine, among them linalool, (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB), n-hexyl acetate, ethyl acetate, ethyl 3-methylbutanoate, 3-oxo-α-ionol and β-damascenone.

Keywords: post-harvest; withering; on-vine; fruttaio; wine aroma; Corvina

1. Introduction

Valpolicella is a wine producing region characterized by the traditional practice of post-harvest withering for the production of dry and sweet red wines, among which Amarone is the most famous [1]. Valpolicella is located in the north-east of Italy close to Verona city, with Corvina, Corvinone and Rondinella grapes being the traditional varieties employed for local wines [1]. At ripening, grapes are harvested and stored in a specific warehouse traditionally called a "fruttaio", where they undergo slow dehydration [2]. The duration of withering varies depending on the wine type being produced, and it is generally monitored by assessing grape weight loss. In the case of Amarone or Recioto, withering generally lasts 2–3 months, with a weight loss of approximately 30% of the initial weight [3]. In the case of other wines such as Valpolicella Classico superiore as well as different IGT wines, a milder withering is usually carried out, lasting 4–8 weeks with weight loss of 10–15%.

Grape withering has a deep impact on the formation of the characteristic aroma of Amarone wine [4,5]. During the withering process, an increase in sugar content due to water loss is not

the only transformation taking place. Phenolic and aromatic composition of grapes and wines is also affected [6–12], and skin wall composition as well as grape mechanical properties are also modified [13,14]. Interestingly, some of these processes are not due to dehydration but are the result of ongoing metabolic activities in the berry, resulting in peculiar gene expression patterns contributing to changes secondary metabolism [15–17].

Different grape withering techniques have been developed in the past, depending to local environment, grape variety and technical issues, entering in the local tradition and history as community heritage [18]. The different techniques can be divided into natural, forced and on-vine withering [18]. An example of a natural withering technique is the exposure of grapes to the sun, while in the forced method, grape dehydration is obtained using ventilated rooms like in the case of a modern fruttaio where withering conditions like temperature, humidity and air flow are controlled. On-vine withering can be obtained by practicing late harvest, cane cutting, or peduncle twist [18]. Though withering in Valpolicella is traditionally made in a fruttaio, there is an increasing interest to explore other postharvest methods to support traditional practices, in particular for mild withering processes requiring a short duration.

The aim of this research was to evaluate the effect of the two withering systems, in "fruttaio" and on-vine with peduncle twist, on the volatile profile of wines. The results were compared with those of wines obtained from not-withered grapes. Wines were also assessed after a period of aging, to evaluate the influence of the different withering practices on aging patterns of the resulting wines.

2. Results

2.1. Volatile Compounds in Young Wines

A total of 53 volatile compounds have been identified and quantified in wine samples (Table 1), including five alcohols, 3 C_6 alcohols, 10 esters, three acids, 18 terpenes, seven norisoprenoids, seven benzenoids. The analysis of the variance (ANOVA) made between the three modalities, showed statistically significant ($p < 0.05$) differences for 35 compounds. Wines from withered grapes in fruttaio and on-vine were characterized by higher content in terpinen-4-ol, β-citronellol, 1,4-cineole, 3-oxo-α-ionol, vinylguaiacol, ethyl acetate, benzyl alcohol and ethyl vanillate. At the same time, samples fruttaio and on-vine compared to control showed lower amounts of 1-pentanol, 1-hexanol, *cis*-3-hexenol, *trans*-3-hexenol, isoamyl acetate, *n*-hexyl acetate, phenylethyl acetate ethyl 3-methylbutanoate, ethyl hexanoate, ethyl octanoate, hexanoic acid, octanoic acid, *cis*-linalool oxide and β-damascenone. Compared to each other, statistical differences of the two withering techniques were observed for 19 volatile compounds, among them 1-butanol, 1-hexanol, *trans*-3-hexenol, ethyl butanoate, β-citronellol, limonene, α-phellandrene, terpinolene, benzyl alcohol and methyl vanillate were found in higher concentration in fruttaio samples, while on-vine samples showed higher content of 2-butanol, ethyl acetate, octanoic acid, β-damascenone, TDN and 4-vinyl guaiacol.

Table 1. Concentration (μg/L) of free compounds in young wine samples. Mean, standard deviation (SD) and ANOVA.

		Control		Fruttaio		On-Vine		
Compounds	**Odor Threshold [1]**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	***p*-Value**
Alcohols								
2-Butanol		3283.8 [b]	±207.9	2203.1 [a]	±80.3	3467.7 [b]	±93.1	<0.0001
1-Butanol		269.7 [a]	±6.7	506.3 [b]	±48.8	228.0 [a]	±48.3	0.000
1-Pentanol		58.3 [b]	±5.3	43.0 [a]	±1.5	42.8[a]	±1.4	0.002
Isoamyl alcool	30,000	35,372.3 [a]	±1853.6	33,820.8 [a]	±922.9	34,951.5 [a]	±2051.8	0.541
Phenylethyl Alcohol	14,000	16,240.6 [a]	±721.1	20,779.8 [a]	±466.8	16,568.9 [a]	±6073.6	0.290
C6 Alcohols								
1-Hexanol	8000	3292.5 [c]	±171.9	2292.4 [b]	±84.2	1951.1 [a]	±70.7	<0.0001
trans-3-Hexen-1-ol		39.4 [b]	±2.4	32.0 [b]	±6.59	21.4 [a]	±2.0	0.006
cis-3-Hexen-1-ol	400	520.3 [b]	±26.9	92.7 [a]	±13.2	65.5 [a]	±7.3	<0.0001

Table 1. *Cont.*

Compounds	Odor Threshold [1]	Control		Fruttaio		On-Vine		
		Mean	SD	Mean	SD	Mean	SD	*p*-Value
Acetate Esters								
Ethyl acetate	12,000	19,537.4 ^{a}	±1896.3	49,654.9 ^{c}	±4413.3	33,649.2 ^{b}	±7566.5	0.001
Isoamyl acetate	30	10,732.9 ^{b}	±1503.1	3216.1 ^{a}	±631.4	1877.3 ^{a}	±293.0	<0.0001
n-Hexyl acetate	1800	279.3 ^{b}	±41.8	22.3 ^{a}	±1.5	6.47 ^{a}	±1.09	<0.0001
Phenylethyl acetate	2400	106.2 ^{b}	±11.0	35.3 ^{a}	±4.5	30.7 ^{a}	±1.9	<0.0001
Ethyl Esters								
Ethyl butanoate	20	254.7 ^{b}	±14.7	265.6 ^{b}	±28.1	210.1 ^{a}	±16.4	0.036
Ethyl 3-methylbutanoate	3	215.4 ^{b}	±32.5	64.6 ^{a}	±14.1	36.7 ^{a}	±6.3	<0.0001
Ethyl hexanoate	14	548.5 ^{b}	±33.4	331.0 ^{a}	±9.5	302.1 ^{a}	±24.9	<0.0001
Ethyl octanoate	5	452.1 ^{b}	±50.2	254.3 ^{a}	±10.7	249.6 ^{a}	±27.9	0.000
Ethyl decanoate	200	45.6 ^{a}	±6.2	50.6 ^{a}	±5.5	38.6 ^{a}	±6.5	0.132
Ethyl lactate	100,000	1087.9 ^{b}	±49.6	690.6 ^{a}	±34.7	647.8 ^{a}	±64.2	<0.0001
Fatty Acids								
3-Methylbutanoic acid	250	511.9 ^{b}	±38.5	402.3 ^{a}	±6.6	449.1 ^{a}	±19.0	0.005
Hexanoic acid	2080	2623.6 ^{c}	±130.17	1286.5 ^{a}	±103.3	1683.5 ^{b}	±36.2	<0.0001
Octanoic acid	2560	2044.8 ^{c}	±184.0	820.0 ^{a}	±36.8	1066.7 ^{b}	±39.8	<0.0001
Terpenes								
cis-Linalool oxide	3000	6.57 ^{b}	±3.12	1.29 ^{a}	±0.78	1.33 ^{a}	±0.19	0.038
trans-Linalool oxide	6000	0.415 ^{a}	±0.211	0.330 ^{a}	±0.130	0.50 ^{a}	±0.282	0.797
Linalool	25	27.3 ^{b}	±3.3	14.8 ^{a}	±0.5	16.5 ^{a}	±0.2	0.000
Terpinen-1-ol		0.351 ^{a}	±0.065	0.429 ^{a}	±0.162	0.519 ^{a}	±0.066	0.242
Terpinen-4-ol		0.092 ^{a}	±0.021	1.163 ^{b}	±0.200	0.795 ^{b}	±0.173	0.026
Ho-trienol	110	0.060 ^{a}	±0.010	0.047 ^{a}	±0.010	0.075 ^{a}	±0.043	0.478
α-Terpineol	250	13.9 ^{b}	±1.3	6.54 ^{a}	±0.09	7.09 ^{a}	±0.30	<0.0001
Nerol	400	3.95 ^{a}	±0.53	4.89 ^{a}	±0.43	3.36 ^{a}	±0.96	0.828
Geraniol	30	6.69 ^{a}	±0.69	6.50 ^{a}	±0.79	6.61 ^{a}	±0.20	0.931
β-Citronellol	100	4.14 ^{a}	±0.18	12.58 ^{c}	±0.44	10.18 ^{b}	±1.87	0.000
p-Menthane-1,8-diol		<LOQ		<LOQ		<LOQ		
α-Phellandrene		0.035 ^{b}	±0.013	0.040 ^{b}	±0.006	0.016 ^{a}	±0.002	0.029
1,4-Cineole	0.54	0.110 ^{a}	±0.013	0.205 ^{b}	±0.023	0.182 ^{b}	±0.024	0.003
1,8-Cineole	1.1	0.215 ^{a}	±0.022	0.153 ^{a}	±0.040	0.176 ^{a}	±0.068	0.336
Limonene		0.228 ^{a}	±0.028	0.388 ^{b}	±0.060	0.225 ^{a}	±0.040	0.006
γ-Terpinen		1.10 ^{a}	±0.51	1.15 ^{a}	±0.09	1.03 ^{a}	±0.19	0.904
p-Cymene		0.083 ^{a}	±0.008	0.145 ^{a}	±0.077	0.093 ^{a}	±0.021	0.289
Terpinolene		0.137 ^{a}	±0.024	0.257 ^{b}	±0.045	0.125 ^{a}	±0.041	0.010
Norisoprenoids								
β-Damascenone	0.05	3.47 ^{c}	±0.27	1.69 ^{a}	±0.13	2.80 ^{b}	±0.10	<0.0001
α-Ionone		2.27 ^{b}	±0.70	0.85 ^{a}	±0.34	1.03 ^{a}	±0.67	0.050
α-Ionol		0.233 ^{a}	±0.019	0.283 ^{a}	±0.026	0.25 ^{a}	±0.010	0.955
Vitispirane		<LOQ		<LOQ		<LOQ		
TPB		0.050 ^{a}	±0.009	0.035 ^{a}	±0.006	0.051 ^{a}	±0.013	0.140
TDN	2	2.64 ^{b}	±0.42	1.57 ^{a}	±0.18	2.79 ^{b}	±0.69	0.040
3-oxo-α-Ionol		1.58 ^{a}	±0.24	2.65 ^{c}	±0.20	2.10 ^{b}	±0.23	0.003
Benzenoids								
4-Ethyl guaiacol	33	<LOQ		<LOQ		<LOQ		
4-Vinyl guaiacol	1100	7.36 ^{a}	±0.44	9.40 ^{b}	±0.53	11.63 ^{c}	±1.63	0.006
2,6-Dimethoxyphenol		0.108 ^{a}	±0.018	0.015 ^{a}	±0.007	0.022 ^{a}	±0.001	0.428
Benzyl Alcohol		128.3 ^{a}	±4.9	229.1 ^{c}	±15.1	173.1 ^{b}	±24.7	0.001
Vanillin	200	2.13 ^{a}	±0.84	2.04 ^{a}	±0.95	0.888 ^{a}	±0.672	0.204
Methyl vanillate		4.37 ^{a}	±0.09	4.86 ^{b}	±0.11	4.49 ^{a}	±0.05	0.001
Ethyl vanillate		16.4 ^{a}	±1.1	21.6 ^{b}	±2.0	31.4 ^{c}	±5.2	0.004

Values in the same row with different letters indicate statistically significant differences, $p < 0.05$; [1] Data from: Ferreira et al. (2000) [19], Francis et al. (2005) [20], Sacks et al. (2012) [21] and Antalick et al. (2015) [22]. <LOQ: Values below the limit of quantification.

Eighteen glycosidically bound compounds have been quantified (Table 2). The ANOVA showed significant differences for eight of these compounds. Compared to control, fruttaio samples and on-vine showed significantly lower concentrations in *cis*-3-hexenol, benzyl alcohol precursor and higher content of methyl vanillate and ethyl vanillate precursors. Comparing fruttaio and on-vine samples, statistically significant differences have been observed for six glycosidically bound compounds. Fruttaio samples were richer in 1-hexanol, *trans*-3-hexenol, geraniol and vanillin precursors, while on-vine samples showed higher concentration only for the phenylethyl alcohol glycosidically bound precursor.

Principal component analysis (PCA) showed that 65.9% of the total variance was explained by the first and second component (Figure 1). In fact, first principal component (PC-1) explained 45.8% of the total variance, while PC-2 explained 20.1%. Samples were separated into three clusters according to their withering technique. PC-1 mostly discriminated withered samples from not-withered. Control samples were characterized principally by esters, fatty acids, C_6 compounds 1-hexanol, *trans*-3-hexenol, and *cis*-3-hexenol precursor. Instead withered compounds were characterized by terpenes like β-citronellol, linalool, terpinen-4-ol, 1,4-cineole; by the norisoprenoid 3-oxo-α-ionol; and by several benzenoids like phenylethyl alcohol, benzyl alcohol, methyl vanillate and its glycosidic precursor. PC-2 permitted to discriminate on-vine from fruttaio samples, the major drivers of this diversity were the ethyl butanoate, ethyl decanoate, ethyl vanillate, and the bound precursor of geraniol.

