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Novel Low-Alcohol Sangria-Type Wine Products with Immobilized Kefir Cultures and Essential Oils

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Abstract: Low-alcohol wines (ranging from <0.5 to 10.5% vol) are novel products that have been steadily gaining scientific and commercial attention. Over the past few years, consumer interest in healthier foods has augmented the development of novel functional products containing probiotic microorganisms, while the urge for a reduction in chemical preservatives has shifted the food and wine industry's interest to natural alternatives, such as essential oils (EOs). In the present study, low-alcohol (~6% vol) wines with (wet or dried) immobilized kefir cultures on fruit pieces, and essential oils (*Citrus medica* or *Cinnamomum zeylanicum*) were produced and evaluated for their properties. The viability of the immobilized kefir cultures on apple and pear pieces was not affected by the addition of EOs, and levels >7 logcfu/g were maintained after 2 h of immersion in wines. HS-SPME GC/MS analysis revealed characteristic compounds originating from the chemical composition of the added EOs in the final products. Principal component analysis (PCA) revealed that the relative content of terpenes, alcohols and carbonyl compounds played a major role in the discrimination of low-alcohol wine products. EO addition affected the products' sensory evaluation and resulted in significantly higher aroma and taste density compared to control samples. Notably, all novel Sangria-type wine variants were approved during preliminary sensory evaluation.

Keywords: low-alcohol wines; immobilized kefir cells; essential oils; *Citrus medica*; *Cinnamomum zeylanicum*; volatiles

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1. Introduction

In the past few years, low-alcohol wines (ranging from <0.5 to 10.5% vol) have been steadily gaining scientific and commercial attention, as they are preferred by consumers for a variety of reasons (healthier habits, modern lifestyle, economic motives, etc.) [1]. Typically, pre- and post-fermentation treatments are required for low-alcohol wine-making, but since their outcome is hard to predict, the use of kefir culture has recently been proposed [2,3]. Kefir culture is derived from a homemade, highly nutritional and healthy fermented milk product, in which a wide spectrum of yeasts, lactic acid and, occasionally, acetic acid bacteria co-exist [4], many species of which have previously been associated with probiotic or beneficial properties for the consumer [5,6]. For this reason, kefir beverages are considered to be of high added value and may be characterized as "functional foods", a term which includes a variety of products that may potentially positively affect body functions, reduce the risk of disease or promote well-being in general upon regular consumption [7]. However, in order to perform alcoholic and malolactic fermentation [3] or, ideally, positively affect the consumer's health [8], adequate numbers of kefir cultures are required. For this reason, immobilization strategies and the use of dried cultures are usually recommended, as they are associated with many advantages and offer compatibility with industrial and commercial needs [9,10].

Nevertheless, microbial spoilage may occur in wine at various pre- or post-fermentation stages and is usually caused by yeast (of the genera *Brettanomyces*, *Candida*, *Hanseniaspora*,

Pichia, *Metschnikowia*, *Saccharomycodes*, *Schizosaccharomyces* and *Zygosaccharomyces*) [11,12] and bacterial species (of the genera *Lactobacillus*, *Oenococcus*, *Pediococcus*, *Weissella*, *Acetobacter*, *Gluconobacter* and *Gluconoacetobacter*) [13,14]. The addition of chemical preservatives, especially sulphur dioxide (SO_2), is a common practice in wineries worldwide for preventing the growth of spoilage microorganisms and their negative actions [15–17]. However, the excessive use of SO_2 has raised a major concern, both from oenologists and the public, since it may negatively affect the wine's aroma [18,19] or even result in various adverse health effects [20–22].

As a result, in the past few years, increased attention has been drawn on the use of natural preservatives like essential oils (EOs) as antimicrobial and antioxidant agents in numerous food matrices [23–26]. In general, EOs are secondary metabolites synthesized by aromatic and medicinal plants. They are volatile, highly soluble in alcohol and organic solvents and consist of multiple chemical compounds, such as alcohols, esters, ketones, terpenes, terpenoids and phenolics, though their composition may vary depending on harvesting season, geographical region, oil extraction methods, etc. [27]. Cinnamon (*Cinnamomum zeylanicum*) EO from bark has revealed antibacterial [28,29], antidiabetic [30], antilipidemic [31], antioxidant [32] and anti-inflammatory actions [30], while, the biological activities of citron (*Citrus medica*) EO are similar [33–36]. Various studies have examined the suitability of these EOs for application in foods (such as meat products [37,38], yogurt [26,39], fruits [40–42], etc.) as natural antimicrobial and antioxidant compounds. Recently, their antimicrobial activity was evaluated in storage of low-alcohol wines and in deliberately contaminated wine products [28]. However, their effect on beneficial cell cultures, such as kefir, has not yet been examined.

Considering the above and following the increasing consumer interest in more natural and healthier food products, the potential of a new functional Sangria-type low-alcohol wine product containing immobilized kefir cultures on fruit pieces and EOs was investigated in the present study. Data indicating the effective survival of immobilized kefir culture (in counts capable of conferring a beneficial effect) and improved product quality are presented.

2. Materials and Methods

2.1. Kefir Culture Immobilization and Production of Dried Cultures

Kefir culture, previously isolated from a traditional kefir drink [2], was grown in diluted grape must of the Muscat Hamburg variety (Tyrnavos Cooperative Winery and Distillery, Tyrnavos, Greece) at 30 °C for 24 h. The medium consisted of ~30–35 g/L sugars supplemented with 0.1% *w*/*v* $MgSO_4 7H_2O$, 0.1% *w*/*v* $(NH_4)_2SO_4$, 0.2% *w*/*v* KH_2PO_4 and 0.4% *w*/*v* yeast extract. Prior to use, the pH was adjusted to 6.2 by adding 1N NaOH, and the medium was sterilized at 121 °C for 15 min.

For the immobilization process, kefir biomass was harvested by centrifugation (6000 rpm, 20 min, 4 °C), washed twice with sterile $^1/_4$ Ringer's solution (LabM, St. Albans, UK) and resuspended in the same solution. Apple or pear pieces (~575 g) were then submerged in 1 L of kefir culture solution for 10 h at room temperature, and the immobilized cells were washed twice with $^1/_4$ Ringer's solution. Immobilized thermally dried and freeze-dried kefir cultures on apple and pear pieces were produced, as recently described [2,3] and stored at 4 °C until further use.

