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Assessment of Antimicrobial Efficiency of *Pistacia lentiscus* and *Fortunella margarita* Essential Oils against Spoilage and Pathogenic Microbes in Ice Cream and Fruit Juices

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Abstract: Nowadays, the use of antimicrobial natural agents as alternative food preservatives represents an intriguing case. The purpose of this study was to investigate possible antimicrobial activity of *Pistacia lentiscus* and *Fortunella margarita* essential oils (EOs) and to evaluate their commercial potential in the food industry. The main constituents identified by GC/MS in *Pistacia lentiscus* EO were a-pinene (67.7%), myrcene (18.8%), and β-pinene (3.0%), whereas limonene (93.8%) and myrcene (2.7%) were the dominant compounds in *Fortunella margarita* EO. The antimicrobial properties were initially assayed and the minimum inhibitory, non-inhibitory, and minimum lethal concentration values against the *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas fragi*, *Aspergillus niger*, and *Saccharomyces cerevisiae* were determined using a previously published model, combining absorbance measurements with the common dilution method and non-linear regression analysis to fit the data. Their efficiency was further validated in ice cream containing 0.2% (*w*/*w*) *Pistacia lentiscus*, 0.006% (*w*/*w*) *Fortunella margarita* EOs and 2% (*w*/*w*) aqueous residue of *F. margarita* EO deliberately inoculated with 4 logcfu/g *Escherichia coli*, *Listeria monocytogenes* or *Pseudomonas fragi*, separately. Similarly, the activity of the oils was monitored in fruit juice (lemon, apple, and blackcurrant) containing 0.2% (*w*/*w*) *Pistacia lentiscus*, 0.006% (*w*/*w*) *Fortunella margarita* EOs and 2% (*w*/*w*) aqueous residue of *F. margarita* EO deliberately spiked with 100 spores/mL of *Aspergillus niger* or 4 logcfu/mL of *Saccharomyces cerevisiae*, separately. The results showed that microbial viable counts in the supplemented products ranged at significantly lower levels compared to the control samples during storage. Overall, the data indicated that both EOs constitute effective antimicrobial sources with many potent applications in the food industry.

Keywords: essential oils; spoilage; foodborne pathogens; ice cream; fruit juices; *Pistacia lentiscus*; *Fortunella margarita*

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1. Introduction

Preservatives are used in the food industry to increase the shelf life, ensure safety, and retain quality characteristics at high standards within the expiration date of the products. Nevertheless, concerns have been raised by consumers and the scientific community on the safety of the widely used chemical preservatives that are routinely added to the majority of manufactured foods in order to prevent the growth of spoilage and pathogenic microorganisms. As antimicrobial natural alternatives, essential oils (EOs) of plant origin have been under the microscope of scientists for the last two decades [1,2]. A properly designed strategy for the incorporation of EOs into foods (formulation strategies, efficiency,

and organoleptic quality issues) is a key factor in the development of novel products. Their incorporation into a food matrix is considered an additional inherent factor for preventing the growth of pathogens or delaying a spoilage effect. In this context, several attempts have been focused on the successful addition of EOs and plant extracts to various foods [3,4], but only a very limited number of products are available on the market, mainly due to their strong taste, or the interaction of bioactive substances with food components, and their intense aroma [5].

Pistacia, a genus of flowering plants of the *Anacardiaceae family*, consists of around twenty species, five of which are the most common: *P. vera, P. atlantica, P. terebinthus, P. khinjuk*, and *P. lentiscus.* Diverse phytochemical ingredients, including terpenoids, phenolic compounds, fatty acids, and sterols, have been extracted and identified from different parts of the *Pistacia* species. A-Pinene has been reported as the main compound of some samples, such as *P. vera* [6–8] *P. terebinthus* [9–11], *P. lentiscus* [12–14], and *P. atlantica* [15,16]. In addition to α-pinene, limonene, α-terpinolene, and ocimene are other important components extracted from different parts of *Pistacia* plants. However, the chemical composition of EOs and extracts may differ on geographical location, climate, cultivation practices, extraction methods, etc. [17].

Extracts and EOs isolated by *Pistacia* species have demonstrated significant antibacterial activity against a variety of Gram-positive and Gram-negative bacteria, as well as molds, such as EOs of *P. lentiscus* and *P. atlantica* var. *kurdica* against *Helicobacter pylori*, and the *P. lentiscus* against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis.* Similarly, *P. lentiscus* leaf and gum EOs, various extracts of *P. khinjuk* leaf, and *P. vera* gum EO exhibited significant antifungal activity [8,12,18].