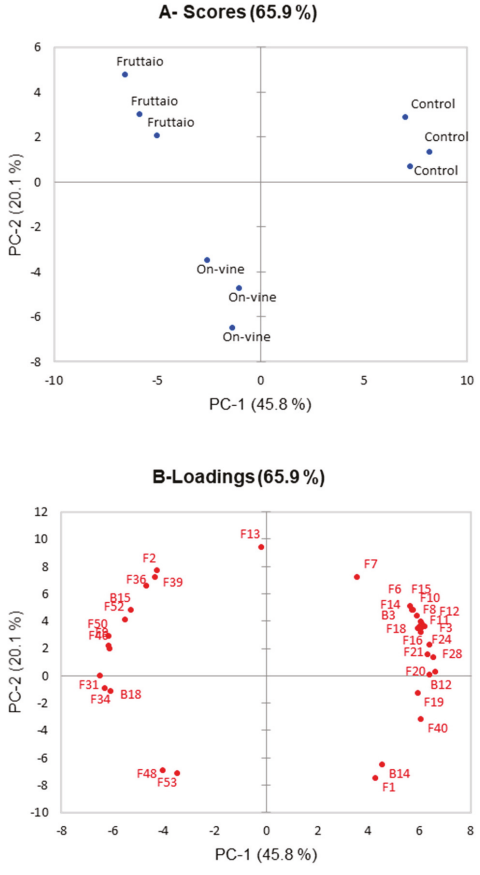

Figure 1. Principal component analysis showing aged wine samples scores (**A**) and loadings (**B**), only loadings with score value > 0.75 were shown. Loadings plot number correspond to: (F1) 2-Butanol, (F2) 1-Butanol, (F3) 1-Pentanol, (F6) 1-Hexanol, (F7) *trans*-3-Hexenol, (F8) *cis*-3-Hexenol, (F9) Ethyl acetate, (F10) Isoamyl acetate, (F11) n-Hexyl acetate, (F12) Phenylethyl acetate, (F13) Ethyl butanoate, (F14) Ethyl 3-methylbutanoate, (F15) Ethyl hexanoate, (F16) Ethyl octanoate, (F18) Ethyl lactate, (F19) 3-Methylbutanoic acid, (F20) Hexanoic acid, (F21) Octanoic acid, (F24) Linalool, (F28) α-Terpineol, (F31) β-Citronellol, (F34) 1,4-cineole, (F36) Limonene, (F39) Terpinolene, (F40) β-Damascenone, (F46) 3-oxo-α-Ionol, (F48) 4-Vinyl guaiacol, (F50) Benzyl alcohol, (F52) Methyl vanillate, (F53) Ethyl vanillate, (B3) bound cis-3-Hexenol, (B12) bound Benzyl alcohol, (B14) bound Phenylethyl alcohol, (B15) bound Vanillin, (B18) bound Ethyl vanillate.

Table 2. Concentration (μg/L) of glycosidically-bound compounds in young wine samples. Mean, standard deviation (SD) and ANOVA.

	Control		Fruttaio		On-Vine		
Compounds	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	***p*-Value**
Alcohols							
Phenylethyl alcohol	284.9 ^{b}	±22.5	191.7 ^{a}	±11.0	282.0 ^{b}	±47.8	0.017
C6 alcohols							
1-Hexanol	203.8 ^{b}	±15.8	216.5 ^{b}	±29.8	159.6 ^{a}	±17.5	0.043
trans-3-Hexen-1-ol	1.80 ^{a}	±0.28	3.27 ^{b}	±0.41	2.03 ^{a}	±0.29	0.003
cis-3-Hexen-1-ol	25.4 ^{b}	±2.4	12.2 ^{a}	±1.6	11.0 ^{a}	±1.6	0.000
Terpenes							
cis-Linalooloxide	1.93 ^{a}	±0.71	2.15 ^{a}	±0.53	2.06 ^{a}	±0.64	0.909
trans-Linalooloxide	2.17 ^{a}	±0.72	2.74 ^{a}	±0.88	2.49 ^{a}	±0.61	0.666
Linalool	5.71 ^{a}	±1.40	6.82 ^{a}	±0.82	6.91 ^{a}	±0.94	0.378
Terpinen-4-ol	0.068 ^{a}	±0.020	0.070 ^{a}	±0.024	0.069 ^{a}	±0.032	0.999
α-Terpineol	2.00 ^{a}	±0.52	2.19 ^{a}	±0.02	2.42 ^{a}	±0.20	0.343
β-Citronellol	0.163 ^{a}	±0.048	0.747 ^{b}	±0.051	0.130 ^{a}	±0.030	0.051
Nerol	15.1 ^{a}	±0.6	16.4 ^{a}	±1.8	11.3 ^{a}	±4.0	0.120
Geraniol	20.7 ^{a}	±1.7	24.1 ^{b}	±1.4	19.0 ^{a}	±1.1	0.013
Norisoprenoids							
3-oxo-α-Ionol	5.44 ^{b}	±0.43	2.80 ^{a}	±1.41	4.40 ^{ab}	±1.56	0.102
α-Ionol	0.035 ^{a}	±0.017	0.015 ^{a}	±0.010	0.030 ^{a}	±0.018	0.329
Benzenoids							
Vanillin	0.175 ^{a}	±0.095	9.41 ^{c}	±1.36	1.60 ^{b}	±0.22	0.001
Methyl vanillate	4.01 ^{a}	±0.21	5.17 ^{b}	±0.29	4.92 ^{b}	±0.02	0.001
Ethyl vanillate	0.365 ^{a}	±0.129	0.737 ^{b}	±0.209	0.747 ^{b}	±0.188	0.065
Benzyl Alcohol	303.7 ^{b}	±58.2	112.4 ^{a}	±10.7	163.9 ^{a}	±21.5	0.002

Values in the same row with different letters indicate statistically significant differences ($p < 0.05$). <LOQ: Value below the limit of quantification.

2.2. Volatile Composition of Aged Wine

After model aging, wines were different for 28 volatile compounds (Table 3) and seven bound compounds ($p < 0.05$) (Table 4). The fruttaio and the on-vine samples showed significant differences ($p < 0.05$) for 17 compounds, three of which were glycosidically bound precursors. The PCA analysis (Figure 2) after wine model aging showed a total variance of 63.1%. Three clusters were formed corresponding to the three conditions studied: control, fruttaio and on-vine. The compounds that most characterized these three groups were basically the same that were obtained in wine samples before model aging: the class of esters, acids and alcohols for control samples, terpenes and benzenoids for withered samples.

Table 3. Concentration (μg/L) of free compounds in aged wine samples. Mean, standard deviation (SD) and ANOVA.

	Control		Fruttaio		On-Vine		
Compounds	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	***p*-Value**
Alcohols							
2-Butanol	3019.8 ^{b}	±237.4	1980.9 ^{a}	±291.9	3276.2 ^{b}	±112.6	0.001
1-Butanol	247.4 ^{a}	±37.2	452.1 ^{b}	±16.7	206.9 ^{a}	±50.3	0.000
1-Pentanol	17.9 ^{a}	±0.6	15.0 ^{a}	±5.2	14.2 ^{a}	±1.9	0.398
Isoamyl alcool	60,167.0 ^{a,b}	±1467.0	55,052.5 ^{a}	±5134.7	63,751.1 ^{b}	±2291.3	0.051
Phenylethyl Alcohol	20,612.4 ^{a}	±2721.8	25,084.0 ^{ab}	±2609.2	28,513.4 ^{b}	±4720.3	0.083
C6 Alcohols							
1-Hexanol	3199.2 ^{c}	±22.3	2231.4 ^{b}	±208.7	1787.8 ^{a}	±102.2	<0.0001
trans-3-Hexen-1-ol	51.9 ^{c}	±2.6	40.4 ^{b}	±1.3	26.2 ^{a}	±7.4	0.001
cis-3-Hexen-1-ol	609.6 ^{b}	±7.7	110.0 ^{a}	±39.5	74.0 ^{a}	±11.1	<0.0001

Table 3. *Cont.*

	Control		Fruttaio		On-Vine		
Compounds	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	***p*-Value**
Acetate Esters							
Ethyl acetate	19,178.22 ^{a}	±7696.5	32,697.7 ^{b}	±2026.1	38,187.8 ^{b}	±5575.2	0.015
Isoamyl acetate	3732.0 ^{b}	±188.2	1435.2 ^{a}	±852.1	760.8 ^{a}	±300.4	0.001
n-Hexyl acetate	82.8 ^{b}	±3.1	8.9 ^{a}	±2.1	8.0 ^{a}	±2.8	0.000
Phenylethyl acetate	55.2 ^{b}	±1.9	29.0 ^{a}	±10.2	23.8 ^{a}	±2.2	0.002
Ethyl Esters							
Ethyl butanoate	255.2 ^{a}	±12.2	296.2 ^{b}	±20.1	231.7 ^{a}	±24.2	0.018
Ethyl 3-methylbutanoate	56.6 ^{a}	±2. 9	56.2 ^{a}	±3.5	53.2 ^{a}	±7.5	0.685
Ethyl hexanoate	491.2 ^{c}	±18.2	357.1 ^{b}	±32.5	300.4 ^{a}	±29.1	0.000
Ethyl octanoate	245.1 ^{b}	±7.7	153.0 ^{a}	±37.2	138.9 ^{a}	±14.4	0.003
Ethyl decanoate	18.8 ^{a}	±1.7	16.0 ^{a}	±6.6	12.1 ^{a}	±3.1	0.246
Ethyl lactate	3704.2 ^{b}	±158.1	3656.8 ^{b}	±99.3	3194.4 ^{a}	±352.9	0.065
Fatty Acids							
3-Methylbutanoic acid	706.1 ^{b}	±115.7	306.1 ^{a}	±8.8	448.0 ^{ab}	±194.8	0.025
Hexanoic acid	3753.0 ^{b}	±77.1	1833.3 ^{a}	±156.2	1878.8 ^{a}	±235.0	<0.0001
Octanoic acid	2901.9 ^{b}	±140.7	1288.8 ^{a}	±183.5	1405.4 ^{a}	±166.7	<0.0001
Terpenes							
cis-Linalool oxide	7.85 ^{a}	±0.53	8.57 ^{ab}	±0.59	9.60 ^{b}	±0.66	0.031
trans-Linalool oxide	4.84 ^{a}	±0.30	6.07 ^{b}	±0.18	6.06 ^{b}	±0.28	0.002
Linalool	15.8 ^{a}	±3.17	23.1 ^{a}	±3.1	18.80 a	±3.3	0.142
Terpinen-1-ol	<LOQ		<LOQ		<LOQ		
Terpinen-4-ol	11.7 ^{a}	±2.7	17.5 ^{b}	±2.0	13.4 ^{a,b}	±1.8	0.044
Ho-trienol	0.013 ^{a}	±0.006	0.018 ^{a}	±0.003	0.022 ^{a}	±0.003	0.115
α-Terpineol	34.2 ^{a}	±3.3	37.5 ^{a}	±3.9	34.6 ^{a}	±3.3	0.511
Nerol	1.47 ^{a}	±0.57	1.33 ^{a}	±0.75	2.29 ^{a}	±0.06	0.147
Geraniol	2.97 ^{a}	±0.14	4.31 ^{a}	±1.99	2.64 ^{a}	±1.02	0.319
β-Citronellol	2.71 ^{a}	±2.37	7.92 ^{b}	±1.60	4.38 ^{a}	±0.99	0.027
p-Menthane-1.8-diol	0.417 ^{b}	±0.060	0.214 ^{a}	±0.069	0.404 ^{b}	±0.054	0.011
α-Phellandrene	0.802 ^{a}	±0.023	0.518 ^{a}	±0.237	0.620 ^{a}	±0.076	0.218
1,4-Cineole	0.202 ^{a}	±0.169	0.286 ^{a}	±0.041	0.323 ^{a}	±0.045	0.398
1,8-Cineole	0.188 ^{a}	±0.032	0.307 ^{a}	±0.024	0.362 ^{a}	±0.018	0.341
Limonene	0.408 ^{a}	±0.178	0.497 ^{a}	±0.026	0.430 ^{a}	±0.038	0.596
γ-Terpinen	1.01 ^{a}	±0.13	0.294 ^{a}	±0.131	0.992 ^{a}	±0.040	0.664
p-Cymene	0.162 ^{a}	±0.061	0.252 ^{b}	±0.016	0.155 ^{a}	±0.018	0.034
Terpinolene	0.115 ^{a}	±0.036	0.175 ^{b}	±0.015	0.098 ^{a}	±0.013	0.017
Norisoprenoids							
β-Damascenone	3.49 ^{a}	±0.05	3.08 ^{a}	±0.18	3.58 ^{a}	±0.10	0.418
α-Ionone	0.282 ^{a}	±0.128	0.448 ^{a}	±0.099	0.480 ^{a}	±0.287	0.441
α-Ionol	0.130 ^{a}	±0.0317	0.140 ^{a}	±0.094	0.295 ^{a}	±0.096	0.554
Vitispirane	5.07 ^{a}	±4.66	8.33 ^{a}	±0.51	7.72 ^{a}	±0.89	0.367
TPB	0.080 ^{b}	±0.013	0.049 ^{a}	±0.006	0.055 ^{a}	±0.008	0.015
TDN	4.25 ^{a}	±1.34	4.20 ^{a}	±0.68	4.50 ^{a}	±0.35	0.906
3-oxo-α-Ionol	17.6 ^{a,b}	±4.2	11.6 ^{a}	±2.8	20.7 ^{b}	±3.4	0.080
Benzenoids							
4-Ethyl guaiacol	0.190 ^{a}	±0.049	0.295 ^{a}	±0.152	0.153 ^{a}	±0.193	0.500
4-Vinyl guaiacol	15.5 ^{a}	±4.4	21.3 ^{a}	±0.7	20.2 ^{a}	±5.1	0.235
2,6-Dimethoxyphenol	0.078 ^{a}	±0.014	0.110 ^{a}	±0.029	0.208 ^{a}	±0.043	0.220
Benzyl Alcohol	153.8 ^{a}	±27.3	275.0 ^{c}	±12.1	215.2 ^{b}	±16.3	0.001
Vanillin	5.36 ^{a}	±1.04	7.26 ^{b}	±0.37	6.39 ^{ab}	±0.61	0.050
Methyl vanillate	4.83 ^{a}	±0.50	5.96 ^{b}	±0.37	5.79 ^{b}	±0.34	0.030
Ethyl vanillate	34.4 ^{a}	±6.0	56.7 ^{b}	±3.7	69.7 ^{b}	±3.4	0.012

Values in the same row with different letters indicate statistically significant differences ($p < 0.05$). <LOQ: Value below the limit of quantification.