2.2. Novel Low-Alcohol Sangria-Type Wines Preparation

Low-alcohol wine of ~6% vol was produced by free kefir culture as previously described [2], using diluted grape must of the Muscat Hamburg variety. After fermentation, wine was clarified by centrifugation (9000 rpm, 20 min, 4 °C). *C. medica* and *C. zeylanicum* EOs were then added separately (0.01% *v*/*v* of each oil) or in combination (0.005% *v*/*v* of each oil) into the low-alcohol wine samples. Wines with no EO addition were used as controls, and all samples were stored at 4 °C until further use. Immobilized wet or dried (thermally dried or freeze-dried) kefir cultures on apple or pear pieces (after rehydration)

were then introduced into the wines at a final concentration of ~30% *w*/*w*, resulting in novel Sangria-type products.

2.3. Microbial Enumeration

Due to the absence of chemical preservatives, all wine samples stored at 4 °C were initially checked for common wine-spoilage microorganisms (*O. oeni*, *P. pentosaceus*, *G. cerinus*, *D. bruxellensis*, *C. zemplinina*, *H. uvarum*, *P. guilliermondii* or *Z. bailii*), as previously described [28].

After preparation of the Sangria-type products, the survival of immobilized kefir cells was monitored at frequent intervals (0.5, 1 and 2 h). In brief, 5 g of immobilized cells were drained, blended with 45 mL of sterile $^{1}/_{4}$ Ringer's solution and subjected to serial dilutions; counts of total mesophilic flora were then determined on Plate Count Agar. Results were expressed as logcfu/g, and the viability of the cultures was calculated as recently reported [43].

2.4. Chemical Analyses

The pH and total and volatile acidity values of all products were determined after the addition of immobilized kefir cultures into the wine products, as previously reported [2].

Samples of novel low-alcohol wines (10 g) were analyzed for the content of minor volatiles using the HS-SPME GC/MS technique [6890N GC, 5973NetworkedMS MSD (Agilent Technologies, Santa Clara, CA, USA)], as previously described [44]. All analyses were carried out in triplicate, and the mean values are presented (standard deviation for all values was ±5%).

2.5. Preliminary Sensory Evaluation

Preliminary sensory evaluation for aroma, taste and overall acceptability of the new wine products with immobilized kefir cultures and EOs was performed by a mixed panel of random wine tasters [45]. The Sangria-type products were evaluated on a 0–5 scale; wine products with apple or pear pieces (no culture addition) were used as controls. Tasters were offered water and crackers to clean their palates between samples.

2.6. Statistical Analysis

Analysis of variance (ANOVA) was employed for determination of statistical differences of all treatments (state of immobilized kefir culture: wet/thermally dried/freeze-dried, immersion time in the final product: 0 h/1 h/2 h and EO enrichment: *C. medica*/*C. zeylanicum*/mix were considered as factors) and their interactions. All analyses were performed using Duncan's multiple range test with a 95% confidence level. The concentrations of minor volatile compounds (HS-SPME GC/MS) were used as variables in principal component analysis (PCA), and XLSTAT 2015.1 was used to compute the algorithm.

3. Results and Discussion

3.1. Viability of Immobilized Kefir Cultures in the Novel Sangria-Type Low-Alcohol Wines

Fruit pieces are known to be suitable immobilization carriers for kefir culture [44,46] and, as expected, total counts of wet immobilized kefir culture on apple and pear pieces exceeded 8 logcfu/g. Application of drying processes (thermal or freeze-drying), however, resulted in significantly ($p < 0.05$) reduced counts compared to the wet culture [2,3], but still >7.5 logcfu/g in all cases (data not shown). Reduced cell viability after a drying treatment is not uncommon [47–49], but cell immobilization is considered to be a promising strategy for the maintenance or even enhancement of kefir culture viability during processing and storage [2,3].

The development of new Sangria-type low-alcohol wines supplemented with EOs and immobilized kefir culture on fruit pieces presupposes the survival of kefir cells. For this reason, dried (thermally or freeze-dried) immobilized kefir cells (on apple or pear pieces) were directly immersed in low-alcohol wines (~6% vol) supplemented with EOs

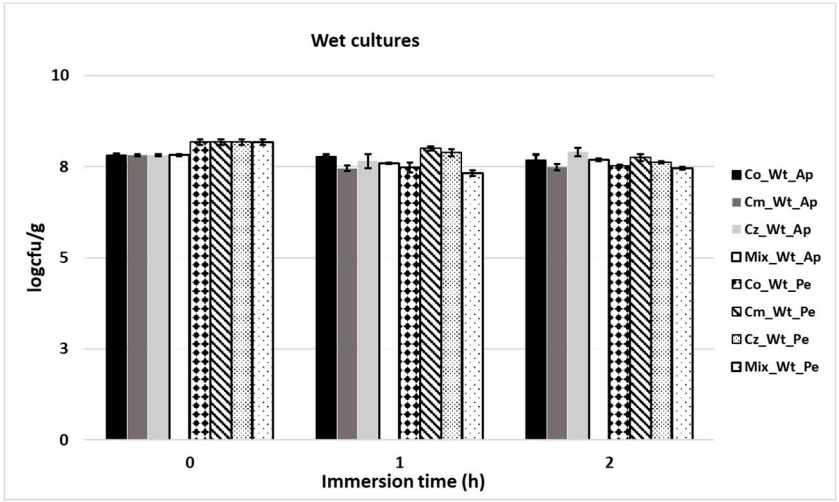
(0.01% *v*/*v* *C. zeylanicum*, 0.01% *C. medica* or a mixture of both at 0.005% each), at a final proportion of ~25% *w*/*w* in the reconstituted wine product, and their populations were monitored for 2 h. Likewise, wet immobilized kefir cells (on apple or pear pieces) were directly immersed in low-alcohol wines with EOs and served as controls. Results showed that the state of immobilized kefir culture (wet or dried) and the immersion time in EO-supplemented low-alcohol wines significantly affected ($p < 0.05$) kefir total counts, and a strong interaction ($p < 0.05$) among the factors was observed (Figures 1 and 2). Interestingly, and despite the documented antimicrobial action of *C. zeylanicum* and *C. medica* EOs [28], the viability of immobilized kefir culture after initial immersion (timepoint of 0 h) was not affected in most cases or resulted in a slight reduction compared to the initial populations. Adaptation and recovery of populations at a later storage stage [50] or even a count increase (up to 1 logcfu/g) have been documented [51]. That is a very important aspect of kefir culture's survival ability, as numerous benefits of kefir consumption have been related to its microbial composition [52–54]. The kefir culture used in this study was originally isolated from a homemade traditional kefir drink and is a part of the microbial collection of the Laboratory of Applied Microbiology and Biotechnology, Department of Molecular Biology and Genetics, Democritus University of Thrace (Alexandroupolis, Greece). Its microbial populations have been previously investigated with DNA next generation sequencing, and the existence of various species with potential health-promoting effects has been verified [2]. For instance, *L. kefiri* strains have been associated with mucus adhesion ability [55,56], in vivo cholesterol and triglyceride level reduction [57,58] and immunomodulation properties [59]. Additionally, *K. marxianus* strains are known to be associated with cholesterol-lowering effects, bile salt hydrolase activity [60] and immunomodulatory properties [61], while *K. lactis* and *S. cerevisiae* strains have been associated with promoting health and well-being, in general [62,63]. In the present study, kefir populations $>10^9$ cfu were established in a typical wine serving [64], thus achieving the minimum recommended cell concentration to confer beneficial health effects on the consumer [65].