Fortunella spp. belongs to the *Citrus* genus and the Rutaceae family. *Fortunella* plants, including *F. japonica*, *F. bawangica*, *F. margarita*, are often grown in the southern part of China, Japan, Philippines, Marocco, Corfu, and Corsica. Chemical analysis of EOs of *F. margarita* revealed that the dominant compound was limonene (84.2–96.3%), followed by myrcene (1.3–12.9%) and germacrene D (0.3–2.4%) [19,20].

In addition to milk, ice cream contains a wide variety of other ingredients, such as cream and sugar. It has a pH close to neutral (6–7) and it can be stored for long time periods [21]. Microbiological contamination can usually be controlled during the production stages of pasteurization and freezing, but not always effectively [22], as microbiological hazards identified in the final products can be introduced after pasteurization through the addition of contaminated ingredients and improper handling procedures. Pasteurization conditions can differ depending on the type of ice cream to be produced. This is especially important when making soft ice cream because the final stage of production occurs at the point of sale, where large containers of ice cream mix and flavoring agents are stored for dispensing as soft serve or frozen desserts. *Escherichia coli*, *Listeria monocytogenes*, and *Pseudomonas* spp. are some of the pathogens that can survive in ice cream, even at low temperatures [23].

Fruit juices are rich in simple carbohydrates and complex nitrogen sources. The critical factors affecting juice spoilage caused by microorganisms include intrinsic and extrinsic factors. Intrinsic factors consist of pH, oxidation-reduction potential, water activity, availability of nutrients, the presence of antimicrobial compounds, and competitive microflora. Extrinsic factors encompass storage conditions, such as temperature, relative humidity, time, and packaging material [24,25]. Yeasts and fungi have the ability to grow at low pH, high sugar content, and high-water activity. *Saccharomyces cerevisiae* and *Aspergillus niger* are the most common spoilage microorganisms in fruit juices [26,27]. Fungal spoilage in fruit juices can lead to unpleasant odors, taste alteration, and discoloration. Conidia or mycelial fragments can contaminate fruit juices and some fungi produce mycotoxins that pose a major threat to human health.

Hence, the aim of the present study was to assess the industrial potential of *Pistachia lentiscus* and *Fortunella margarita* EOs as natural antimicrobial agents against common spoilage bacteria and yeasts/molds in ice cream and juices. Data suggesting a significant

extension of the product's shelf life and repression of microbial growth in spiked samples are presented.

2. Materials and Methods

2.1. Extraction of EOs

Pistachia lentiscus was kindly provided by Chios Mastic Gum Growers Association L.L.C. (Chios, Greece). The air-dried resinous gum was collected by hand from the plant or from its surrounding area on Chios Island. The *P. lentiscus* EO was produced in small experimental distillation equipment under vacuum in VIORYL SA research laboratories (Afidnes, Greece), as described before [28].

The chopped peel of *Fortunella margarita* fruits (collected between January and March on the island of Corfu) was handled and processed immediately for hydrodistillation, as described before [19] and the aqueous residue after hydrodistillation was collected.

2.2. Microbial Strains

Escherichia coli ATCC 25922 and *Listeria monocytogenes* NCTC 10527 serotype 4b were grown in Brain Heart Infusion broth (LABM, Heywood, UK) at 37 °C for 24 h. Similarly, *Pseudomonas fragi* 211 (kindly provided by Prof. Nychas G.J.E., Agricultural University of Athens, Greece) was grown in BHI broth at 25 °C for 24 h. *Aspergillus niger* 19111 (kindly provided by Prof. Nychas G.J.E., Agricultural University of Athens) was grown on malt extract agar for seven days at 37 °C. *Saccharomyces cerevisiae* uvaferm NEM (Lallemand, Montreal, QC, Canada) was grown in YPD broth (yeast extract, 10 g/L; peptone, 20 g/L; and dextrose, 20 g/L) at 28 °C for three days.