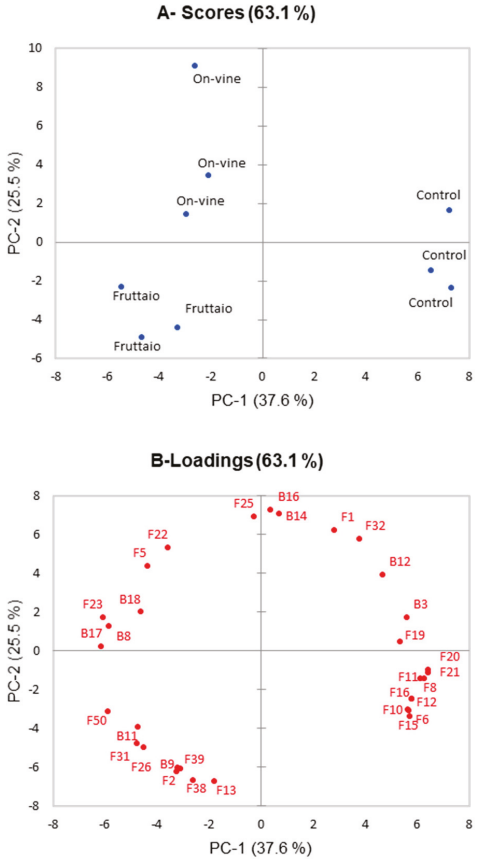

Figure 2. Principal component analysis showing aged wine samples scores (**A**) and loadings (**B**), only loadings with score value >0.75 were shown. Loadings plot number correspond to: (F1) 2-Butanol, (F2) 1-Butanol, (F5) Phenyl ethyl alcohol, (F6) 1-Hexanol, (F8) *cis*-3-Hexenol, (F10) Isoamyl acetate, (F11) n-Hexyl acetate, (F12) Phenylethyl acetate, (F13) Ethyl butanoate, (F15) Ethyl hexanoate, (F16) Ethyl octanoate, (F19) 3-Methylbutanoic acid, (F20) Hexanoic acid, (F21)Octanoic acid, (F22) *cis*-Linalool oxide, (F23) *trans*-Linalool oxide, (F25)Terpinen-1-ol, (F26) Terpinen-4-ol, (F31) β-Citronellol, (F32) p-Menthane-1,8-diol, (F38) p-Cymene, (F39) Terpinolene, (F50) Benzyl alcohol, (B3) bound *cis*-3-Hexenol, (B8) bound α-Terpineol, (B9) bound β-Citronellol, (B11) bound Geraniol, (B12) bound Benzyl alcohol, (B14) bound Phenylethyl alcohol, B16) bound 3-oxo-α-Ionol, (B17) bound Methyl vanillate.

Table 4. Concentration (μg/L) of bound compounds in aged wine samples. Mean, standard deviation (SD) and ANOVA.

	Control		Fruttaio		On-Vine		
Compounds	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	***p*-Value**
Alcohols							
Phenylethyl alcohol	121.4 ^{ab}	±7.8	96.2 ^{a}	±21.9	146.9 ^{b}	±9.4	0.015
C6 alcohols							
1-Hexanol	84.6 ^{a}	±6.7	84.8 ^{a}	±14.0	82.8 ^{a}	±7.4	0.965
trans-3-Hexen-1-ol	0.588 ^{a}	±0.035	0.852 ^{b}	±0.163	0.750 ^{ab}	±0.065	0.054
cis-3-Hexen-1-ol	22.6 ^{b}	±1.6	11.2 ^{a}	±5.2	13.8 ^{a}	±0.8	0.011

Table 4. *Cont.*

	Control		Fruttaio		On-Vine		
Compounds	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	***p*-Value**
Terpenes							
cis-Linalooloxide	2.23 ^{a}	±0.26	2.41 ^{a}	±0.46	2.79 ^{a}	±0.32	0.229
trans-Linalooloxide	2.83 ^{a}	±0.74	3.05 ^{a}	±0.71	3.68 ^{a}	±0.64	0.366
Linalool	<LOQ		<LOQ		<LOQ		
Terpinen-4-ol	<LOQ		<LOQ		<LOQ		
α-Terpineol	0.182 ^{a}	±0.029	0.977 ^{b}	±0.253	0.977 ^{b}	±0.140	0.009
β-Citronellol	0.175 ^{a}	±0.004	1.19 ^{b}	±0.20	0.000 ^{a}	±0.096	<0.0001
Nerol	3.60 ^{a}	±0.60	4.68 ^{a}	±0.20	3.95 ^{a}	±0.73	0.128
Geraniol	4.08 ^{a}	±1.03	6.95 ^{b}	±0.56	5.20 ^{ab}	±1.63	0.060
Norisoprenoids							
3-oxo-α-Ionol	2.59 ^{a}	±0.22	2.11 ^{a}	±0.37	3.17 ^{b}	±0.24	0.011
α-Ionol	<LOQ		<LOQ		<LOQ		
Benzenoids							
Vanillin	3.76 ^{a}	±0.07	3.61 ^{a}	±0.30	3.76 ^{a}	±0.16	0.595
Methyl vanillate	3.94 ^{a}	±0.09	5.22 ^{b}	±0.31	5.02 ^{b}	±0.28	0.001
Ethyl vanillate	0.317 ^{a}	±0.132	0.737 ^{a}	±0.135	0.668 ^{a}	±0.209	0.134
Benzyl Alcohol	130.5 ^{b}	±6.2	65.7 ^{a}	±3.4	105.9 ^{ab}	±8.5	0.023

Values in the same row with different letters indicate statistically significant differences ($p < 0.05$). <LOQ: Value below the limit of quantification.

3. Discussion

Post-harvest withering plays a central role in determining the compositional and sensory characteristics of Valpolicella red wines [1,3]. From a quantitative point of view, the main physiological change associated with this traditional practice is water loss, that is carried out up to an average of 30% weight loss depending on wine style. This has major implication for grape composition, most notably increased concentration of metabolites such as sugars, phenolics, and certain aroma compounds, directly influencing composition of the resulting wine. Additional important consequences of increased sugar levels are related to changes in yeast metabolism, which can further impact wine composition. However, it has been recently shown that post-harvest withering is not simply a dehydration process, with many complex metabolic transformations beyond simple concentration taking place inside the berry, inducing important modifications in the pool of grape secondary metabolites, including volatile compounds [17]. In consideration of this complex scenario, one of the purposes of the present study was to investigate how and to what extent withering of the grapes affects the volatile composition of the resulting wine. Second, and most important, this study had the objective to assess the potential of an alternative withering approach to modulate Corvina wine volatile composition. Although post-harvest withering is traditionally carried out in warehouses (locally called "fruttaio"), there is an ongoing interest towards the exploration of alternative strategies that can be applied to obtain a suitable degree of over-ripening or withering, also with the aim of producing alternative wine types and styles [2]. Among these, cane-cut on-vine has been shown to positively influence wine aroma and phenolic composition [6–12]. In the present study, an alternative approach to on-vine withering, still based on blocking xylem flow but not involving cane cutting, was investigated in comparison with conventional fruttaio withering. As sugar levels at grape crush were similar for both withering modalities, any difference is expected to result from differences in grape composition in terms of secondary metabolites or interaction with yeast.

3.1. Influence of Grape Withering on Volatile Composition of Corvina Wines

Analysis of free and glycosidically-bound volatile compounds of the wines at bottling showed that withering of the grapes significantly affected wine aroma compounds, influencing the concentrations of various classes of volatiles. Free compounds can have a direct influence on wine aroma while the

bound compounds can act as an aroma reservoir that is released during aging. At a general level, fermentation-derived volatiles such as esters, higher alcohols, and acids, as well as grape-related compounds such as certain norisoprenoids, were mostly associated with non-withered grapes, whereas withering resulted in higher wine content in terpenes and benzenoids (Figure 1 and Table 1). Among compounds known to impact red wine aroma, acetate esters (i.e., isoamyl acetate) and ethyl fatty acid esters (i.e., ethyl hexanoate and octanoate) were strongly influenced by withering, which resulted in a significant decrease in the concentration of nearly all the analyzed esters. Esters are related to red wine's fruity character [23] and are formed during alcoholic fermentation involving amino acid metabolism in the case of acetates, and fatty acid metabolism for ethyl esters [24]. The production of esters by yeast is influenced by several factors, and different studies have reported an influence of grape maturity [25] and levels of withering [4,9,26] on wine ester content, suggesting and influence of must sugar content on ester production. In agreement with these reports, wines from withered Corvina grapes, which at harvest displayed additional 2 Brix compared to control grapes, showed lower ester content. In particular, acetate esters were more impacted, in spite of the fact that higher alcohol content, a precursor of acetates, was not so different across treatments and in some cases was even greater in withered samples. Ester/alcohol ratios were calculated to establish esterification rates of the different esters, and in the case of acetates it appeared clear that acetylation was much higher in fermentation of non-withered grapes (Figure 3). Likewise, a higher acetylation rate was also observed for the control wine in the case of the ethyl ester of the branched chain fatty acid 3-methylbutanoic acid, also derived from amino acid metabolism. Conversely, although concertation of ethyl esters was higher in control samples, esterification of the corresponding fatty acid was similar in all treatments, so that it can be inferred that wine ester levels were determined by concentration of the corresponding fatty acid. It can be therefore assumed that, under our experimental conditions, withering impact on esters was due on one hand to reduced acetylation and on the other hand to reduced production of fatty acids, which would be in agreement with the observations of Saerens et al. (2008) [27]. In the case of ethyl fatty acid esters, the reduced availability of short chain fatty acid precursors could be due to the greater availability of unsaturated fatty acids in musts from withered grapes [28], which would result in reduced medium chain fatty acids biosynthesis [29]. An increase in available lipids can also reduce the expression of the *ATF1* gene and therefore lower acetyl transferase activity catalyzing the acetylation reaction [30]. Interestingly, ethyl acetate showed a completely different trend, its concentration increasing significantly in wines from withered grapes. Although acetyl transferase activities are expected to play a role in ethyl acetate formation by *S. cerevisiae*, recent observations indicated that acetyl transferases other than Atf1 and Atf2 contribute significantly to production of this ester [31], which could explain its different response to withering.

C_6 alcohols were also found to discriminate, with a high level of significance, control wines from the two withering modalities. C_6 alcohols contribute to the "leafy" and "herbaceous" odors of wines [32]. In control samples *cis*-3-hexenol showed and odor active value (OAV, calculated as ration between concentration and odor threshold) higher than one therefore potentially contributed to wine aroma (OAV = 1.3). Instead, in withered samples, the C_6 alcohols had OAV values lower than one. C_6 alcohols are formed during berry crushing by enzymatic oxidation of grape unsaturated fatty acids, initiated by grape lipoxygenase enzymes [33]. Zenoni et al. (2016) [17] reported a decrease in the expression of lipoxygenase genes during withering of Corvina, which could explain the decrease in C_6 alcohols observed here. However, other studies indicated an opposite trend [8,16], an increase in C_6 aldehydes and alcohols during postharvest grape dehydration of Malvasia grape was reported [8], suggesting that more complex patterns could occur.

Various terpenes were affected by withering, although trends varied depending on the specific molecule. The importance of monoterpene alcohols and cyclic terpenes to Corvina wines aroma was recently described, in particular for linalool [34,35]. In the present study, linalool was the main monoterpene alcohol detected and its concentration was significantly decreased by withering, in agreement with previous findings [17,36]. Considering that in control wines linalool had an OAV

= 1.1, a possible contribution to wine aroma characteristics can be expected, whereas in wines from withered grapes this was not the case (OAV = 0.59 and 0.66 for fruttaio and on-vine respectively). Terpenes are produced in grapes through both the 1-deoxy-D-xylulose-5-phosphate/methylerythritol phosphate (DOXP/MEP) pathway and the mevalonic acid (MVA) pathway. In Corvina the influence of withering on these pathways is complex, with upper steps of the pathway being downregulated but late biosynthetic steps upregulated [16]. In addition to free forms of terpenes, grapes also contain non-volatile glycosylated forms of these compounds, which in Corvina can contribute significantly to terpenes level in finished through enzymatic and chemical hydrolysis during vinification [35,37]. Although in the present study differences in glycosidically-bound terpenes in the finished wines were relatively small, we observed generally higher concentrations of bound terpenes in withered wines (Table 2). Contrary to linalool, citronellol, the second most abundant monoterpene alcohols detected, increased with withering. In non-aromatic grapes such as Corvina, formation of citronellol is connected to the ability of yeast to reduce available geraniol including the portion derived from hydrolysis of geraniol glyosidic precursors [37,38]. Bound geraniol in finished wines increased in one of the withering modalities, supporting a possible contribution of bound geraniol to free citronellol levels. In wines from withered Pinot noir, Moreno et al. (2008) [9] also observed increased wine citronellol content. Among other terpenes, withering was consistently associated with increased contents of linalool oxides, limonene and 1,4-cineole. Small increases in the content of terpinen-4-ol in wine were also observed with withering, in agreement with the observations of Zenoni et al. (2016) [17]. The contribution of linalool oxides, limonene, 1,4-cineole and terpinen-4-ol to wine aroma seemed to be limited because their concentrations were found to be lower than the respective odor thresholds.

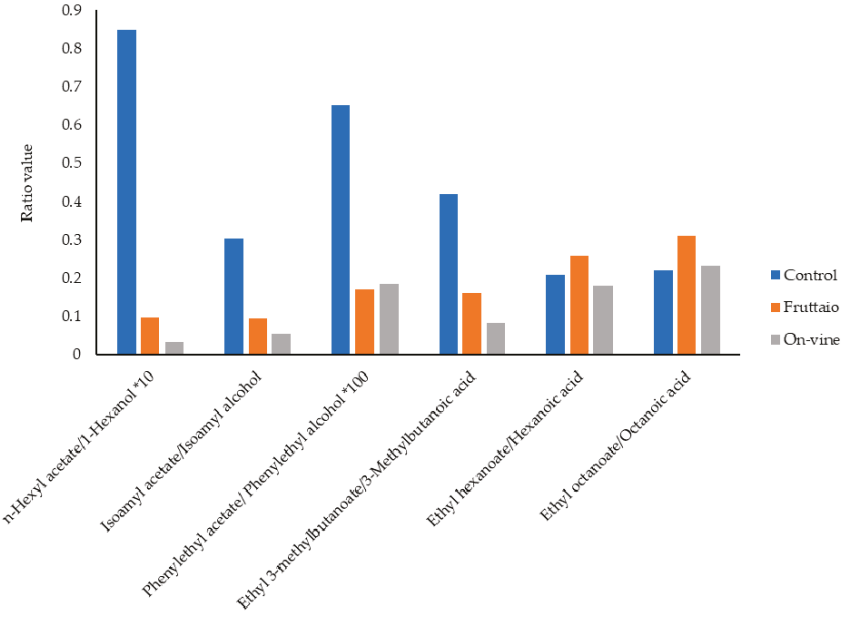

Figure 3. Ratio between esters and related precursors.