As previously demonstrated, no food-spoilage microorganisms were detected in the low-alcohol wine samples used for the Sangria-type products, despite the absence of chemical preservatives after wine manufacture [28]. In general, it should be noted that various species present in kefir culture are known for their antimicrobial activity. Specifically, *L. kefiri* strains have been associated with protective or inhibitory effects against food-spoilage microorganisms and other pathogens (such as *Salmonella*, *E. coli*, *C. difficile*, etc.) [54]. Similarly, *Saccharomyces* and *Kluyveromyces* species (the most abundant kefir yeasts) are known for their antimicrobial and antioxidant properties [66]. The antimicrobial activity of various EOs against food-borne pathogens and food-spoilage microbes is also well documented [28,67–70]. Thus, the combined inhibitory effect of EOs along with kefir culture could be utilized for food microbial safety enhancement and shelf-life prolongation [71].

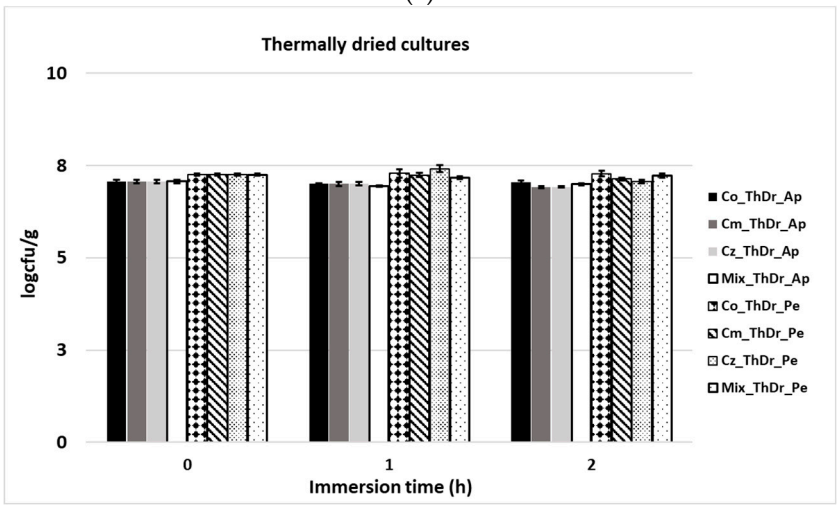
3.2. Chemical Analyses

3.2.1. pH and Acidity Values

In all cases examined, pH (3.9–4.0) was at a level typically observed in low-alcohol wines (Table 1). Volatile acidity was significantly affected ($p < 0.05$) by the state of the immobilized kefir cells (wet or dried cultures), and higher values were recorded in wine products with wet cultures (0.51–0.57 g acetic/L) compared to wine products with freeze-dried immobilized cultures (0.39–0.45 g acetic/L). Regarding wines with thermally dried immobilized kefir cultures, the volatile acidity ranged from 0.48 to 0.63 g acetic/L (depending on EO treatment applied), but remained within values commonly found in low-alcohol wine products [2,3]. Likewise, wine products with freeze-dried or thermally dried immobilized kefir cultures had increased total acidity values compared to wet cultures, but the increase was not significant in all cases and remained at typical levels for wines.



(**a**)



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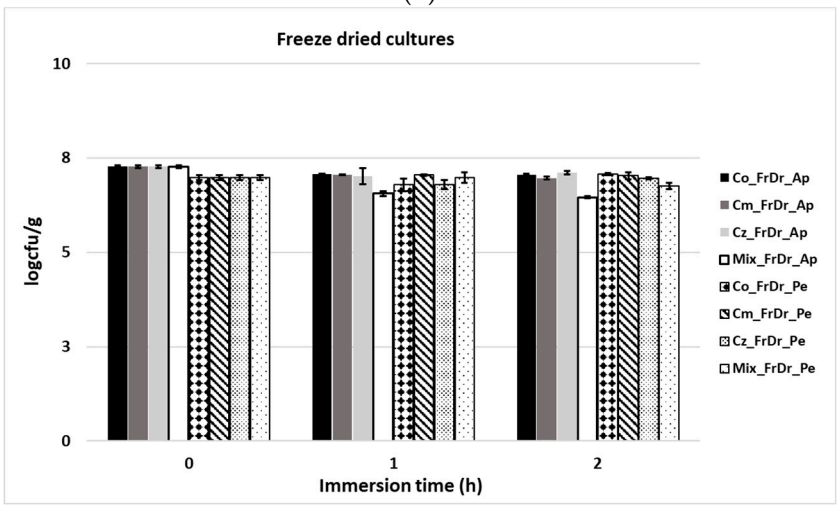

(**c**)

Figure 1. Total cell counts (logcfu/g) of (**a**) wet immobilized kefir cultures, (**b**) thermally dried immobilized kefir cultures, (**c**) freeze-dried immobilized kefir cultures in low-alcohol wine products supplemented with essential oils (EOs). Co: low-alcohol wines without EOs addition, Cm: low-alcohol wines with 0.01% *v/v Citrus medica* EO, Cz: low-alcohol wines with 0.01% *v/v Cinnamon zeylanicum* EO, Mix: low-alcohol wines with *Citrus medica* and *Cinnamon zeylanicum* EOs (0.005% each). The immobilization support is shown at the end of the sample codes. Ap: immobilized kefir cultures on apple pieces, Pe: immobilized kefir cultures on pear pieces.