2.3. Novel Ice Cream Products Supplemented with P. lentiscus and F. margarita EOs

Ice cream production was carried out on a pilot line with a batch capacity of 40 kg of ice cream by EMFI SA (Athens, Greece). In a double-walled tank, the raw materials were mixed under stirring. The water was added first, followed by the sugar (10% *w*/*w*), which was initially mixed with the stabilizers (locust bean gum, guar gum) and emulsifiers (carrageenan, pectin) for a total of 0.5% (*w*/*w*). Then, 10% *w*/*w* glucose was added, and when the temperature reached 45 °C, 10% (*w*/*w*) fats (milk, cream, and butter) were also added. The mixture was pasteurized at 80–85 °C for 40 s before being cooled to 4 °C and homogenized in a PS valve (GEA Mechanical Equipment Italia S.p.A, Italy) homogenizer at a pressure of 140–200 bar. The homogenized mixture was kept in a refrigerated tank at 4 °C for 4 days. Finally, *P. lentiscus* EO 0.2% (*w*/*w*), *F. margarita* EO 0.006% (*w*/*w*) and 2% (*w*/*w*) aqueous residue of *F. margarita* EO were incorporated in the mixture, which was then placed in the pilot ice cream machine for 24 h, at −23 °C for preparation of the new products.

2.4. Novel Fruit Juices Supplemented with P. lentiscus and F. margarita EOs

Mixture 26% (*w*/*v*) of concentrated lemon juice (48°brix), concentrated apple juice (70°brix), gooseberry concentrate (65°brix), and citric acid monohydrate 0.1% (*w*/*v*) were mixed in a 1 L glass beaker, followed by the addition of 739 mL water. The beaker was then placed in a boiling water bath for 25–30 s with the water level below the rim of the beaker, in order to pasteurize the mixture at 85 °C. After that, the mixture was allowed to cool to room temperature before adding flavorings, and *P. lentiscus* EO 0.2% (*w*/*w*), *F. margarita* EO 0.006% (*w*/*w*) and 2% (*w*/*w*) aqueous residue of *F. margarita* EO. Fruit juices were provided by EMFI SA.

2.5. Analytical Procedures

2.5.1. GC/MS Analysis

The GC/MS analysis of the obtained EOs was performed in a GC-MS (GC: 6890 A, Agilent Technologies, Carlsbad, CA, USA; MSD: 5973, Agilent Technologies) using a Factor Four VF 1ms column (25 m, 0.2 mm i.d., 0.33 m film thickness, Agilent Technologies). An

amount of 0.1 μL of EO was directly injected and a 1:100 split ratio was applied. The oven temperature was set at 50 °C for 1 min, followed by a temperature gradient of 2.5 °C/min. When the temperature reached 160 °C, it was kept steady for 20 min. Then, a step of 5 °C/min was applied until the oven temperature reached 250 °C, where it was kept for 15 min. Helium was used as a carrier gas with a constant flow rate of 1 mL/min. Injector and transfer line temperatures were set to 200 °C and 250 °C, respectively. The mass spectrometer operated in the electron impact mode with the electron energy set to 70 eV. Volatile identification was completed according to the standard method of Kováts Indices and mass spectra comparison to Willey/NIST 0.5 and in-house created libraries (VIORYL S.A.).

2.5.2. Antimicrobial Assays

Screening of *P. lentiscus* and *F. margarita* EOs for Antimicrobial Activity by the Disc Diffusion Assay

The antibacterial activity of the *P. lentiscus* and *F. margarita* EOs, as well as the aqueous residue of *F. margarita* EO, was initially tested using the disk diffusion assay on BHI agar plates, as described previously [29]. Ciproxin (5 g) (Oxoid Ltd., Hampshire, UK) was used as a positive inhibitory control for *E. coli*, *L. monocytogenes*, and *P. fragi*, whereas sterile water was used as a negative control [29].

The same procedure was also followed for screening the activity against *A. niger* and *S. cerevisiae* using malt and YPD agar plates, respectively [29]. Amphotericin B (10 μg) (Mast Group Ltd., Merseyside, UK) and sterile water were used as positive inhibitory and negative controls, respectively.