Comparted to terpenes, norisoprenoids, were affected to a smaller extent by withering, with concentration of the potent odorant β-damascenone decreasing. The formation of this compound during winemaking is associated with multiple pathways involving acid- or yeast-mediated hydrolysis of different precursors [39,40]. The negative influence of withering on damascenone wine content could be due to complex factors and requires further investigation, also considering that other

norisoprenoids such as 3-oxo-α-ionol had an opposite behavior and were found in higher concentrations in withered samples.

The benzenoids ethyl vanillate, and benzyl alcohol were found in higher concentrations in withered samples, unlike previously reports by Bellincontro et al. (2016) [4], albeit with more intense withering.

Considering that wines made from withered grapes are generally destined to age, the volatile profile evolution of withered and non-withered samples was investigated by means of a model aging protocol [34]. Data showed that after model aging, the three sample modalities showed differences for a smaller number of compounds compared to young wines. Variations in compound concentrations are reported in Table 5. Esters remained one major factor discriminating wines from withered grapes after aging (Table 3 and Figure 2), with control wines exhibiting higher levels of ethyl fatty acids and acetate esters. Trends during aging were, however, different, with withered wines typically showing reduced losses of even increases in some cases compared to control. Esters can be formed or degraded according to wine pH and the ester/acid ratio. As a consequence, esters produced in a higher amount by yeast during fermentation, such as isoamyl acetate, tend to decrease during aging while branched-chain fatty acid esters increase [41–43]. Considering that the pH of the different samples was similar, we can conclude that by reducing esters formation during fermentation, withering resulted in reduced ester losses. One exception to this was observed for ethyl hexanoate, for which the rate of hydrolysis was similar in all treatments.

Table 5. Composition of Corvina wines made from not withered grapes, withered on-vine and withered in "fruttaio".

	Brix	pH	Alcohol % vol
Control	21	2.96 ± 0.05	13.6 ± 0.2
On-vine	23.2	2.92 ± 0.08	15.0 ± 0.2
Fruttaio	23.2	3.03 ± 0.06	15.2 ± 0.2

Aging patterns of certain terpenes also showed differences that could be associated with withering. For example, linalool in control wines during aging decreased from being sensorially active (OAV = 1.1) to an OAV < 1. Instead, in withered wines, linalool concentration tended to increase with aging. Particularly in the Fruttaio samples, after aging linalool had an increase of 8.3 μg/L, 1.5 times higher than young Fruttaio wines. This increase could be due to a higher content of glycosylated precursors in withered samples. However, the analysis of bound compounds in young wines did not show significant differences between samples that could explain the observed differences. It may be that different precursors forms of linalool exist in our samples that were not quantified with the employed method. *cis*-Linalool oxide increased more markedly in wines from withered grapes, and this could be attributed to acid hydrolysis of glycosidic precursors (diendiol) [44], and 3,7-dimethyloct-1-ene-3,6,7-triol (triol) [45]. This last pathway seemed more consistent in this sample set, as both *cis*- and *trans*-linalool oxide bound precursors did not decrease with aging. 1,8-Cineole also displayed substantially different behaviors during aging between wines from withered and non-withered grapes, with concentration increasing during aging only in withered wines. This could be due to the fact that young wines from withered grapes exhibited significantly higher content of tepinen-4-ol, which we have recently shown to be a precursor to 1,8-cineole in Corvina wines [34].

β-Damascenone evolution with aging also highlighted a major difference associated with withering. Evolution of β-damascenone in Corvina wines during aging is characterized by a complex trend with an initial increase followed by a decline [34], reflecting simultaneous release from precursors (until available) followed by degradation through various reactions [46,47]. In the present study, the concentration of β-damascenone after model aging remained stable in control wines, and increased in withered samples reaching, in the case of on-vine samples, the level of control (Figure 4). Samples withered in fruttaio showed the most important increase of β-damascenone.

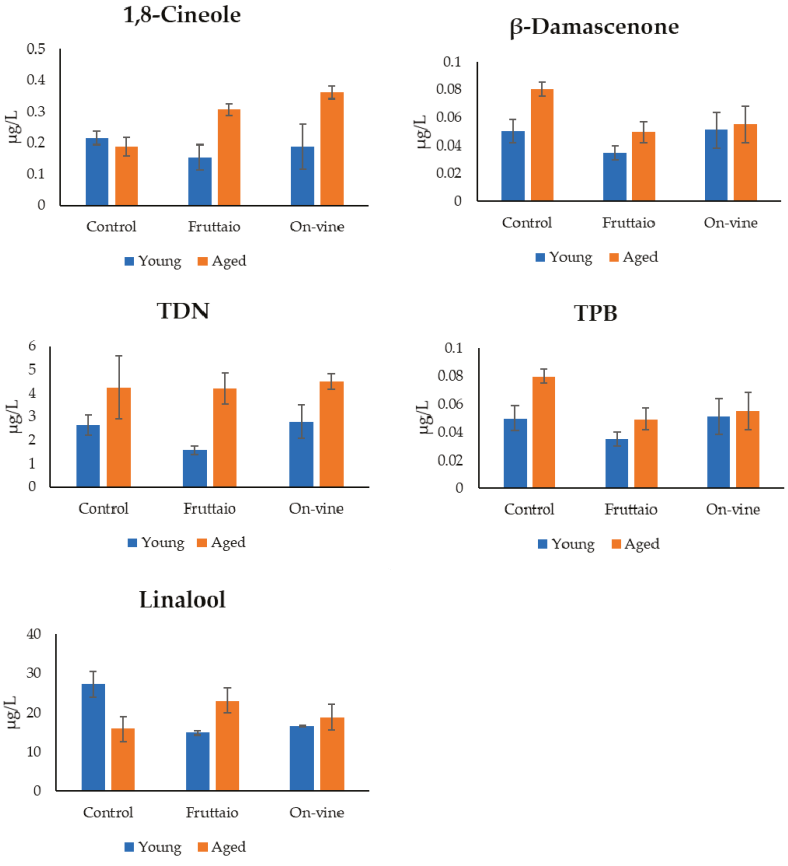

Figure 4. 1,8-Cineole, β-damascenone, (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB),1,1,6-Trimethyl-1,2-dihydronapthalene (TDN) and linalool content in young and model aged wines.

The (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB) also showed interesting differences in aged wines. A significant important increase has been observed after aging only in control samples. A slight increase occurred in samples withered in fruttaio while on-vine samples did not show any changes with aging. TPB has a tobacco aroma at low concentrations and geranium like odor at higher concentrations [48]. Its concentration in Corvina wine has been correlated with wine ageing [34]. It has been suggested that in red wine rich in tannins, TPB could react with polyphenols, resulting in a lower concentration like in Shiraz and Cabernet Sauvignon (Janusz 2003). It is reported that withered wines have a higher polyphenol content [10,12] while Corvina is known to be poor in polyphenols, so a TPB–tannin reaction may explain the lower content found in aged withered samples.

TDN variation was higher in fruttaio samples; however, it should be noticed that at the end of model aging samples of all the three modalities, it reached the same concentration level. TDN has a kerosene-like aroma, the concentration in wine was reported to be influenced by grape sun exposure, wine age, pH, and storage temperature [49,50]. Our data suggested that TDN concentration in wine was not affected by grape withering, the level of TDN formed in withered wine could depend on the TDN precursors accumulated just before the start of the withering process, harvest or peduncle twist.

The occurrence of aroma notes related to TDN is often associated with aged wines; however, in the control and on-vine samples, an OAV of 1.32 and 1.39, respectively, was observed already in the young wine, indicating a possible sensory contribution.

3.2. Influence of Withering Modality on Volatile Composition of Corvina Wine

In comparison with the differences due to withering, those associated with the withering modality were quantitatively less important and restricted to a small number of volatiles. In young wines, small but statistically significant increases in the concentration of different wine esters and citronellol were observed in fruttaio withering compared to on-vine withering, whereas linalool, β-damascenone, and α-ionol were mostly associated with on-vine withering. The trends observed for glycosylated volatiles were different, as in this case fruttaio wines exhibited a higher content of bound terpenes such as geraniol and nerol, whereas benzenoids and certain norisoprenoids were more abundant in on-vine withering. Several studies have investigated the influence of on-vine over-ripening or even on-vine withering on grape composition, but only in a few cases these were compared with other methods of withering. Zamboni et al. (2008) [16] provided interesting insights in differences existing at a transcriptomic level between on-vine and off-vine (fruttaio) withering of Corvina, indicating that differences in transcripts associated with secondary metabolites were minor [16]. However, on-vine withering did not involve any blockage of vascular tissues, so results are hard to compare with the present study.

Interestingly, some differences between the two withering modalities could be observed after aging. For example, the above-mentioned trend of reduced ester loss was lower in the case of on-vine withering, to the point that some esters actually increased during aging of wines from on-vine withering. Increases in certain grape-derived compounds were also dependent on withering, as in the case of 3-oxo-α-ionol, p-menthane-1,8-diol. Overall, it appeared that the two different withering conditions induced similar types of changes, mostly modulating the extent of such changes.

4. Materials and Methods

4.1. Chemicals

Octan-2-ol (97%), 1-hexanol (99%), *cis*-3-hexenol (98%), *trans*-3-hexenol (97%), vanillin (99%), 2,6-dimethoxyphenol (99%), linalool (97%), terpinen-4-ol (≥95%), α-terpineol (90%), nerol (≥97%), geraniol (98%), linalool oxide (≥97%), β-citronellol (95%), p-cymene (99%), terpinolene (≥85%), γ-terpinene (≥97%), limonene (97%), 1,8-cineole (99%), 1,4-cineole(≥98.5%), β-damascenone (≥98%), isoamyl alcohol (98%), benzyl alcohol (≥99%), 2-phenylethanol (≥99%), ethyl acetate (99%), ethyl butanoate (99%), ethyl 3-methyl butanoate ((≥98%), isoamyl acetate (≥95%), ethyl hexanoate (≥95%), phenylethyl acetate (99%), n-hexyl acetate (≥98%), ethyl lactate (≥98%), ethyl octanoate (≥98%), ethyl decanoate (≥98%), hexanoic acid (≥99%), octanoic acid (≥98%), α-phellandrene (95%), p-menthane-1,8-diol (97%), 3-methylbutanoic acid (99%), α-ionone (90%), 1-pentanol (99%), 1-butanol (≥99%), 2-butanol (≥99%), ethyl guaiacol (≥99%), vinyl guaiacol (≥98%), methyl-vanillate (99%) and ethyl vanillate (99%), were supplied by Sigma Aldrich (Milan, Italy). Dichloromethane (≥99.8%) and methanol (≥99.8%), were provided by Honeywell (Seelze, Germany). Sodium chloride (≥99.5%) was supplied by Sigma Aldrich (Milan, Italy).

4.2. Wine Samples

Wine samples were produced in the experimental facility of Masi Agricola. Corvina grapes from the 2017 vintage were obtained from a single 3 ha vineyard (45°29′22.9″ N 10°46′20.5″ E) located in the town of Lazise, 25 km west of Verona. The vineyard site was flat, with an altitude of 70 m asl. Vines had 12 years of age and were trained with a double arch cane system, with an average of 60,000 gems/ha and a yearly production of 11–12 tons/ha. Upon achievement of a sugar level of 21 Brix (27 of September), three experimental modalities were applied. Control grapes were hand harvested, placed in 7 kg harvest bins and transferred to the experimental winery where they were directly vinified as described later. A second batch, labelled "fruttaio", was harvested on the same day and the harvest bins were placed in a non-conditioned withering warehouse until November 4, when the berries had achieved a sugar content of 23.2 °Brix. Average conditions in the warehouse over the same period for

the previous 10 years indicated a gradual temperature decrease (from 16 °C to 7 °C) and a progressive increase in relative humidity (from 55% to 80%). A third modality, labelled "on-vine", was obtained by applying a peduncle twist in order to block vascular tissue and induce grape dehydration (Figure 5). Weather conditions in the vineyard area during the on-vine withering period were obtained from the Arpa Veneto meteorological database (http://www.arpa.veneto.it/). These conditions were in line with the typical conditions of the area, and were as follows: average daily minimum temperature of 7.5 °C), average daily maximum temperature of 20.6 °C, average daily mean temperature of 13.1 °C, average daily minimum relative humidity 44%, average daily maximum relative humidity of 97%, total precipitations 19 mm (1 rainy day). Upon achievement of a sugar content of 23.2 Brix (25 October), grapes were hand harvested as for the other modalities and were vinified. All vinifications were carried out in triplicate. For each vinification, 100 kg of grapes were destemmed and crushed, and the obtained musts were added with 100 mg/L of potassium metabilsulfite. Fermentations were conducted 75 L steel tanks by inoculation with the the proprietary *S. cerevisiae* yeast MASY03 (Microbion, Castel d'Azzano, Italy). At the end of fermentation, potassium metabisulphite was added in order to reach 30 mg/L of free SO_2, wines were then filtrated at 1 micron and bottled. Sample bottles were stored at 16 °C until analysis. Data concerning grapes at harvest and wine at bottling are summarized in Table 5.

Figure 5. Plier for the peduncle twist (**A**), and grape after peduncle twist (**B**). Photo courtesy of Masi.

4.3. Wines Model Aging

Model aging was carried out as described by Slaghenaufi et al. (2019) [35], by placing 115 mL of wine in glass vial and crimped leaving 0.8 mL of headspace corresponding to 2 mg/L of oxygen. Vials were then crimped and sealed with Araldite glue and stored at 40 °C for 12 weeks.

4.4. Volatile and Glycosidically-Bound Compound Analysis

Volatile and glycosidically-bound compounds have been analyzed as described by Slaghenaufi et al. (2019) [35] with minor modification. In total, 50 mL of sample was added with 20 µL of internal standard solution (2-octanol at 42 mg/L in ethanol) and diluted with 50 mL of distilled water. The solution was then loaded on a BOND ELUT-ENV, SPE cartridge, containing 1 g of sorbent (Agilent Technologies, Santa Clara, CA, USA), previously activated with 20 mL of methanol and equilibrated with 20 mL of water. The cartridge was then washed with 15 mL of water. Free volatile compounds were eluted with 10 mL of dichloromethane, and then concentrated under gentle nitrogen stream to 200 µL prior to GC injection. Bound compounds were recovered with 20 mL of methanol. Methanol was then evaporated under vacuum. Bound compounds were then dissolved in 5 mL of citrate buffer (pH 5). were added to dissolve bound compounds to that 200 µL of an enzyme preparation AR2000 (DSM, Brussels, Belgium, prepared at 70 mg/mL in citrate buffer) were added and incubated at 37 °C for 24 h under shaking (150 rpm).