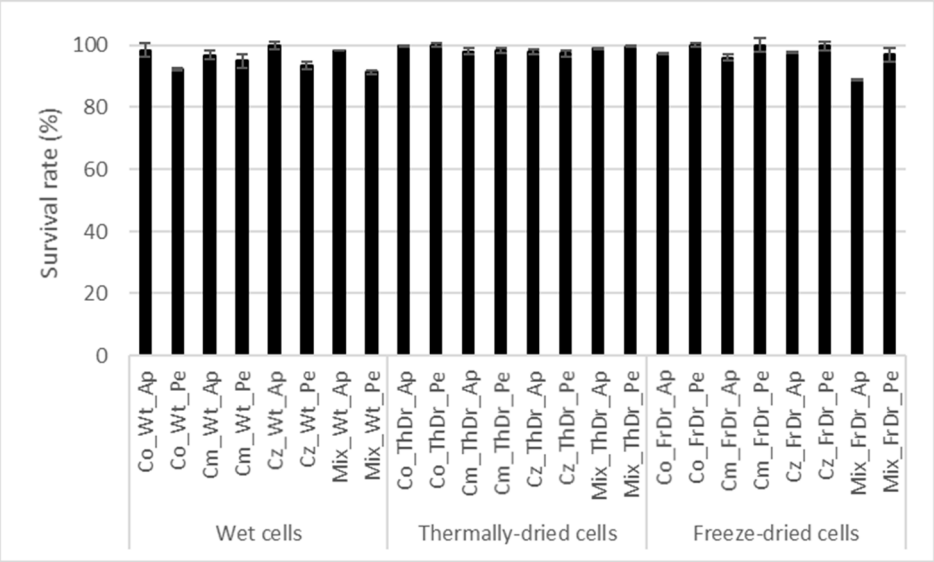

Figure 2. Survival rate (%) of wet, thermally dried and freeze-dried immobilized kefir cultures in low-alcohol wine products supplemented with essential oils (EOs) after 2 h of immersion. Co: low-alcohol wines without EOs addition, Cm: low-alcohol wines with 0.01% *v/v Citrus medica* EO, Cz: low-alcohol wines with 0.01% *v/v Cinnamon zeylanicum* EO, Mix: low-alcohol wines with *Citrus medica* and *Cinnamon zeylanicum* EOs (0.005% each). The immobilization support is shown at the end of the sample codes. Ap: immobilized kefir cultures on apple pieces, Pe: immobilized kefir cultures on pear pieces.

Table 1. Oenological parameters of low-alcohol wine products with essential oils and wet, thermally dried or freeze-dried immobilized kefir culture on fruit pieces.

	Wine Sample	Total Acidity (g Tartaric/L)	Volatile Acidity (g Acetic/L)	pH	Alcohol (% vol)
Wet kefir cultures	Co_Wt_Ap	2.6 ± 0.1	0.54 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Co_Wt_Pe	2.1 ± 0.1	0.51 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Cm_Wt_Ap	2.0 ± 0.1	0.57 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Cm_Wt_Pe	1.8 ± 0.1	0.51 ± 0.02	4.0 ± 0.1	6.0 ± 0.1
	Cz_Wt_Ap	2.1 ± 0.1	0.54 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Cz_Wt_Pe	2.3 ± 0.1	0.54 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Mix_Wt_Ap	2.6 ± 0.1	0.57 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Mix_Wt_Pe	2.0 ± 0.1	0.54 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
Thermally dried kefir cultures	Co_ThDr_Ap	2.3 ± 0.1	0.48 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Co_ThDr_Pe	2.6 ± 0.1	0.51 ± 0.02	4.0 ± 0.1	6.0 ± 0.1
	Cm_ThDr_Ap	3.0 ± 0.1	0.63 ± 0.03	3.9 ± 0.1	6.0 ± 0.1
	Cm_ThDr_Pe	2.0 ± 0.1	0.51 ± 0.02	4.0 ± 0.1	6.0 ± 0.1
	Cz_ThDr_Ap	2.9 ± 0.1	0.54 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Cz_ThDr_Pe	2.7 ± 0.1	0.57 ± 0.03	4.0 ± 0.1	6.0 ± 0.1
	Mix_ThDr_Ap	3.0 ± 0.1	0.57 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Mix_ThDr_Pe	2.3 ± 0.1	0.57 ± 0.03	4.0 ± 0.1	6.0 ± 0.1
Freeze-dried kefir cultures	Co_FrDr_Ap	2.7 ± 0.1	0.45 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Co_FrDr_Pe	2.4 ± 0.1	0.42 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Cm_FrDr_Ap	2.7 ± 0.1	0.39 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Cm_FrDr_Pe	2.1 ± 0.1	0.45 ± 0.03	4.0 ± 0.1	6.0 ± 0.1
	Cz_FrDr_Ap	2.7 ± 0.1	0.39 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Cz_FrDr_Pe	2.1 ± 0.1	0.45 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Mix_FrDr_Ap	2.6 ± 0.1	0.42 ± 0.02	3.9 ± 0.1	6.0 ± 0.1
	Mix_FrDr_Pe	2.1 ± 0.1	0.42 ± 0.02	4.0 ± 0.1	6.0 ± 0.1

Co: low-alcohol wines without EO addition, Cm: low-alcohol wines with 0.01% *v/v Citrus medica* EO, Cz: low-alcohol wines with 0.01% *v/v Cinnamon zeylanicum* EO, Mix: low-alcohol wines with *Citrus medica* and *Cinnamon zeylanicum* EOs (0.005% each). The immobilization support is shown at the end of the sample codes. Ap: immobilized kefir cultures on apple pieces, Pe: immobilized kefir cultures on pear pieces.

3.2.2. HS-SPME GC/MS Analyses

As the development of new foods and beverages with a unique sensory profile is an undeniable consumer desire, EOs are gaining attention for their organoleptic effect on food matrices. For that reason, the aromatic profile of new Sangria-type wine products with EOs and immobilized kefir cultures on fruit pieces (apple or pear) was analyzed using the HS-SPME GC/MS technique. In general, esters, organic acids, terpenes, alcohols and carbonyl compounds are considered important aroma and flavor constituents. In total, 99 volatile compounds were detected in samples supplemented with EOs (Supplementary Table S1), with the majority of them belonging to esters and terpenes. Noticeably, wine samples with no EO addition revealed similar volatile composition, as recently reported [2,3]. The relative content (%) of the volatile groups identified by HS-SPME GC/MS analysis is presented in Table 2.