Determination of Minimum Inhibitory Concentration (MIC), Non-Inhibitory Concentration (NIC), and Minimum Lethal Concentration (MLC)

The determination of minimum inhibitory (MIC) and non-inhibitory (NIC) concentrations was carried out as recently described [30]. In brief, BHI broths supplemented with various concentrations of EOs (ranging from 20 to 25,000 mg/L) were inoculated with *E. coli*, *L. monocytogenes* or *P. fragi* and incubated at 37 °C for 24 h or at 25 °C for 24 h for *P. fragi*. Changes in optical density were monitored using a microplate reader (Molecular Devices, VERSAmax, Hayward, CA, USA; Softmaxprov.5.0 software). BHI broths supplemented with ciproxin (ranging from 2 to 20 mg/L) were used as positive inhibitory controls. BHI broths with no inoculum and inoculated BHI broths with no EOs were used as negative controls [29]. The calculation of MIC and NIC values was based on the Lambert–Pearson model (LPM) [31,32].

As the LPM model was not applicable to *A. niger* and *S. cerevisiae* due to conidia flotation and yeast cell sedimentation, the standard protocols described by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) were applied for MIC determination [33,34], using amphotericin B (ranging from 1 to 4 mg/L) as positive inhibitory control [19].

Minimum lethal concentration (MLC) was defined as the lowest concentration, resulting in a negative subculture or presence of only 1 colony-forming unit after incubation (99.9% of the inoculum killed). It was determined by subculturing 100 μL from each well at which no growth was observed onto BHI (for *L. monocytogenes*, *E. coli*, and *P. fragi*) and malt or YPD (for *A. niger* or *S. cerevisiae*) agar plates [29].

Antimicrobial Activity of *P. lentiscus* EO in Ice Creams

Ice creams supplemented with the EOs were deliberately spiked with *E. coli* (4 logcfu/g) (IC_EOs_Ec) or *L. monocytogenes* (4 logcfu/g) (IC_EOs_Lm) or *P. fragi* (4 logcfu/g) (IC_EOs_Pf) and microbial survival was monitored at −20 °C. For comparison reasons, control samples with no EOs and deliberately spiked with *E. coli* (IC_Ec) or *L. monocytogenes* (IC_Lm), or *P. fragi* (IC_Pf) were also included in the study. At various intervals, samples (25 g) were collected to monitor the levels of spoilage and the inoculated strains. The following tests on microbiological analysis were performed: (i) total aerobic counts on plate count agar

(LABM) at 30 °C for 48 h, (ii) coliforms on violet red bile agar (LABM) at 37 °C for 24 h, (iii) *Enterobacteriaceae* on violet red bile glucose agar (LABM) at 37 °C for 24 h, (v) lactobacilli (Gram-positive) on acidified MRS agar (LABM) at 30 °C for 24 h anaerobically (Merck Millipore, Burligton, Massachusetts,USA, Anaerobic Jar 2.5 L, Oxoid ltd, Hampshire, UK, AnaeroGen 2.5L Sachets), (vi) *Escherichia coli (E. coli)* on TBX (LABM) at 42 °C for 24–48 h, (vii) *L. monocytogenes* on Palcam Agar (LABM) supplemented with Palcam Listeria Selective Supplement (LABM) at 37 °C for 24–48 h and (vii) *P. fragi* on brain heart agar (LABM) at 25 °C for 24–48 h.

Antimicrobial Activity of *P. lentiscus and F. margarita* EOs in Fruit Juices

Spoilage of fruit juices supplemented with EOs or without EOs both at room temperature (18–20 °C) and 4 °C was monitored by yeast/mold counts on malt extract agar (LABM) after media incubation at 30 °C, respectively, for 2–5 days. Cell counts below 1 log colony forming units/mL were determined by pour plating 1 mL of fruit juice directly into the culture medium.

Fruit juices containing EOs and fruit juices without EOs were deliberately spiked with *A. niger* (100 spores/mL) or *S. cerevisiae* (4 logcfu/mL), separately and the microbial growth was examined at both room temperature (18–20 °C) and 4 °C. During storage, samples (25 mL) were collected at various intervals for the determination of the levels of *A. niger* or *S. cerevisiae.* Numbers of *A. niger* spores/mL were determined by microscopic enumeration with a cell-counting hematocytometer (Neubauer chamber), while *S. cerevisiae* counts were determined in YPD agar after incubation at 28 °C for 3 days.

2.6. Preliminary Sensory Evaluation

Tasters from EMFI SA evaluated all products based on their organoleptic characteristics. *P. lentiscus* and *F. margarita* EOs were tested in ice cream or in fruit juice at various concentrations.

2.7. Statistical Analysis

All experiments were performed at least in four replicates and the mean values are presented.