A calibration curve was prepared for each analyte using seven concentration points and three replicate solutions per point in model wine (12% *v/v* ethanol, 3.5 gr/L tartaric acid, pH 3.5) [51]. A total of 20 µL of internal standards 2-octanol (42 mg/L in ethanol), was added to the solution. SPE extraction

and GC-MS analysis were performed as described above for the samples. Calibration curves were obtained using Chemstation software (Agilent Technologies) by linear regression, plotting the response ratio (analyte peak area/internal standard peak area) against concentration ratio (analyte added concentration/internal standard concentration). Method characteristics are reported in Table 6. The 3-oxo-α-ionol analysis was semi-quantitative and they were expressed as μg/L of 2-octanol equivalent (internal standard) as for this compound no commercial standard was available.

Table 6. Retention indices, quantification ions of studied compounds.

	Method [1]	LRI [1]	Identification [2]	Quantitation Ion *m/z*	Qualifier Ions *m/z*	LOD (μg/L)	LOQ (μg/L)
1-Butanol	a	1159	RS	56	55	0.02	0.06
2-Butanol	a	1020	RS	59		0.20	0.6
1-Pentanol	a	1256	RS	55	56, 57, 70	0.04	0.11
Isoamyl alcohol	a	1220	RS	57	55, 56, 70	0.02	0.06
Phenylethyl Alcohols	a	1920	RS	91	65, 92, 122	1.95	5.84
1-Hexanol	a	1316	RS	56	55, 69	0.76	2.27
trans-3-Hexen-1-ol	a	1379	RS	67	55, 69, 82	0.40	1.21
cis-3-Hexen-1-ol	a	1391	RS	68	55, 69, 83	1.23	3.68
Ethyl acetate	b	895	RS	88	61, 70	0.5	1.58
Isoamyl acetate	a	1125	RS	70	55, 60, 87	0.03	0.1
n-Hexyl acetate	a	1271	RS	56	55, 61, 84	0.03	0.1
Ethyl 3-methyl butanoate	a	1069	RS	88	57, 60, 85	0.30	0.9
Ethyl butanoate	a	1032	RS	71	88	0.01	0.04
Ethyl hexanoate	a	1240	RS	88	60, 99	5.82	17.47
Ethyl octanoate	a	1430	RS	88	57, 100, 127	0.54	1.63
Ethyl decanoate	a	1640	RS	88	71, 101, 155	0.16	0.49
Ethyl lactate	a	1340	RS	75	88, 90	2.1	6.3
3-Methylbutanoic acid	a	1667	RS	60	87	0.17	0.52
Hexanoic acid	a	1839	RS	60	73, 87	0.15	0.46
Octanoic acid	a	2071	RS	60	73, 101, 115	0.00	0.01
cis-Linalooloxide	b	1437	RS	59	111, 94	0.02	0.07
trans-Linalooloxide	b	1469	RS	59	111, 94	0.02	0.07
Linalool	b	1547	RS	71	121, 93	0.08	0.25
Geraniol	b	1860	RS	93	123, 121, 105	0.06	0.2
β-Citronellol	b	1771	RS	69	82, 81, 67	0.07	0.21
α-Terpineol	b	1701	RS	136	121, 93, 59	0.23	0.7
α-Phellandrene	b	1180	RS	93	136, 91	0.001	0.003
γ-Terpinen	b	1188	RS	121	93, 126	0.03	0.1
Limonene	b	1198	RS	136	139, 125, 111	0.03	0.1
1,4-Cineole	b	1186	RS	154	139, 111, 108	0.003	0.011
1,8-Cineole	b	1217	RS	154	139, 111, 108	0.003	0.011
p-Cymene	b	1271	RS	119	134, 91	0.02	0.06
Terpinolene	b	1283	RS	121	136, 93	0.03	0.09
Terpinen-1-ol	b	1581	LRI MS	136	121, 81	-	-
Terpinen-4-ol	b	1614	RS	71	111, 93, 86	0.02	0.05
p-Menthane-1,8-diol	a	2250	RS	96	88, 139	0.03	0.09
Ho-trienol	b	1585	LRI MS	82	67, 71	-	-
Nerol	b	1812	RS	93	121, 84, 69	0.04	0.12
β-Damascenone	b	1825	RS	69	190, 121, 105	0.01	0.03
α-Ionone	b	1853	RS	121	136, 192	0.02	0.06
α-Ionol	b	1925	RS	95	123, 138	0.04	0.12
3-Oxo-α-ionol	a	2555	LRI MS	108	152	-	-
Vitispirane	b	1523	LRI MS	192	177, 93	-	-
TPB	b	1828	LRI MS	172	157, 142	-	-
TDN	b	1745	LRI MS	157	172, 142	-	-
Benzyl Alcohols	a	1874	RS	106	105, 77, 51	0.03	0.1
Vanillin	a	2572	RS	151	81, 152, 109	0.01	0.02
4-Ethyl guaiacol	a	1988	RS	137	122, 152	0.03	0.09
4-Vinyl guaiacol	a	2212	RS	150	107, 135	0.07	0.21
Ethyl vanillate	a	2665	RS	151	168, 196	2.36	7.09
Methyl vanillate	a	2630	RS	151	123, 182	0.97	2.91
2,6-Dimethoxyphenol	a	2270	RS	154	95, 111, 139	0.01	0.03

[1] Extraction method: a (SPE) and b (SPME) [2] Linear Retention Index (LRI) were determined on DB-WAX polar column, as described by van Den Dool and Kratz (1963) [52]. RS identified using reference standard; LRI MS tentatively identified by comparing the Linear Retention Index and mass spectra with those of literature.

Terpenoids have been analyzed by SPME-GC-MS as described by Slaghenaufi and Ugliano (2018) [34]. In total, 5 mL of wine added with 5 µL of internal standard solution (octen-2-ol at 420 mg/L in ethanol) was placed into a 20 mL vial, together with 5 mL of mQ water (18.2 MΩ-cm) and 3 g of NaCl. The sample was equilibrated for 1 min at 40 °C. Subsequently SPME extraction was performed using a 50/30 µm divinylbenzene–carboxen–polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellafonte, PA, USA) exposed to sample headspace for 60 min at 40 °C. The fiber was then desorbed into the injector port of a HP 7890A (Agilent Technologies) gas chromatographer coupled to a 5977B mass spectrometer. Injection was performed at 250 °C for 5 min in splitless mode. Chromatographic separation was done using a DB-WAX capillary column (30 m × 0.25, 0.25 µm film thickness, Agilent Technologies). Helium was used as carrier gas at 1.2 mL/min of constant flow rate. The temperature of the GC oven was initially kept at 40 °C for 3 min, and then programmed to raise at 230 °C at 4 °C/min, maintained for 20 min. Mass spectrometer operated in electron ionization (EI) at 70 eV with ion source temperature at 250 °C and quadrupole temperature at 150 °C. Acquisition was done in Selected Ion Monitoring (SIM). Quantification was performed using calibration curve obtained by standards addition at 7 different concentration levels in Corvina wine. A total of 5 µL of internal standards 2-octanol (420 mg/L in Ethanol), 5 mL of water and 3 g of NaCl were added to 5 mL of standard solutions. GC-MS analysis was performed as described above for the samples. Linear term for calibration curves were obtained using Chemstation software (Agilent Technologies) by linear regression, plotting the response ratio (analyte peak area/internal standard peak area) against concentration ratio (analyte added concentration/internal standard concentration). The analysis of vitispirane, terpinen-1-ol, TPB, TDN, and ho-trienol was semiquantitative as no standards was available. Results for these molecules were expressed as µg/L of 2-octanol equivalent (internal standard) (Table 6).

4.5. Statistic

Data treatment, ANOVA, Tukey post-hoc test and PCA were performed using XLSTAT 2017 (Addinsoft SARL, Paris, France).

5. Conclusions

The present study allowed to characterize the influence of post-harvest withering of Corvina grapes on the aroma profile of wines. The aromatic contribution given by the withering on-vine or in a traditional withering warehouse (fruttaio) was also been evaluated.

Withering resulted in a lower content in fermentation-derived volatiles such as esters, higher alcohols, and acids, as well as grape-related compounds such as C_6 alcohols, and certain norisoprenoids like β-damascenone. Terpenes showed different behaviors according to the compound. Linalool, the major terpene found in the sample wines analyzed, and *cis*-linalool oxide were negatively influenced by the withering process while β-citronellol and 1,4-cineole showed a different trend and it was found in higher concentrations in withered samples. The same trend was observed for ethyl acetate, ethyl vanillate, benzyl alcohol and vanillin

The aroma profile of wines obtained by whitering in fruttaio was characterized by higher concentrations of esters such as ethyl acetate compared to on-vine withering. Wines withered in fruttaio were also distinguished by higher concentrations of β-citronellol and 3-oxo-α-ionol, while on-vine withering showed higher content of β-damascenone. The withering process as well as the technique employed also influenced the behavior of compounds during aging, showing different variation.

Overall, the results of the present study indicate that on-vine withering with blocked xylem is an interesting alternative to conventional fruttaio withering for the production of wines where a mild withering is requested. Although on-vine withering can only be carried out in years where climatic conditions are suitable, the possibility to explore this kind of withering technique is of interest to reduce the workload of fruttaio facilities and the energy cost associated with their functioning, reducing the environmental impact of the winemaking process.

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Sample Availability: Samples are not available from the authors.

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Characterization of Volatile Component Changes in Jujube Fruits during Cold Storage by Using Headspace-Gas Chromatography-Ion Mobility Spectrometry

Lvzhu Yang [1,2], Jie Liu [1,2], Xinyu Wang [1,2], Rongrong Wang [3,*], Fang Ren [4], Qun Zhang [2], Yang Shan [2] and Shenghua Ding [1,2,*]

1 Longping Branch Graduate School, Hunan University, Changsha 410125, China; ylzhu0115@163.com (L.Y.); jiel95712@163.com (J.L.); wxy25994@163.com (X.W.)
2 Provincial Key Laboratory for Fruits and Vegetables Storage Processing and Quality Safety, Agricultural Product Processing Institute, Hunan Academy of Agricultural Sciences, Changsha 410125, China; zqun208@163.com (Q.Z.); sy6302@sohu.com (Y.S.)
3 College of Food Science and Technology, Hunan Agricultural University, Changsha 410128, China
4 G.A.S. Department of Shandong Hanon Science Instrument Co., Ltd., Jinan 253000, China; amanda@hanon.cc
* Correspondence: sdauwrr@163.com (R.W.); shhding@hotmail.com (S.D.); Tel.: +86-731-84617093 (R.W.); +86-731-84691006 (S.D.)

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Abstract: Volatile components in jujube fruits from *Zizyphus jujuba* Mill. *cv*. Dongzao (DZ) and *Zizyphus jujuba* Mill. *cv*. Jinsixiaozao (JS) were analyzed under different cold storage periods via headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS). Results identified 53 peaks that corresponded to 47 compounds and were mostly alcohols, aldehydes, esters, and ketones. Differences in the volatile components of jujube fruits were revealed in topographic plots and fingerprints. For DZ, 3-pentanone was the characteristic component of fresh fruits. After storage for 15 days, dipropyl disulfide became the most special substance. Moreover, when stored for 30 and 45 days, the fruits had some same volatile components, like 2-pentyl furan and diallyl sulfide. However, for DZ stored for 60 days, esters were the prominent constituent of the volatile components, simultaneously, some new alcohols appeared. For JS, 2-ethyl furan was the representative of fresh fruits, and 2-butoxyethanol content was the most abundant after 15 and 30 days of storage. Different from that in DZ, the content of ester in JS increased after storage for 45 days. Substances such as amyl acetate dimer, methyl salicylate, and linalool greatly contributed to the jujube flavor during the late storage period. Principal component analysis (PCA) showed that fresh samples and refrigerated fruits were effectively distinguished. Heat map clustering analysis displayed the similarity of volatile components in different samples and was in accordance with PCA results. Hence, the volatile components of jujube fruits can be readily identified via HS-GC-IMS, and jujube fruits can be classified at different periods based on the difference of volatile components.

Keywords: jujube fruits; volatile components; headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS); cold storage; principal component analysis (PCA)

1. Introduction

Jujube (*Zizyphus jujuba* Mill) tree belongs to the Rhamnaceae family, which is indigenous to China and is distributed worldwide, in places such as Asia, northern Africa, southern Europe, the Middle East, and the southwestern USA. This tree has a history of more than 4000 years, with over

700 cultivars found in China [1–3]. Phytochemical analytical studies showed that jujube fruits are rich in nutrients, including fiber, sugars, organic acids, amino acids, vitamins, and trace minerals [4]. They also contain high levels of functional components, such as polysaccharides, triterpene acids, phenolics, and cyclic nucleotides, which exhibit multiple health-promoting properties, such as antioxidant and anti-inflammatory properties and liver protection [5–9]. Recognized for its delicious taste and health beneficial properties, jujube fruits have been consumed for thousands of years as ordinary fruits and Chinese traditional medicine.

In addition to their nutritional and biological activity, jujube fruits are favored as food by consumers due to their unique flavor. Fresh jujube fruits show an extraordinary flavor, but they are highly perishable when not handled properly due to their high moisture content, which leads to the loss of their commercial value. Cold storage is a common means of delaying the deterioration of quality and prolonging shelf-life effectively, which can affect the physicochemical properties of jujube fruits. Liu et al. found that the contents of chlorophyll, ascorbic acid, and soluble solids in fresh-cut jujubes without any treatment were reduced after storage at 4 °C for a certain period [10]. Kou et al. [11] reported that when jujube fruits were stored at 0 °C, the contents of total soluble solids, ascorbic acid, and chlorophylls continuously declined, and the anthocyanin content firstly increased and then decreased. Furthermore, Günther et al. [12] studied kiwifruit and found that cold storage also affected flavor. Simultaneously, changes in post-harvest metabolic and anaerobic environment greatly influence the fruit flavor, anaerobic conditions can enhance the flavor quality of fruits by producing certain aromatic compounds during storage [13]. Aroma profiles are important characteristics to evaluate the quality of fruits. Several studies have reported that the volatile compounds of jujube fruits are affected by many factors, such as growth period [14], extraction methods [15], the load [16], and processing methods [17–20]. However, few reports have been associated with changes in volatile compounds of jujube fruits during cold storage, which should be considered. The difference in volatile compounds among different periods of jujube fruit cold storage is unclear. Therefore, the purpose of this study was to monitor the changes in flavor and identify its characteristics at different times.