Table 2. Relative percentage content (%) of the identified volatile compound groups in low-alcohol wine products with immobilized kefir cultures and essential oils, as detected by HS-SPME GC/MS analysis.

Essential Oil Addition	Samples	Compounds Detected					
		Esters	Acids	Terpenes	Alcohols	Carbonyls	Miscellaneous
Citrus medica (0.01% *v*/*v*)	Cm_Wt_Ap	8.4 ^{a}	0.3 ^{a}	89.1 ^{f}	2.1 ^{a}	<0.1 ^{a}	0.1 ^{a}
	Cm_ThDr_Ap	8.5 ^{a}	0.3 ^{a}	86.9 ^{f}	3.8 ^{a}	<0.1 ^{a}	0.4 ^{b}
	Cm_FrDr_Ap	8.5 ^{a}	0.3 ^{a}	87.7 ^{f}	3.4 ^{a}	<0.1 ^{a}	0.1 ^{a}
	Cm_Wt_Pe	4.9 ^{A}	0.2 ^{A}	92.2 ^{D}	2.5 ^{A}	<0.1 ^{A}	0.1 ^{A}
	Cm_ThDr_Pe	3.9 ^{A}	0.2 ^{A,B}	92.7 ^{D}	2.9 ^{A}	<0.1 ^{A}	0.2 ^{A}
	Cm_FrDr_Pe	12.3 ^{B}	0.3 ^{A,B}	84.6 ^{D}	2.5 ^{A}	<0.1 ^{A}	0.2 ^{A}
Cinnamon zeylanicum (0.01% *v*/*v*)	Cz_Wt_Ap	37.9 ^{c}	1.0 ^{b}	33.5 ^{c}	24.8 ^{e}	2.0 ^{b}	0.8 ^{c}
	Cz_ThDr_Ap	42.4 ^{c}	1.0 ^{b}	24.8 ^{b}	18.9 ^{d}	10.9 ^{d}	1.9 ^{f}
	Cz_FrDr_Ap	36.3 ^{c}	2.3 ^{c}	21.7 ^{b}	17.1 ^{d}	21.3 ^{f}	1.2 ^{e}
	Cz_Wt_Pe	30.8 ^{D}	1.2 ^{D}	34.1 ^{B}	29.1 ^{F}	3.3 ^{E}	1.5 ^{E}
	Cz_ThDr_Pe	29.8 ^{D}	1.8 ^{E}	34.4 ^{B}	25.3 ^{E}	5.9 ^{F}	2.8 ^{F}
	Cz_FrDr_Pe	37.6 ^{E}	1.0 ^{C,D}	33.3 ^{B}	16.6 ^{D}	10.3 ^{G}	1.2 ^{D}
EO Mix (0.005% *v*/*v* each)	Mix_Wt_Ap	23.1 ^{b}	0.4 ^{a}	68.1 ^{e}	7.4 ^{b}	0.7 ^{a}	0.4 ^{b}
	Mix_ThDr_Ap	19.0 ^{b}	0.9 ^{b}	65.8 ^{d,e}	7.4 ^{b}	6.0 ^{c}	1.0 ^{d}
	Mix_FrDr_Ap	19.7 ^{b}	1.0 ^{b}	58.8 ^{d}	7.5 ^{b}	12.7 ^{e}	0.4 ^{b}
	Mix_Wt_Pe	19.0 ^{C}	0.5 ^{A,B,C}	72.1 ^{C}	6.8 ^{B}	0.9 ^{B,C}	0.7 ^{B,C}
	Mix_ThDr_Pe	11.8 ^{B}	1.0 ^{C,D}	74.7 ^{C}	10.1 ^{C}	1.2 ^{C,D}	1.2 ^{D}
	Mix_FrDr_Pe	14.6 ^{B,C}	0.8 ^{B,C,D}	70.3 ^{C}	7.7 ^{B,C}	6.0 ^{F}	0.6 ^{B}
No EO addition	Co_Wt_Ap	68.7 ^{d}	4.3 ^{e}	2.9 ^{a}	23.7 ^{e}	0.1 ^{a}	0.4 ^{b}
	Co_ThDr_Ap	76.3 ^{e}	2.2 ^{c}	1.1 ^{a}	18.7 ^{d}	0.4 ^{a}	1.3 ^{e}
	Co_FrDr_Ap	82.1 ^{e}	3.6 ^{d}	1.8 ^{a}	11.4 ^{c}	0.2 ^{a}	0.9 ^{c}
	Co_Wt_Pe	72.5 ^{H}	2.3 ^{F}	8.2 ^{A}	15.7 ^{D}	0.4 ^{A,B}	0.8 ^{C}
	Co_ThDr_Pe	59.5 ^{G}	5.0 ^{G}	2.5 ^{A}	29.8 ^{F}	1.6 ^{D}	1.5 ^{E}
	Co_FrDr_Pe	45.3 ^{F}	10.7 ^{H}	5.6 ^{A}	38.1 ^{G}	0.1 ^{A}	0.2 ^{A}

Co: low-alcohol wines without EO addition, Cm: low-alcohol wines with 0.01% *v*/*v* *Citrus medica* EO, Cz: low-alcohol wines with 0.01% *v*/*v* *Cinnamon zeylanicum* EO, Mix: low-alcohol wines with *Citrus medica* and *Cinnamon zeylanicum* EOs (0.005% each). The state of the cells is shown in the middle of the sample codes (Wt: wet immobilized kefir cultures, ThDr: thermally dried immobilized kefir cultures, FrDr: freeze-dried immobilized kefir cultures). The immobilization support is shown at the end of the sample codes. Ap: immobilized kefir cultures on apple pieces, Pe: immobilized kefir cultures on pear pieces. $^{a–f}$ Significant differences ($p < 0.05$) in the same column between low-alcohol wines with kefir cells immobilized on apple pieces are shown with different letters. $^{A–H}$ Significant differences ($p < 0.05$) in the same column between low-alcohol wines with kefir cells immobilized on pear pieces are shown with different letters. Standard deviation for all values was about ±5%.