Fig P.2.1 software (Fig.P Software Incorporated, Toronto, ON, Canada) was used to calculate standard deviation for MIC and NIC values.

The results were analyzed with analysis of variance (ANOVA) using Duncan's multiple range test to determine significant differences ($p < 0.05$) among results (coefficients, ANOVA tables, and significance ($p < 0.05$) were computed using Statistica v.10.0).

3. Results

3.1. GC/MS Analysis

GC/MS analysis of the EOs is presented in Table 1 [19].

Table 1. Volatile compounds identified in *P. lentiscus* and *F. margarita* EOs by GC/MS analysis and their relative percent (%) area.

Compounds	KRI *	*P. lentiscus* (% Area)	*F. margarita* (% Area)
cis-3-hexenol	811		trace
thujene	915		trace
α-pinene	922	67.71	0.743
camphene	927	0.70	trace
verbenene	937	0.07	
sabinene	953		0.133
β-pinene	958	3.05	0.019
myrcene	973	18.81	2.680
α-phellandrene	981		0.073

Table 1. *Cont.*

Compounds	KRI *	*P. lentiscus* (% Area)	*F. margarita* (% Area)
δ-3 carene	990		0.020
α-terpinene	997		trace
p-cymene	1004		trace
limonene	1011	0.89	93.784
cis-ocimene	1016		0.001
trans-ocimene	1018		0.019
γ-terpinene	1030		0.023
dehydro-p-cymen	1062		trace
terpinolene	1070		0.014
linalol	1086	0.73	0.118
α -campholenic ald	1094	0.26	
trans-p-menthe-2.3-dien-1-ol	1105		0.018
pinocarveol	1113	0.32	
cis-p-menthe-2.8-diene-1-ol	1115		0.017
trans-verbenol	1117	0.07	
cis-verbenol	1120	0.69	
terpinene-4-ol	1152		0.020
α-terpineol	1168		0.026
verbenone	1168	0.32	
8-cumenol	1172		trace
trans-carveol	1177		0.014
decanal	1178		0.015
octyl acetate	1191		0.055
cis-carveol	1197		0.011
carvone	1217		0.023
geraniol	1231		trace
perilla aldehyde	1233		0.019
neryl acetate	1340		0.014
α-cubebene	1344		trace
geranyl acetate	1358		0.111
α-copaene	1366		0.016
β-elemene	1378		0.023
p-menthe-1-en-9-yl acetate	1399		0.007
caryophyllene	1403	0.50	0.010
humulene	1436		0.008
δ-germacrene	1462		1.343
bicyclogermacrene	1479		0.246
α-mourolene	1483		trace
valencene	1501		0.009
δ-cadinene	1504		0.053
B-germacrene	1533		0.039

* Kováts Retention Indices.

In the case of *P. lentiscus* EO, 13 compounds were identified consisting of 94.12% of the total chromatographic area [14]. The most predominant compound was α-pinene, accounting for 67.71%, followed by myrcene (18.81%). At the *F. margarita* EO, 45 compounds were identified [15], representing 99.7% of the total chromatographic area. Limonene was the most abundant compound (93.784%).

3.2. Antimicrobial Assays

The antimicrobial activity of EOs against spoilage and foodborne pathogens of concern for ice cream (*E. coli*, *L. monocytogenes*, and *P. fragi*) or fruit juices (*A. niger* and *S. cerevisiae*) was firstly determined by the disk diffusion method (data not shown). Subsequently, MIC, NIC, and MLC values were evaluated, as their precise determination is critical for the food industry. Indeed, their values shall be determined in order to conclude the optimum amount of antimicrobial agent that is needed to ensure microbial safety as the chemical composition can vary greatly, depending on climate, environmental and cultivation conditions, and

harvesting time [35,36]. Although MIC, NIC, and MLC values were significantly ($p < 0.05$) higher compared to ciproxin and amphotericin B used as controls, the effective growth inhibition of *P. lentiscus* EO against all microorganisms tested (Table 2) was confirmed. Of note, *F. margarita* EO had no antibacterial activity, although it showed considerable activity against fungi and yeast. Similarly, the aqueous residue of *F. margarita* EO had no activity on the microbes tested in our study.