Ion mobility spectrometry (IMS) is an instrumental analytical technique of separating the ions of detected substances based on their ion mobility velocity under atmospheric pressure [21]. It is a convenient and efficient instrument with the advantages of simple sample preparation, easy operation, high sensitivity, and quick analytical speed. Even trace volatile compounds can also be detected in a short time [22,23]. Furthermore, ion mobility notably allows the separation of isomers and isobaric compounds, which cannot be separated even with ultra-high resolution instruments [24]. At first, compounds extracted from the sample enter the ionization chamber directed by a carrier gas, and the analyte is charged after being ionized. Then, a series of reactions occur and reactant ions $[H^+(H_2O)_n]$ are generated. In an IMS instrument, if the proton affinity of the analyte is higher than the proton affinity of water, it will react with the reactant ions. Based on the content of the analyte, their chemical nature or the drift tube temperature, product ions such as protonated monomers or proton-bound dimers were produced [25]. Subsequently, analyte ions enter the drift region through the Bradbury–Nielsen–Shutter. Analyte ions react against the reverse drift gas under the action of the electric field and migrate to the right end to reach the right detector, the drift velocity of ions depends on their charge, mass, and shape [26]. Finally, the Faraday–Plate detects ions and outputs electrical signals. The results are expressed in terms of voltage units. IMS has been used for chemical warfare agents [27], illicit drug detection [28], analysis of explosives [29], and environmental monitoring [30]. The instrument is highly sensitive to high electronegativity and high proton affinity compounds, which can detect a large number of compounds from different chemical families, such as alcohols, aldehydes, aromatics, esters, and ketones [30]. Combining IMS with other instruments is a good way to increase its advantages and produce a good analysis result. In recent years, HS-GC-IMS has been extensively applied to investigate volatile compounds in food science, such as *Tricholoma matsutake* Singer [22], jujube fruits [31], eggs [26], Iberian ham [32,33], and honey [25]. As a consequence, HS-GC-IMS could be used to identify the volatile components of jujube fruits at different periods.

In this study, HS-GC-IMS was used to analyze the variations in the volatile compositions of jujube fruits at different storage periods, and the fingerprints were established to confirm the characteristic substance of each period. The results will provide a new method for studying the flavor of jujube fruits, which will help to rapidly select the best storage time of jujube fruits.

2. Results and Discussion

2.1. Volatile Components Identification of All Samples at Different Storage Periods

The aromatic components of fruits undergo many complicated changes during storage, such as vitamin degradation and phenol oxidation, resulting in changes in fruit flavor. Rodrigo et al. [34] found that the aroma of peaches was related to storage conditions and fruit quality characteristics. As the refrigeration progressed, the aroma of peaches dropped considerably, and the flavor and fruit sweetness, juiciness, and texture were strongly correlated. In this study, the volatile components of jujube fruits at different storage periods were determined by HS-GC-IMS. The samples were ionized in the column and then identified using ion mobility systems based on retention and drift times. The qualitative analysis of volatile components in jujube fruits is shown by numbers in Figure 1, where the ordinate represented the retention time, and the abscissa represented the drift time. A total of fifty-three peaks, forty-seven components were identified from the GC×IMS library (Figure 1 and Table 1), including fourteen alcohols, six aldehydes, nine esters, six ketones, two organic acids, two furans, three pyrazines, four sulfur-containing compounds, linalool oxide, and 2-methoxy-4-cresol. Among them, sulfur-containing compounds, linalool oxide, and 2-methoxy-4-cresol were detected in jujube fruits for the first time. This finding may be ascribed to the differences in detection methods and essential differences of raw materials. The identified components are listed in Table 1, which includes the compound name, CAS number, molecular formula, molecular weight, retention index, retention time, and drift time. Moreover, other substances with signals were detected but could not be determined were not listed. When moving through the drift region, due to the formation of adducts between the analyzed ions and neutral molecules (such as dimers and trimers), multiple signals were observed for a single compound [35]. The compounds of 1-octen-3-one, 3-hydroxy-2-butanone, 1-pentanol, heptanal, amyl acetate, and ethyl propanoate exhibited two peaks due to the presence of both monomer and dimer.

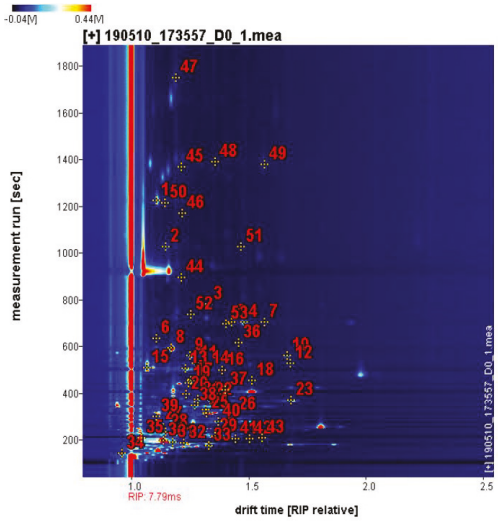

Figure 1. Ion migration spectra of jujube fruits stored for different times at 4 °C. The numbers are identified volatile components.

Table 1. The information on identified compounds of jujube fruits.

NO.	Compound	CAS#	Formula	MW	RI	Rt (Sec)	Dt (RIP Relative)	Comment	Identification Approach
1	Furfuryl alcohol	C98000	$C_5H_6O_2$	98.1	1661.4	1226.886	1.1116		RI, Dt
2	Octan-2-ol	C123966	$C_8H_{18}O$	130.2	1401.0	704.688	1.4313		RI, Dt
3	(*E*)-3-Hexen-1-ol	C928972	$C_6H_{12}O$	100.2	1327.8	562.241	1.2529		RI, Dt
4	2-Butoxyethanol	C111762	$C_6H_{14}O_2$	118.2	1400.9	704.400	1.5715		RI, Dt
5	2-Heptanol	C543497	$C_7H_{16}O$	116.2	1291.4	500.190	1.3920		RI, Dt
6	1-Pentanol	C71410	$C_5H_{12}O$	88.1	1262.0	456.411	1.2576	monomer	RI, Dt
7	1-Pentanol	C71410	$C_5H_{12}O$	114.2	1262.0	456.411	1.5164	dimer	RI, Dt
8	2-Methyl-1-butanol	C137326	$C_5H_{12}O$	88.1	1215.7	397.445	1.2367		RI, Dt
9	2-Hexanol	C626937	$C_6H_{14}O$	102.2	1194.9	374.160	1.2880		RI, Dt
10	3-Methyl-2-butanol	C598754	$C_5H_{12}O$	88.1	1124.2	306.504	1.4417		RI, Dt
11	2-Propanol	C67630	C_3H_8O	60.1	908.5	188.339	1.2264		RI, Dt
12	2-Methyl-1-propanol	C78831	$C_4H_{10}O$	74.1	1090.2	279.704	1.3727		RI, Dt
13	Linalool	C78706	$C_{10}H_{18}O$	154.3	1496.8	896.913	1.2161		RI, Dt
14	Citronellol	C106229	$C_{10}H_{20}O$	156.3	1742.8	1390.307	1.3610		RI, Dt
15	3-Octanol	C589980	$C_8H_{18}O$	130.2	1397.5	697.657	1.4073		RI, Dt
16	(*E*)-2-Octenal	C2548870	$C_8H_{14}O$	126.2	1438.5	779.930	1.3345		RI, Dt
17	(*E*)-2-Heptenal	C18829555	$C_7H_{12}O$	112.2	1326.9	560.684	1.6667		RI, Dt
18	Heptanal	C111717	$C_7H_{14}O$	114.2	1191.5	370.381	1.3412	monomer	RI, Dt
19	Heptanal	C111717	$C_7H_{14}O$	114.2	1193.2	372.270	1.6841	dimer	RI, Dt
20	3-Methylbutanal	C590863	$C_5H_{10}O$	86.1	917.4	190.741	1.1796		RI, Dt
21	(*E*)-2-undecenal	C53448070	$C_{11}H_{20}O$	168.3	1736.9	1378.477	1.5700		RI, Dt
22	5-Methylfurfural	C620020	$C_6H_6O_2$	110.1	1562.0	1027.52	1.4726		RI, Dt
23	1-Octen-3-one	C4312996	$C_8H_{14}O$	126.2	1308.2	527.716	1.2773	monomer	RI, Dt
24	1-Octen-3-one	C4312996	$C_8H_{14}O$	126.2	1308.2	527.716	1.6826	dimer	RI, Dt
25	1-Hydroxypropan-2-one	C116096	$C_3H_6O_2$	74.1	1295.3	506.307	1.2352		RI, Dt
26	3-Hydroxy-2-butanone	C513860	$C_4H_8O_2$	88.1	1298.6	511.779	1.0710	monomer	RI, Dt
27	3-Hydroxy-2-butanone	C513860	$C_4H_8O_2$	88.1	1295.5	506.629	1.3273	dimer	RI, Dt
28	2,3-Butanedione	C431038	$C_4H_6O_2$	86.1	1021.6	233.375	1.1507		RI, Dt
29	3-Pentanone	C96220	$C_5H_{10}O$	86.1	989.2	215.961	1.3600		RI, Dt
30	2-Methyl-3-heptanone	C13019200	$C_8H_{16}O$	128.2	1169.0	347.292	1.2730		RI, Dt
31	Ethyl octanoate	C106321	$C_{10}H_{20}O_2$	172.3	1401.0	704.688	1.4841		RI, Dt

Table 1. *Cont.*

NO.	Compound	CAS#	Formula	MW	RI	Rt (Sec)	Dt (RIP Relative)	Comment	Identification Approach
32	Amyl acetate	C628637	$C_7H_{14}O_2$	130.2	1150.4	329.460	1.3196	monomer	RI, Dt
33	Amyl acetate	C628637	$C_7H_{14}O_2$	130.2	1134.6	315.334	1.3240	dimer	RI, Dt
34	Ethyl propanoate	C105373	$C_5H_{10}O_2$	102.1	942.6	197.947	1.1418	monomer	RI, Dt
35	Ethyl acetate	C141786	$C_4H_8O_2$	88.1	866.3	176.930	1.3333		RI, Dt
36	Hexyl acetate	C142927	$C_8H_{16}O_2$	144.2	1230.8	415.502	1.4044		RI, Dt
37	Ethyl propanoate	C105373	$C_5H_{10}O_2$	102.1	964.6	205.502	1.4486	dimer	RI, Dt
38	Propyl acetate	C109604	$C_5H_{10}O_2$	102.1	968.8	207.090	1.5065		RI, Dt
39	Ethyl isobutanoate	C97621	$C_6H_{12}O_2$	116.2	970.1	207.619	1.5604		RI, Dt
40	Methyl salicylate	C119368	$C_8H_8O_3$	152.1	1732.1	1368.733	1.2162		RI, Dt
41	2-Methylpropanoic acid	C79312	$C_4H_8O_2$	88.1	1562.1	1027.802	1.1494		RI, Dt
42	2-Methylbutanoic acid	C116530	$C_5H_{10}O_2$	102.1	1632.9	1169.846	1.2188		RI, Dt
43	2-Ethylpyrazine	C13925003	$C_6H_8N_2$	108.1	1365.8	634.530	1.1116		RI, Dt
44	2-Ethyl-6-methylpyrazine	C13925036	$C_7H_{10}N_2$	122.2	1345.4	594.936	1.1737		RI, Dt
45	Acetylpyrazine	C22047252	$C_6H_6N_2O$	122.1	1654.4	1212.857	1.1463		RI, Dt
46	2-Pentyl furan	C3777693	$C_9H_{14}O$	130.2	1253.1	444.179	1.2477		RI, Dt
47	2-Ethylfuran	C3208160	C_6H_8O	96.1	975.6	209.900	1.0454		RI, Dt
48	Dimethyldisulphide	C624920	$C_2H_6S_2$	94.2	1074.6	268.202	1.1262		RI, Dt
49	Dimethyl sulfide	C75183	C_2H_6S	62.1	737.5	142.102	0.9636		RI, Dt
50	Dipropyl disulfide	C629196	$C_6H_{14}S_2$	150.3	1356.5	616.330	1.4603		RI, Dt
51	Diallyl sulfide	C592881	$C_6H_{10}S$	114.2	1118.4	301.687	1.1088		RI, Dt
52	Linalool oxide	C60047178	$C_{10}H_{18}O_2$	170.3	1417.2	737.207	1.2585		RI, Dt
53	2-Methoxy-4-cresol	C93516	$C_8H_{10}O_2$	138.2	1921.7	1749.150	1.1921		RI, Dt

MW: molecular mass; RI: retention index; Rt: retention time; Dt: drift time.

2.2. Differential Analysis of the Topographic Plots of Volatile Components in Jujube Fruits at Different Storage Periods

For an intuitive observation and comparison, topographic plots were used to characterize the substances of different jujube fruits during storage. From the 3D topographic plot (Figure 2), it can be clearly observed that with prolonged storage periods, the content of some compounds decreased, and new substances were formed. For DZ, some of the volatile components in the yellow circles disappeared after 15 days of storage but new peaks appeared after 60 days. Moreover, new peaks with high retention time were found after 45 days of storage, as indicated by the black circles (Figure 2A). For JS, more new peaks appeared when the storage time was extended to 45 days (Figure 2B). Hence, compared with fresh jujube fruits, volatile components were formed during the storage process [36]. During fruit ripening, the production of aroma volatiles (especially esters) was regulated by ethylene signaling pathways. Moreover, the production of volatile aroma components was strongly hampered in ethylene-suppressed fruits [37]. It could be inferred that after a certain time of refrigeration, the increase in ethylene synthesis led to more aroma components.