In low-alcohol wines with immobilized kefir cells on apple pieces, the state of kefir cultures (wet, thermally or freeze-dried) and the EO addition (*C. medica*, *C. zeylanicum*, EO mix or no EO) significantly affected ($p < 0.05$) organic acids, terpenes, alcohols, carbonyls and miscellaneous content. The content of esters, however, was affected only by EO addition, while strong interactions ($p < 0.05$) between the two factors were observed in

most cases. Similarly, esters, organic acids, alcohols, carbonyls and miscellaneous content in low-alcohol wines with immobilized kefir cells on pear pieces was significantly affected ($p < 0.05$) by both factors, while strong interactions ($p < 0.05$) were noted in all cases. The content of terpenes, on the other hand, was significantly affected ($p < 0.05$) only by the addition of EOs.

Esters are considered crucial aroma contributors, enhancing the aroma complexity of wines and mainly contributing pleasant fruity and floral notes [72]. In contrast to methyl esters, which are naturally present in grapes, ethyl esters are mainly secondary metabolites produced via the conjugation of alcohols and fatty acids during the alcoholic fermentation process, and most of them have low odor-detection thresholds, while they can mask the unpleasant aroma scents of fatty acids [73]. In the present study, 31 ester compounds were identified and, among them, ethyl octanoate (responsible for sweet, banana- and pear-like aroma notes), ethyl hexanoate (producing banana and apple aromas), 2-phenylethyl acetate (responsible for fruity rose aroma) [74] and ethyl 9-decenoate (providing quince-like and floral notes) [75] were the most abundant. Other esters adding fruity aromas, such as banana-, pineapple-, strawberry- and apple-like scents (isoamyl acetate, butyl acetate, ethyl propionate, hexyl acetate, ethyl butanoate, ethyl 2-methylbutyrate, ethyl heptanoate, ethyl nonanoate, ethyl decanoate), floral attributes (ethyl benzoate, ethyl tetradecanoate), oily and fatty notes (ethyl dodecanoate, ethyl linoleate), as well as creamy (ethyl hexadecanoate) or sweet, honey and floral scents (ethyl phenylacetate) were also detected in lower concentrations [72,74]. Additionally, cinnamyl acetate, benzyl benzoate and hydrocinnamyl acetate were present only in wine variants with *C. zeylanicum* EO, in line with its chemical composition [28,76], and these volatile odorants exhibit sweet, fruity, flowery and balsamic fragrances [77,78]. Other ester compounds linked with cinnamon EO addition in wines were benzyl acetate, ethyl benzoate, ethyl cinnamate, ethyl hydrocinnamate and isoeugenyl acetate, which are known for cinnamon-, sweet-balsamic, floral, fruity and honey-like odors [77,79], and their concentration was lower compared to the previously mentioned substances.

Terpenes and their derivative compounds were the most numerous and the most abundant identified volatile odorants, resulting in significantly ($p < 0.05$) higher content, especially in wine samples supplemented with *C. medica* EO (for both fruit carriers). Notably, 35 terpenes and derivative compounds were detected in wine samples with EO addition versus only 8 relative compounds in the control samples. In samples supplemented with 0.01% *v*/*v* *C. medica* EO, linalool was the most prevalent volatile compound, ranging from 70.9 to 80.9% (of the total volatile content) between different samples and contributing floral, citrus-like, sweet and muscat-like odors [74,77,78]. It was followed by ethyl octanoate (2.7–7.5%), geraniol (3.8–6.3%), nerol (1.0–2.2%), α-terpineol (1.5–2.5%) and limonene (0.6–2.2%), which also added mostly floral, sweet, citrus-like and green scents [74,78,80]. In contrast, apart from linalool, wines with *C. zeylanicum* EO addition were characterized by α-terpineol, caryophyllene oxide (revealing woody, spicy notes), caryophyllene (known for sweet, woody, green, spicy odor) and α-humulene (having woody odor) [78,79] in relatively high content. Other compounds also known to contribute woody (β-myrcene, δ-cadinene), fruity (β-phellandrene, α-terpinene, γ-terpinene, citral, α-farnesene, citral, (-)-spathulenole), floral (o-cymene, linalool oxide, (E)-nerolidol) or spicy notes (α-phellandrene, carvacrol) were also detected in small quantities [78,79,81,82].

Long-chain fatty acids are naturally present in grapes. They generally have high odor threshold values and thus present a modest contribution to the aroma of wines [77,83]. They are mainly produced from autoxidation of the saturated lipid constituents of fruits, while short-chain fatty acids are metabolism products produced by yeast and bacteria [74,77]. Despite their low impact on wine aroma, they are important precursor compounds for the formation of more powerful odorants, such as esters, aldehydes and alcohols [84]. Octanoic acid, known for its oily, fatty acid, rancid, soapy and cheesy odor character [74], was the major identified fatty acid and was present in all wine samples. Other fatty

acids presenting cheesy, vinegar, sour, fatty and rancid aromas (hexanoic, decanoic and hexadecanoic acid) [74,79] were also detected in lower concentrations.

Higher alcohols are also considered important wine flavor contributors and may be produced as byproducts of yeast alcoholic fermentation or synthesized through degradation of the relevant amino acids [85,86]. Higher alcohols (especially C6 and C9) usually generate herbal, strong and pungent odors, but at low concentrations (<300 mg/L), are associated with a wine's complexity, elegance and high quality [86]. In the present study, higher alcohols content ranged from 26.2 to 324.9 mg/L, and 2-phenylethanol (providing a characteristic floral, rose-, honey-like bouquet) [74] was present in all wine samples; its concentration varied (1.1–129.4 mg/L) depending on EO treatment. Likewise, isoamyl and amyl alcohols were also identified in all wine variants in high quantities, producing burnt, cocoa, floral, malty [78] and oily, sweet, dark chocolate and malty scents, respectively [79,87]. Noticeably, eugenol, cinnamyl alcohol and hydrocinnamyl alcohol were detected only in wines with cinnamon EO, revealing a sweet, spicy, clove-like (eugenol and hydrocinnamyl alcohol) or sweet, balsam, cinnamon-like (cinnamyl alcohol) fragrance [79,88]. Other alcohols responsible for fruity, creamy (2,3 butanediol), herbal, grassy, woody (1-hexanol), green, bitter, alcohol (isobutanol) or jasmine-, lemon-like (1-octanol) bouquets [74,79] were detected in most wine samples, but in low quantities.