Table 2. MIC, NIC, and MLC (mg/L) of *P. lentiscus and F. margarita* EOs against *E. coli*, *L. monocytogenes*, *P. fragi*, *A. niger*, and *S. cerevisiae*. Ciproxin and amphotericin B were used as positive controls.

Microbial Species	*P. lentiscus*			*F. margarita*			Ciproxin			Amphotericin B		
	MIC	NIC	MLC	MIC	NIC	MLC	MIC	NIC	MLC	MIC	NIC	MLC
E. coli	4584 ± 17	4291 ± 9	17,200	-	-	-	0.984 ± 0.001	0.956 ± 0.002	4	-	-	-
L. monocytogenes	516 ± 17	129 ± 9	2150	-	-	-	0.979 ± 0.001	0.968 ± 0.001	4	-	-	-
P.fragi	1542 ± 26	145 ± 9	6880	-	-	-	0.955 ± 0.001	0.940 ± 0.002	8			
A. niger	2150	-	9300	4785	-	18,600	-	-	-	1	-	4
S. cerevisiae	2150	-	9300	4785	-	18,600	-	-	-	1	-	4

3.3. P. lentiscus EO as Potential Biopreservative in Ice Cream

To study the survival inhibitory effect of *P. lentiscus* EO against spoilage and foodborne pathogens in ice cream, samples supplemented with the EOs were spiked with *E. coli* (IC_EOs_Ec), *L. monocytogenes* (IC_EOs_Lm) or *P. fragi* (IC_EOs_Pf) and then cell survival under freezing conditions (−20 °C) was monitored. The results are presented in Figure 1a,b,c.

Supplementation with the EOs resulted in a gradual decrease in the *E. coli*, *L. monocytogenes* and *P. fragi* counts until the 7th week of storage by about 3 logcfu/g, in contrast to the control samples, in which cell counts were maintained at higher levels ($p < 0.05$). Of note, no other microbial species other than the inoculated strains were detected.

3.4. P. lentiscus and F. margarita EOs as Potential Biopreservatives in Fruit Juices

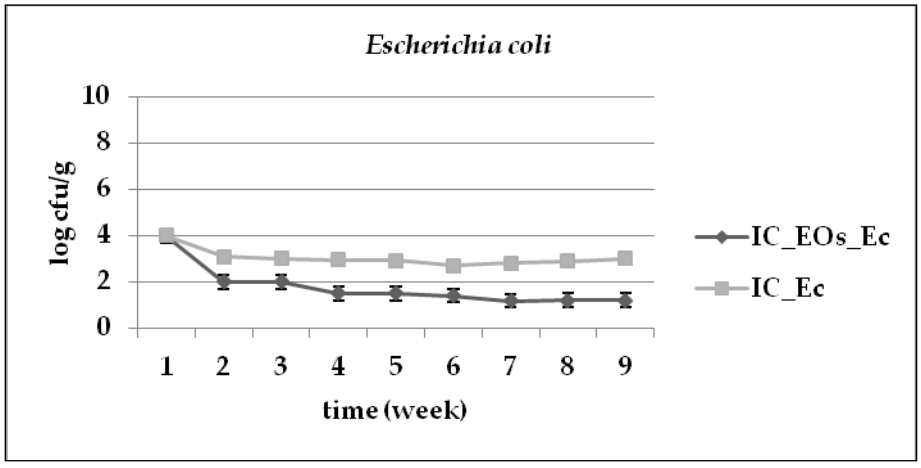
Spoilage of fruit juices supplemented with EOs or without EOs both at room temperature (18–20 °C) and 4 °C was monitored. The results are shown in Figure 2a,b. Supplementation of the juices with *P. lentiscus* and *F. margarita* EOs resulted in prolongation of the product's shelf life, as spoilage was observed on the 3rd day at room temperature and after the 10th day at 4 °C, while on the control samples, spoilage was observed on the 1st day at room temperature and on the 6th day at 4 °C.

To study the growth inhibitory effect of *P. lentiscus* and *F. margarita* EOs against spoilage microbes in fruit juices, juice samples supplemented with the EOs were spiked with *A. niger* (FR_EOs_An) or *S. cerevisiae* (FR_EOs_Sc) and cell survival was monitored during storage at both room temperature (18–20 °C) or 4 °C (Figure 3a,b). EOs addition resulted in a decrease in *A. niger* levels at both room temperature and 4 °C, ranging in <100 spores/mL, in contrast to the control samples, at which spores numbers remained higher ($p < 0.05$).