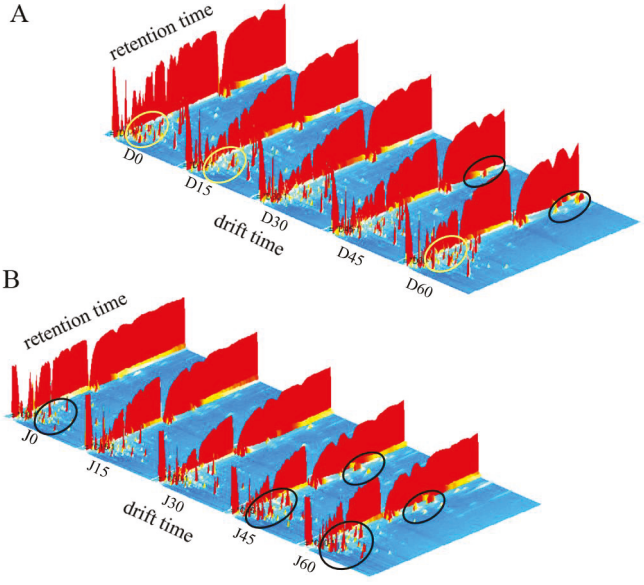

Figure 2. 3D-topographic plots of jujube fruits at different times. Jujube fruits are Dongzao shown in (**A**), D0, D15, D30, D45, and D60 represent refrigeration for 0, 15, 30, 45, and 60 days, respectively. Jujube fruits are Jinsixiaozao shown in (**B**), J0, J15, J30, J45, and J60 represent refrigeration for 0, 15, 30, 45, and 60 days, respectively.

Considering that the 3D spectrum was rough, the overhead view was used for comparison, as represented in Figure 3. Different volatile organic components (VOCs) were different points in the picture, which was highly convenient for observation and analysis. The red vertical line at the abscissa was the reactive ion peak (RIP) at normalized drift times for DZ and JS of 7.79 and 7.81, respectively. The differential contrast model was used to compare the differences among the samples. The fresh sample was selected as the reference, and the spectrum of the other samples deducted the reference. The background after deduction was white showed that the VOCs were the same. Red spots indicate that the content of the substance was higher than the reference, and blue spots indicate that the content was lower than that of the reference. Compared with that of untreated fruits, more red spots are located in the retention time range of 900–1400 s of DZ after storage, and the VOCs changed

inconspicuously in the retention time range of 100–600 s at the topographic plot (Figure 3A). For JS, many red spots appeared, and the entire retention time was covered. Especially after 45 days, most of the signals were much higher than that of fresh fruits. This finding indicated that the VOCs of two species varied at different refrigeration times and were affected by variety when the fruits were under similar conditions. Further ripening of the jujube fruits during storage promoted the synthesis of certain volatile components. The formation of fruit flavor was a dynamic process in which the fruit continuously synthesized volatile aroma substances [38]. In the post-harvest ripening stage of bananas, the ester content in the yellow ripening period increased, and the alcohol content in the over-ripe period increased [39].

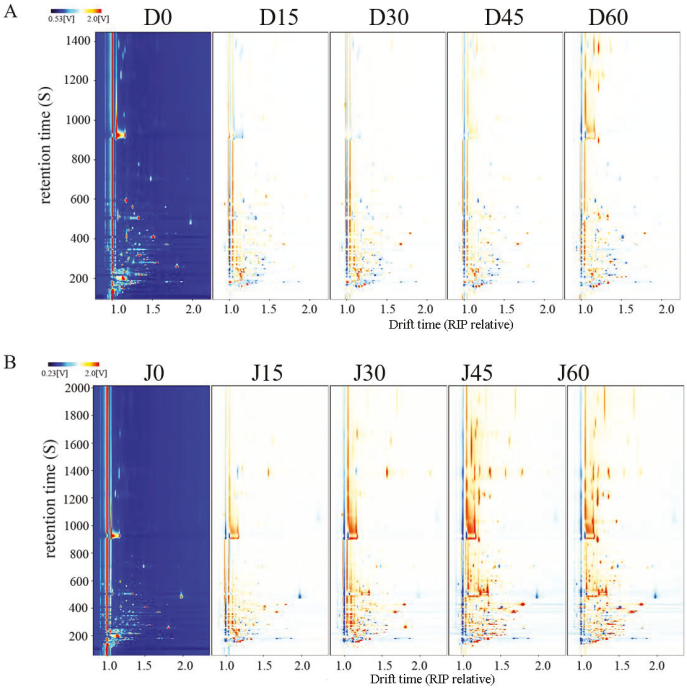

Figure 3. 2D-topographic plots of jujube fruits at different times. Jujube fruits are Dongzao shown in (**A**), D0, D15, D30, D45, and D60 represent refrigeration for 0, 15, 30, 45, and 60 days, respectively. Jujube fruits are Jinsixiaozao shown in (**B**), J0, J15, J30, J45, and J60 represent refrigeration for 0, 15, 30, 45, and 60 days, respectively.

2.3. Fingerprints of VOCs in Jujube Fruits at Different Storage Periods

Although the topographic plots showed the tendency of volatile components, it is difficult to make an accurate judgment for the dense material on the map. The use of fingerprint was a good way to solve this problem. According to the peak signal of the topographic plots, the fingerprints of jujube fruits were formed (Figures 4 and 5). In the fingerprints, each row represents the entire signal peak of one sample, and each column represents the same substance in different samples. Each cell represents the content of a substance at different times. Colors represent the content of volatile compounds. The brighter the color, the higher the content. Two compounds with the same name in the fingerprints were the monomers and their dimers. The drift time of dimers was increased due to their proton affinity and higher content [40]. By utilizing the fingerprints, the VOCs between different samples can be compared intuitively, moreover, the dynamic changes of each substance can be revealed. The unidentified substances are represented by numbers in the fingerprints. During the whole storage

period, the volatile components detected in JS were more than those in DZ, which may be caused by the differences in the varieties and genetic factors of the two jujube fruits.

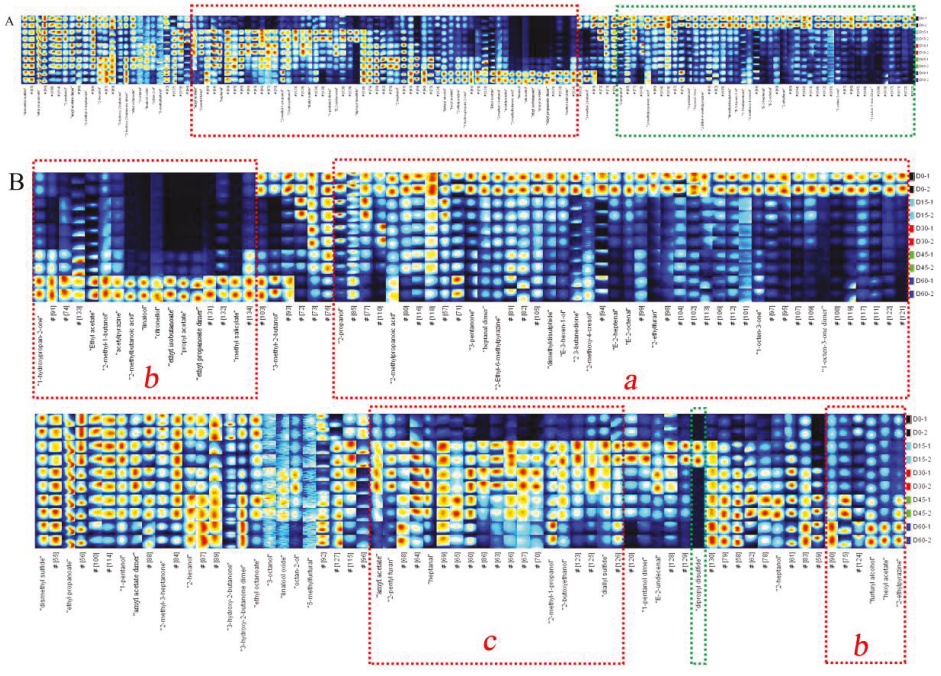

Figure 4. Fingerprint of volatile compounds of Dongzao samples. (**A**) Fingerprint of all Dongzao, (**B**) two parts of the fingerprint.

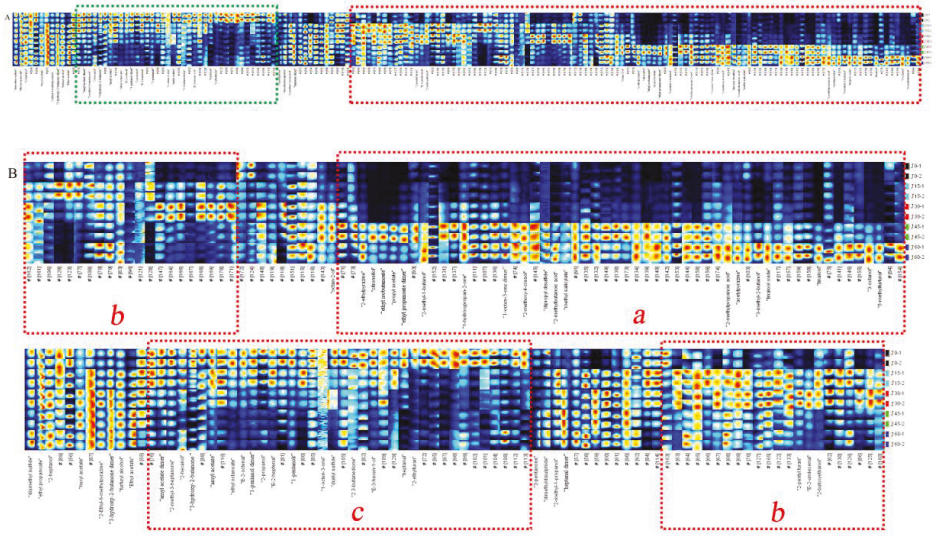

Figure 5. Fingerprint of volatile compounds of Jinsixiaozao samples. (**A**) Fingerprint of all Jinsixiaozao, (**B**) two parts of the fingerprint.

The VOCs of the jujube fruits constantly changed during storage. By comparing the intensity of spot for the profiles of VOCs at different stages, changes in the substances during storage (increased, decreased, disappeared or fluctuated) can be determined. Substances in the green-framed areas of Figures 4A and 5A were the most abundant in fresh jujube fruits but dramatically decreased or even disappeared during the later period of storage. For DZ, 2-propanol, 3-methyl-2-butanol, 3-pentanone, and heptanal dimer were detected, while amyl acetate, ethyl octanoate, (*E*)-2-octenal, and 1-pentanol dimers were detected in JS. Simultaneously, it can be clearly seen that, compared to other periods, the relative amount percentage of these substances was the highest in fresh fruits during the entire storage period (Table S1). This finding may be due to the degradation of these substances during refrigeration.

By contrast, with prolonged storage time, some new compounds appeared, which showed strong signal intensity and bright color (the red-framed areas of Figures 4A and 5A). These substances included linalool, ethyl isobutanoate, propyl acetate, ethyl ester, and methyl salicylate. This finding may be attributed to the complex physiological metabolism in fruits during storage, which mainly were a series of reactions, such as fatty acid, amino acid, and carbohydrate metabolism. Esters, which are mainly derived from the lipoxygenase pathway and amino acid metabolism are associated with the "fruity" attributes of fruit flavor, and its levels typically increase in the later periods of the ripening process [41]. Moreover, peaches stored after pre-storage were sweeter and had higher levels of propyl acetate, amyl acetate, and 2-methyl-1-butanol than control fruits [42]. The VOCs of the unframed part in the fingerprints presented few changes during the whole storage period, indicating that these substances were relatively stable during the cold storage of jujube fruits.

2.4. Changes of VOCs during Different Storage Periods

Changes in volatile components during different storage periods have been observed from the fingerprints. To compare the changes of each substance clearly, the fingerprint of each jujube fruit was divided into two parts based on the characteristic volatiles presented in different parts (Figures 4B and 5B). The representative VOCs detected in fresh DZ and JS were found in the region *a* of Figure 4B and region *c* of Figure 5B, respectively. The same ingredients included 1-octen-3-one, 2-ethylfuran, (*E*)-2-octenal, (*E*)-2-heptenal, 2,3-butanedione, (*E*)-3-hexen-1-ol, and heptanal. Chen et al. [43] studied ten different varieties of Chinese jujube fruits and found that aldehydes had the highest content and contributed to the aroma of fresh jujube fruits, moreover, (*E*)-2-hexenal, hexanal, (*Z*)-2-heptenal, benzaldehyde, and (*E*)-2-nonenal were the common volatile components in fresh jujube fruits. This finding differed from our results, presumably due to differences in varieties and detection methods. Heredity can determine precursors, enzymes, and their activity in the formation of flavor components in jujube fruits. Compared with fresh jujube fruits, these parameters decreased significantly after 15 days of storage in DZ and 30 days in JS. Aldehydes significantly affected the flavor of fresh jujube fruits, which generated from the oxidation of fatty acids and the metabolism of amino acids [44]. They can affect the overall aroma of samples at low content for their low odor threshold values [45]. (*E*)-2-Heptenal was characterized by soap and almond flavor, (*E*)-2-octenal was associated with roasted, cucumber, nutty, and fatty characteristics, and heptanal has a fishy, nutty and sweet apricot note flavor [46].

Many different changes of VOCs have been observed during storage. As shown in Figure 4B, the contents of 3-methyl-2-butanol, heptanal dimer, 2-ethyl-6-methylpyrazine, dimethyldisulphide, (*E*)-3-hexen-1-ol, (*E*)-2-octenal, (*E*)-2-heptenal, 2-ethylfuran, and 1-octen-3-one dimer in fresh DZ were much higher than that after storage. During storage, their contents continuously declined, (*E*)-2-octenal, (*E*)-2-heptenal, 2-ethylfuran, and 1-octen-3-one dimer vanished after storage. It showed that 2-methylpropanoic acid, 3-pentanone, 2,3-butanedione, 2-methoxy-4-cresol, and 1-octen-3-one were important for the flavor of fresh DZ. The contents of 3-pentanone and 2,3-butanedione were initially the highest in DZ, which decreased significantly after a certain period of storage and increased slightly after 60 days, while in JS, 3-pentanone with low content in fresh fruits. Low-carbon saturated ketones have a special aroma, and many ketones have been found in cheese, which are the main

volatile constituents of cheese with a unique flavor [47]. In addition, it can be observed that the change of 2-methylpropanoic acid was consistent with 3-pentanone and 2,3-butanedione. However, 2-methylbutanoic acid was different from them, it appeared with a strong signal at 60 days, which was not detected during the storage time of 0–45 days. Additionally, its relative amount percentage was up to 100% when stored for 60 days, before this period, this value was basically less than 10% (Table S1). Interestingly, only these two short-chain organic acids were identified in the two cultivars of jujube fruits. In JS, they appeared in storage for 45 days and then enhanced (Figure 5B). Organic acid is derived from the oxidation of fatty acids, which affects the flavor of jujube fruits [48]. During ripening, fruits undergo an esterification reaction, consuming a large amount of acid. When the jujube fruits are ripe, the amount of acids becomes low [49]. However, long-term storage resulted in the oxidation and decomposition of fatty acids. Besides, it should be noted that high content of organic acids can damage fruit quality and deteriorate the flavor of jujube fruits. In addition, processing methods also affect the content of organic acids. Chen et al. reported that acids were the major group among all the volatile chemicals in dried jujube fruits. Moreover, pre-treatment of jujube fruits with 5% CO_2 can induce a decrease in acid content during drying, which is mainly caused by a decrease in lauric acid and nutmeg acid content [18]. 2-Methoxy-4-cresol was a newly detected component in the VOCs of jujube fruits, which appeared with high content in fresh DZ but not found in fresh JS and showed a pleasant clove-like flavor [50]. For DZ, its signal intensity was almost invisible after storage for 15–30 days, then gradually strengthened. In JS, it appeared after 45 days with a bright color in the fingerprint plot.