In general, carbonyl compounds comprise the most abundant group of volatiles detected in red grape varieties [84]. According to the results, a few carbonyl compounds were detected in all samples, but significantly higher ($p < 0.05$) relative amounts were observed in wine variants containing *C. zeylanicum* EO compared to wines with no EO addition or wines with *C. medica* EO (for both fruit carriers). Among them, cinnamaldehyde and benzenepropanal were the major aldehydes, as they are important constituents in cinnamon oil [76]. Cinnamaldehyde has a sweet, spicy, cinnamon, honey-like odor contribution, and benzenepropanal has a honey-like, floral and roasted fragrance [79,87]. Other aldehydes, such as hexanal (fresh, green), nonanal (fatty, soapy, rose, floral, citrus-like), benzaldehyde (sweet, cherry, roasted, almond-like) and decanal (floral, citrus notes) [74,79,88,89] were also identified in low quantities.

A few miscellaneous compounds with minimal sensorial impact were also identified, in accordance with our previous studies in low-alcohol wines [2,3].

Each aromatic compound present in wine has its own odor threshold and, notably, most of them are usually found in concentrations below that value [90]. As a result, they do not solely influence the development of the product's fragrance [72] nor can the aroma be considered as the sum of contributions made by each individual compound [91]. Instead, the final aromatic bouquet relies on synergistic or additive or even suppressive interactions, depending on the compounds' presence and concentrations. Moreover, the alcohol content of the wine may alter the organoleptic perception of the product, while parameters, such as variations in an individual's perception or sensitivity should not be ignored [72,91].

3.2.3. Principal Component Analysis

As shown in Figure 3, PCA of minor volatiles detected by HS-SPME analysis revealed that the addition of EOs rather than the state of the kefir culture (wet or dried) had a significant effect. In particular, wine samples with 0.01% *v/v C. medica* EO, presenting a relatively high abundance of terpenes and their derivative compounds (Supplementary Figure S1), were clustered at the top left part of the plot. In contrast, wine samples with 0.01% *v/v C. zeylanicum* EO were characterized by a relatively high amount of alcohols and carbonyl compounds (Supplementary data), which correlated positively most with PC1. As a result, the mixed EOs addition resulted in a diagonal distribution, affected by both trends. All wine variants with no EO addition (controls) were clustered at the bottom left corner of the plot.

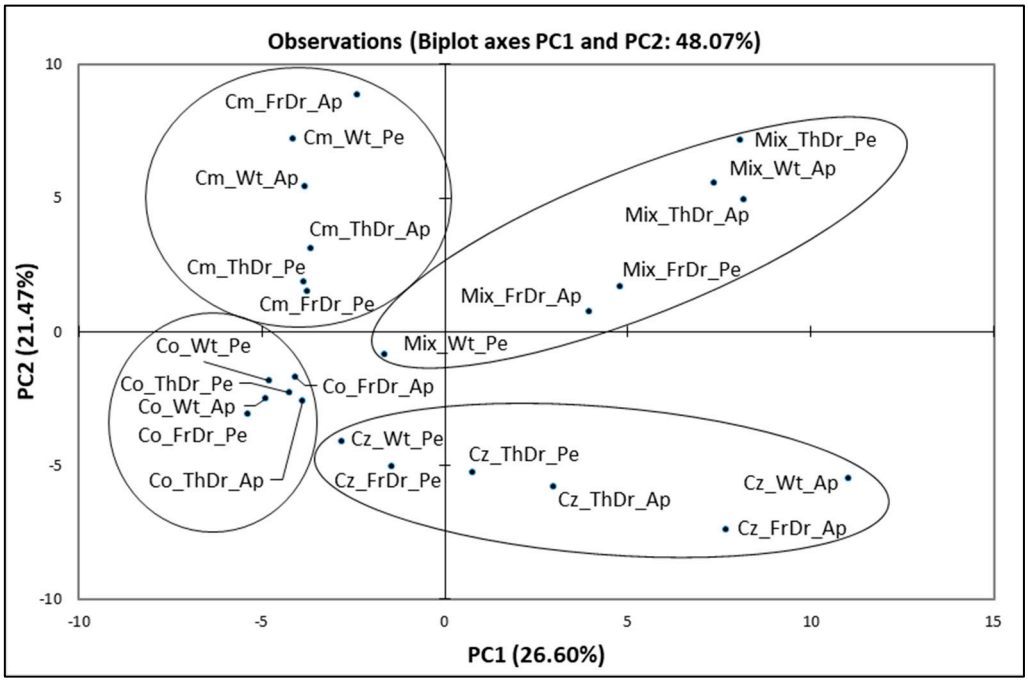

Figure 3. Score plot of the principal component analysis (PCA) of total identified minor volatiles in low-alcohol wine variants with essential oils (EOs) and immobilized kefir cultures. Co: low-alcohol wines without EOs addition, Cm: low-alcohol wines with 0.01% *v*/*v* *Citrus medica* EO, Cz: low-alcohol wines with 0.01% *v*/*v* *Cinnamon zeylanicum* EO, Mix: low-alcohol wines with *Citrus medica* and *Cinnamon zeylanicum* EOs (0.005% *v*/*v* each). The state of the cells is shown in the middle of the sample codes (Wt: wet immobilized kefir cultures, ThDr: thermally dried immobilized kefir cultures, FrDr: freeze-dried immobilized kefir cultures). The immobilization support is shown at the end of the sample codes (Ap: immobilized kefir cultures on apple pieces, Pe: immobilized kefir cultures on pear pieces).

3.3. Preliminary Sensory Evaluation

The sensory attributes (aroma, taste, overall evaluation) of the new Sangria-type wines represent important criteria for determining their acceptability by consumers. According to the results (Table 3), the aroma, taste density and overall evaluation were significantly ($p < 0.05$) affected by the addition of EOs, while the state of kefir cultures (wet, thermally or freeze-dried) had no effect on the scores. In particular, wines with EOs had significantly ($p < 0.05$) higher scores (3 to 4 rating) regarding aroma and taste density compared to the wines with no addition of EOs (control samples). The majority of wine variants supplemented with EOs were characterized by fruity and piquant scents, while the samples without EOs mostly had wine-like aromas. Concerning taste, addition of EOs resulted in a sweet/bitter taste in most wine variants, which, in the case of *C. medica* supplementation, was associated with an unpleasant aftertaste, while wines supplemented with *C. zeylanicum* EO were characterized by an enjoyable cola-like aftertaste. As for wines with no EOs added, the taste was slightly sour with a typical wine aftertaste. In contrast to the aroma and taste density, wines without EOs were highly graded regarding overall evaluation, probably because they more closely resembled the consumers' familiar perception of the typical Sangria wine [92]. Products with *C. zeylanicum* EO closely followed the tasters' preference, but samples with *C. medica* EO gathered mixed criticism, possibly due to the bitter notes detected upon tasting. After all, factors like the appearance (color, shape, etc.) and general expected perception of a product (e.g., Sangria wine) constitute the basic characteristics responsible for the identification and selection of the product and strongly affect concepts, such as the desirability and selection by consumers [93].

Table 3. Sensory evaluation of new Sangria-type wines with immobilized kefir cultures on fruit pieces and essential oils.

Wine Products Supplemented with EOs		Aroma Density	Taste Density	Overall Evaluation
Wet kefir culture	Co_Wt_Ap	2.8 ± 0.4	2.8 ± 0.4	3.6 ± 0.7
	Co_Wt_Pe	2.7 ± 0.5	3.1 ± 0.2	3.5 ± 0.6
	Cm_Wt_Ap	3.8 ± 0.8	3.2 ± 0.4	3.3 ± 0.9
	Cm_Wt_Pe	4.0 ± 0.9	4.3 ± 0.5	2.9 ± 0.9
	Cz_Wt_Ap	3.3 ± 0.4	3.4 ± 0.5	3.3 ± 0.7
	Cz_Wt_Pe	3.5 ± 0.8	2.9 ± 0.2	3.4 ± 0.5
	Mix_Wt_Ap	4.2 ± 0.4	3.5 ± 0.8	3.3 ± 0.9
	Mix_Wt_Pe	3.5 ± 0.6	3.3 ± 0.5	3.3 ± 0.7
Thermally dried kefir culture	Co_ThDr_Ap	2.6 ± 0.7	2.9 ± 0.5	3.0 ± 0.6
	Co_ThDr_Pe	2.9 ± 0.4	2.8 ± 0.7	3.7 ± 0.8
	Cm_ThDr_Ap	3.6 ± 0.6	3.5 ± 0.6	2.6 ± 0.7
	Cm_ThDr_Pe	3.6 ± 0.6	3.2 ± 0.6	2.9 ± 0.9
	Cz_ThDr_Ap	2.9 ± 0.5	3.1 ± 0.8	3.1 ± 0.7
	Cz_ThDr_Pe	3.6 ± 0.5	3.4 ± 0.4	3.4 ± 0.6
	Mix_ThDr_Ap	3.2 ± 0.6	3.1 ± 0.5	3.0 ± 0.8
	Mix_ThDr_Pe	3.5 ± 0.7	3.3 ± 0.4	3.2 ± 0.7
Freeze-dried kefir culture	Co_FrDr_Ap	2.6 ± 0.7	2.9 ± 0.5	3.4 ± 0.9
	Co_FrDr_Pe	2.4 ± 0.7	2.8 ± 0.5	3.1 ± 0.7
	Cm_FrDr_Ap	3.4 ± 0.9	3.3 ± 0.6	2.7 ± 0.6
	Cm_FrDr_Pe	3.4 ± 0.7	3.7 ± 0.8	2.5 ± 0.9
	Cz_FrDr_Ap	3.7 ± 0.7	3.8 ± 0.5	2.8 ± 0.6
	Cz_FrDr_Pe	3.6 ± 0.7	3.4 ± 0.5	2.8 ± 0.8
	Mix_FrDr_Ap	4.2 ± 0.3	3.6 ± 0.8	3.0 ± 0.8
	Mix_FrDr_Pe	3.3 ± 0.5	3.3 ± 0.4	3.0 ± 0.9

Co: low-alcohol wines without EOs addition, Cm: low-alcohol wines with 0.01% *v*/*v* *Citrus medica* EO, Cz: low-alcohol wines with 0.01% *v*/*v* *Cinnamon zeylanicum* EO, Mix: low-alcohol wines with *Citrus medica* and *Cinnamon zeylanicum* EOs (0.005% each). The immobilization support is shown at the end of the sample codes. Ap: immobilized kefir cultures on apple pieces, Pe: immobilized kefir cultures on pear pieces.

Serving fruit pieces (containing immobilized cells) and low-alcohol wines with EOs in different containers, with the testers' direct involvement in the process of reconstituting the final Sangria-type product, was characterized as highly intriguing. Thus, attributes like the style of presentation could be exploited, in terms of sensory marketing, as it can influence the consumers' perception, judgement and behavior, affecting their satisfaction and resulting in indirect product promotion [94]. All Sangria-type products with immobilized kefir cultures and EOs were characterized as highly original and were accepted by the tasting panel, indicating a great market potential for these products.

4. Conclusions

In the current study, novel Sangria-type low-alcohol wines supplemented with immobilized kefir cultures on fruit pieces and EOs were produced and evaluated. High cell viability was retained after immersion of immobilized kefir cells for 2 h in low-alcohol wines supplemented with *Citrus medica* EO (0.01% *v*/*v*), *Cinnamon zeylanicum* EO (0.01% *v*/*v*) or mixed EOs (0.005% *v*/*v* each), and adequate numbers (enough to confer potential beneficial activity) were retained in all cases. Fluctuations in pH, as well as total and volatile acidity values, were observed depending on the state (wet or dried) of the cells used, but were among typical levels for wines. HS-SPME GC/MS analysis and PCA applied on results revealed that EO supplementation and the relative content of terpenes, alcohols and carbonyl compounds played a major role in the discrimination of the novel low-alcohol wine products. Notably, EO supplementation resulted in higher aroma and taste density, and all new wine products were accepted by the sensory panel.

In conclusion, data supporting the development of a novel Sangria-type low-alcohol wine with immobilized kefir cells on fruit pieces and EOs with great market potential are

presented. However, more research is still required for aspects, such as minimizing the organoleptic impact of EOs on the product, as well as effectively prolonging its shelf life.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres14020038/s1, Table S1: Minor volatiles (mg/L) detected by HS-SPME GC/MS analysis in Sangria-type low-alcohol wines with immobilized kefir cultures and essential oils.; Figure S1: Biplot showing the projection of the minor volatiles on the first two principal components of the PCA.

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