Some volatile compounds, mostly including esters, were rare in fresh samples of two jujube fruits but abundant in the later storage period, which were the most important volatile components at the end of storage (Figures 4B and 5B), including hexyl acetate, ethyl acetate, ethyl propanoate, propyl acetate, ethyl isobutanoate, and methyl salicylate. Most of these compounds show a typical fruity or floral flavor, which may greatly affect the aroma of fruits. Zhou et al. found that low-temperature treatment could prevent the loss of aromatic esters during the ripening of 'Nanguo' pears at room temperature [51]. During the cold storage period, although complete ripening of the jujube fruits was inhibited, ripening proceeded at a slower pace, thus generating more esters. It can be found that the relative amount percentage of ethyl propanoate, propyl acetate, ethyl isobutanoate, and methyl salicylate was lower in the two cultivars of jujube at 0–30 days of storage and increased after 45 days. Moreover, it is worth noting that long-term storage at low temperatures inhibits the activity of enzymes, such as lipoxygenase and alcohol acyltransferase, which are the key enzymes in the aroma metabolism of fruits esters, resulting in a reduction in fruit flavor [52]. The jujube fruits in this investigation were refrigerated for 45–60 days, showed a good flavor. However, some substances had an adverse effect on the flavor of jujube fruits when the content was too high. The odor of high content of ethyl acetate was not described as "fruity" but rather as an off-flavor. Its elevated levels are usually associated with the over-ripeness and/or anaerobic metabolism of the fruit [53].

Two alcohols, including linalool and citronellol, showed the same variation tendency of the whole storage period, which was not detected at the initial stages of storage but appeared at a later time. For DZ they were detected with high signal intensity when the storage time was 60 days, but they appeared earlier in JS, showing a high content after 45 days of storage. This condition might have occurred because, with the prolongation of storage time, the glycoside precursors and other precursors for linalool and citronellol synthesis were formed in the jujube fruits. He et al. found that the content of linalool was closely related to the temperature of lemon-flavored hard tea during storage. At room temperature, its content increased slightly but decreased significantly when the temperature increased [54]. An interesting phenomenon was that the monoterpene alcohol of transgenic citrus peels could induce resistance against fungal invasion [55]. Therefore, whether jujube fruits were infected by fungi in the late storage period was uncertain, and the increased in linalool and citronellol content may be related to the self-defensive mechanism of fruits.

Meanwhile, 2-ethylpyrazine and acetylpyrazine with weak signal in fresh jujube fruits, and eye-catching signals have emerged in the fingerprints until storage for 45 days. Pyrazines were the

characteristic flavor of the Maillard reaction and were often found in dried jujube fruits, which show a roasted or nutty flavor [56]. During drying, heat treatment could accelerate the progress of the Maillard reaction, resulting in more furans and pyrazines were formed. In this study, jujube fruits were stored at low temperature, which did not provide a good condition for the formation of pyrazines compared with drying.

Some substances with the highest content during the middle of storage. Amyl acetate, 2-pentyl furan, 2-butoxyethanol, diallyl sulfide, heptanal, 1-pentanol dimer, and (*E*)-2-undecenal were the characteristic VOCs when DZ was stored at 4 °C for 15–30 days. Among these compounds, 2-pentyl furan, 2-butoxyethanol, and (*E*)-2-undecenal were the characteristic VOCs in JS in the same period. Heptanal, 2-methyl-1-propanol, and 2-butoxyethanol remained in DZ and were maintained at a high content during the subsequent storage period. In addition, the content of furfuryl alcohol continued to increase during storage, reaching a maximum of 60 days. Linalool and citronellol were synthesized in large quantities at the late storage stage. Most alcohols showed increased contents in the later stages, indicating that aldehydes were reduced and converted into corresponding alcohols at this stage, thus promoting the esterification of alcohols with acids produced by anaerobic respiration during storage. However, the signal intensity of 2-butoxyethanol disappeared later in JS, but the heptanal dimer was largely accumulated.

Dipropyl disulfide was only detected when the storage period was 15 days in DZ samples, but its content increased in JS after storage for 45 days. Three other sulfide compounds, namely, dimethyl sulfide, diallyl sulfide, and dimethyldisulphide were identified. The signal of dimethyl sulfide was observed in the whole storage process, and minor changes in signal intensity were observed in all samples. Diallyl sulfide and dimethyldisulphide were the characteristic VOCs of fresh JS and DZ, respectively. Sulfur-containing compounds are widely found in vegetables [57]. Diallyl sulfide was the characteristic flavor of garlic, dipropyl disulfide was associated with onion, and dimethyldisulphide was abundant in the volatile components of cabbage. Sulfur-containing compounds commonly arise from sulfur-bearing precursors, and in jujube fruits, sulfur-containing amino acids primarily exist, such as methionine. Low content of sulfur compounds can enhance the aromatic flavor of jujube fruits. Pyrazines and sulfur-containing compounds had low odor thresholds, and they acted with other compounds to enhance the overall aroma in jujube fruits [58,59]. Some substances, such as ethyl propanoate, amyl acetate dimer, 2-methyl-3-heptanone, and 3-hydroxy-2-butanone, existed in all periods of two jujube fruits, and the signal had minor changes.

2.5. PCA of Jujube Fruits at Different Storage Periods

PCA is a multivariate statistical analysis method that uses multiple variables to linearly transform to select a few significant variables. The main features are extracted for linear analysis by reducing the dimensionality of the data, and the main information is retained in several unrelated principal components [60]. Generally, when the cumulative contribution rate reaches 60%, PCA model is selected as the separation model [61]. Li et al. found the volatile components of different tissue parts of tomato showed disparate distributions among four varieties by PCA [62]. In this study, PCA was performed to analyzed the variation of 53 identified volatile compounds in the two cultivars of jujube. Firstly, all data were normalized to calculate the covariance matrix and its eigenvalues and eigenvectors, which derived from the corresponding peak area of each volatile component of jujube fruits. Then, the principal component was determined, and the corresponding contribution rate was calculated. Finally, a classification program was adopted in a smaller space to illustrate the relationship of jujube fruits during different storage periods. The results were shown in Figures 6 and 7, a clear separation trend of jujube fruits at different storage periods in two principal components can be observed.

As shown in Figures 6 and 7, the first principal component (PC1) and the second principal component (PC2) explained 82% and 84.6% of the total variables of the model in DZ and JS, respectively. A remarkable difference was found between fresh jujube fruits and refrigerated ones in volatile components. On the axis, a large distance is found between fresh DZ and the other samples, the four

other samples in different storage periods can be distinguished easily. Among them, the ones stored for 15 days and 30 days are closer (Figure 6A), which indicates that the volatile components of DZ in these two periods are similar. In JS, changes of the sample are distributed from right to left in the PCA, as the storage time was extended (Figure 7A). Jujube fruits stored for 0–30 days are distributed in the right quadrant, while the ones stored for 45–60 days are in the left quadrant with a higher similarity. Based on the PCA, the jujube fruits at different periods of two cultivars are separated well. Additionally, some information on the volatile compounds was lost during the statistical re-modeling. As shown in the loading plots (Figures 6B and 7B), the length of the arrow reflects the extent of information loss, the shorter the arrow, the more information lost [20]. For example, linalool oxide and 5-methyfurfural suffered the most with the loss of their information of DZ, but in JS, dimethyl sulfide loss was the highest.

To get more details, the biplots were used (Figures 6C and 7C). From the biplots, it can be clearly seen that 2-methoxy-4-cresol, heptanal and some ketones (1-octen-3-one and 2,3-butanedione) were positively related to the fresh DZ. However, for JS, 3-hydroxy-2-butanone contributed a lot to the flavor of the fresh fruits. When storage for 15 days, (*E*)-undecenal and 2-penty furanl were closely related to the DZ, 2-butoxyethanol was positively related to JS. In the biplots, the relationship between specific volatile components and jujube fruits in a certain period was demonstrated. While at the end of the storage, most esters were positively related to the jujube fruits, while 2-methylbutanoic acid and 2-methylpropanoic acid were found to be positively related to JS. These results were in accordance with the above fingerprints.

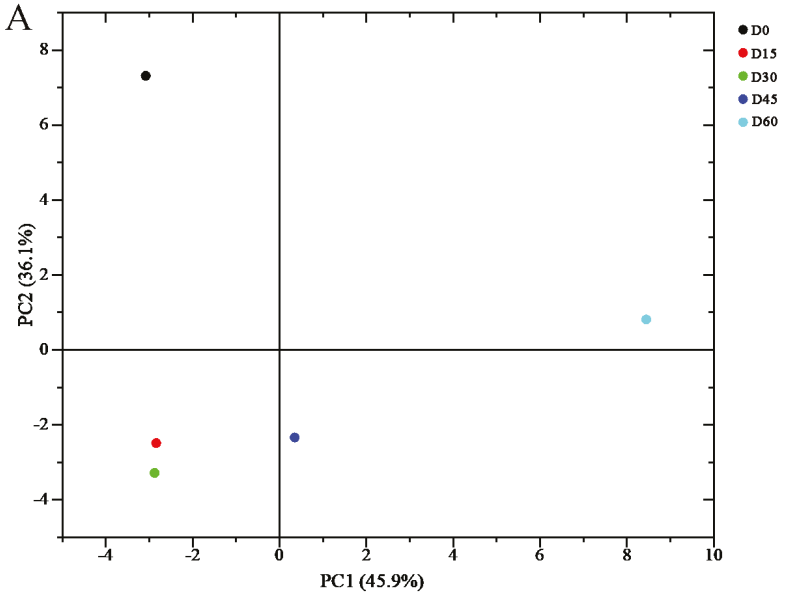

Figure 6. *Cont.*

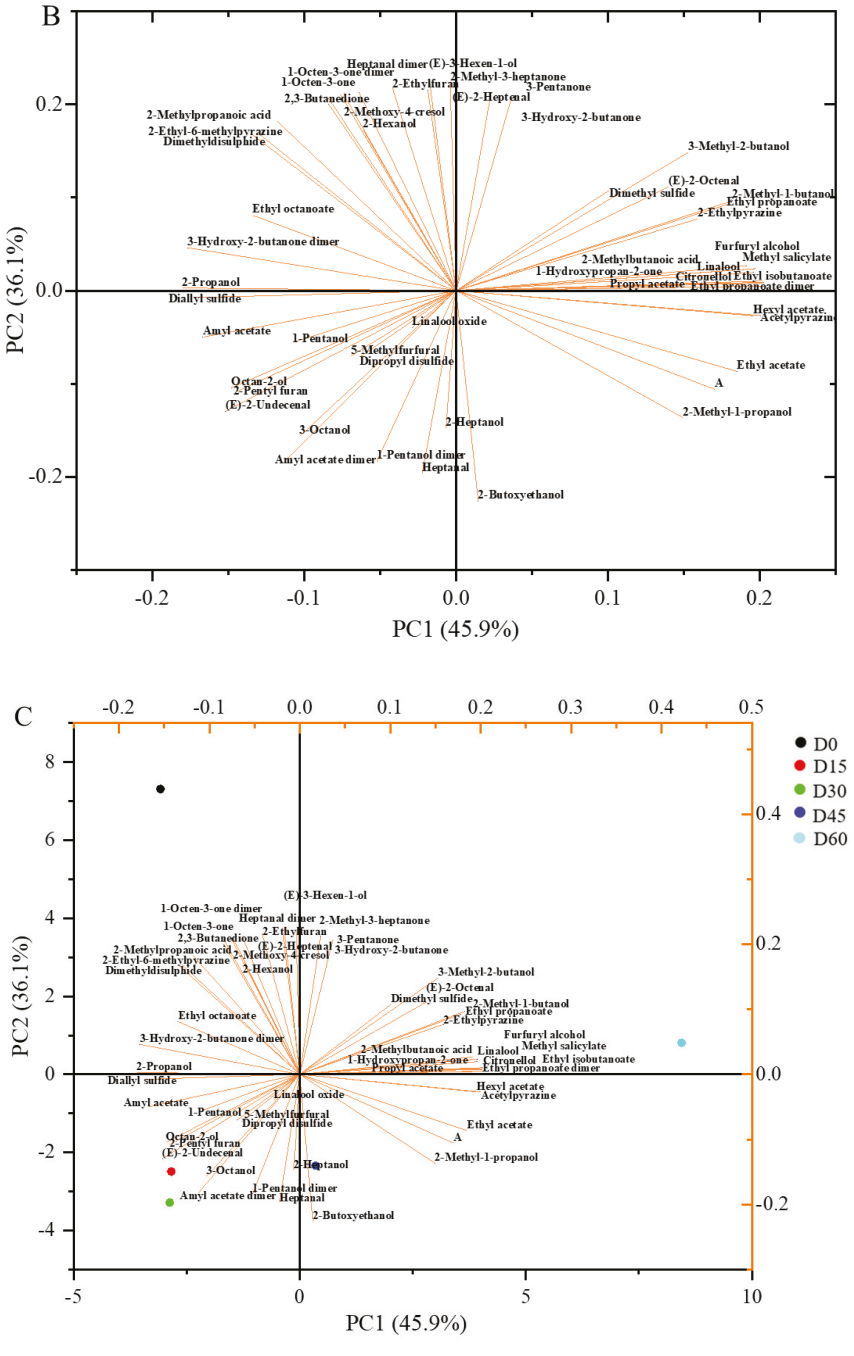

Figure 6. PCA analysis of Dongzao. (**A**) score plot of the first two principal components, D0, D15, D30, D45, and D60 represent refrigeration for 0, 15, 30, 45, and 60 days, respectively. (**B**) loading plot of different variances, (**C**) biplot of PCA.