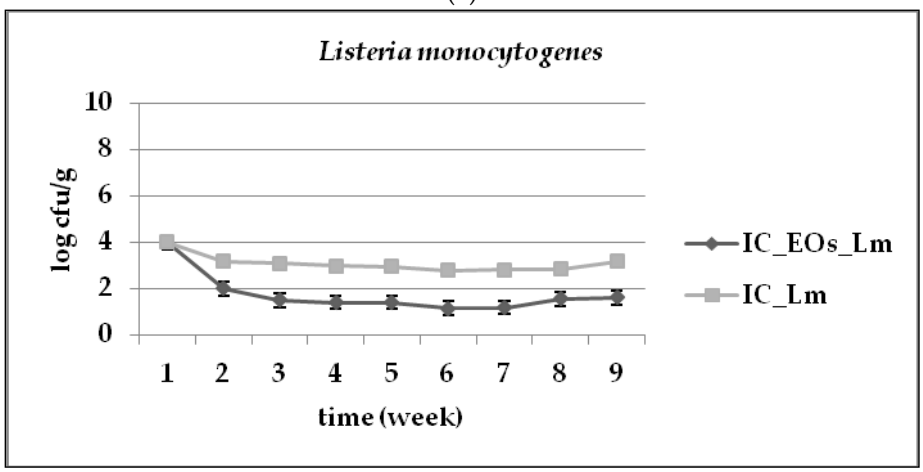
When the same juice was inoculated with *S. cerevisiae*, no significant changes ($p > 0.05$) were observed at the yeast levels in relation to the initial inoculum at room temperature, whereas at 4 °C, the concentration of *S. cerevisiae* decreased by 3 logcfu/mL ($p < 0.05$). It is worth noting that in the control samples yeast counts were significantly higher ($p < 0.05$).

3.5. Preliminary Sensory Evaluation

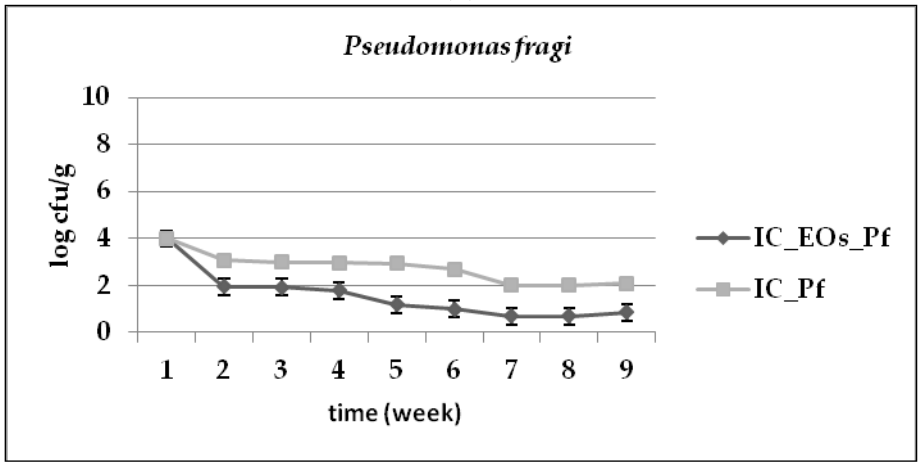
The vast majority of products were rejected because they had a bad, intense, and bitter taste. However, the ice cream or juice produced by combining *P. lentiscus* and *F. margarita* EOs in concentrations of 0.2 % (*w*/*w*) and 0.006% (*w*/*w*) in the final product, demonstrated a unique character with promising commercial prospects. The products were also manufactured on a larger scale to verify the organoleptic properties during industrial production processes. The organoleptic test results of the industrially produced product were similar to those of the laboratory and pilot production (data not shown). Of note, 2% (*w*/*w*) aqueous residue of *F. margarita* EO was considered necessary as flavor enhancer.



(**a**)



(**b**)



(**c**)

Figure 1. Counts of (**a**) *E. coli* (Ec), (**b**) *L. monocytogenes* (Lm) and (**c**) *P. fragi* (Pf) in spiked samples of ice cream (IC) supplemented with *P. lentiscus* and *F. margarita* EOs during storage at −20 °C. Spiked samples of ice cream with no EOs served as controls.

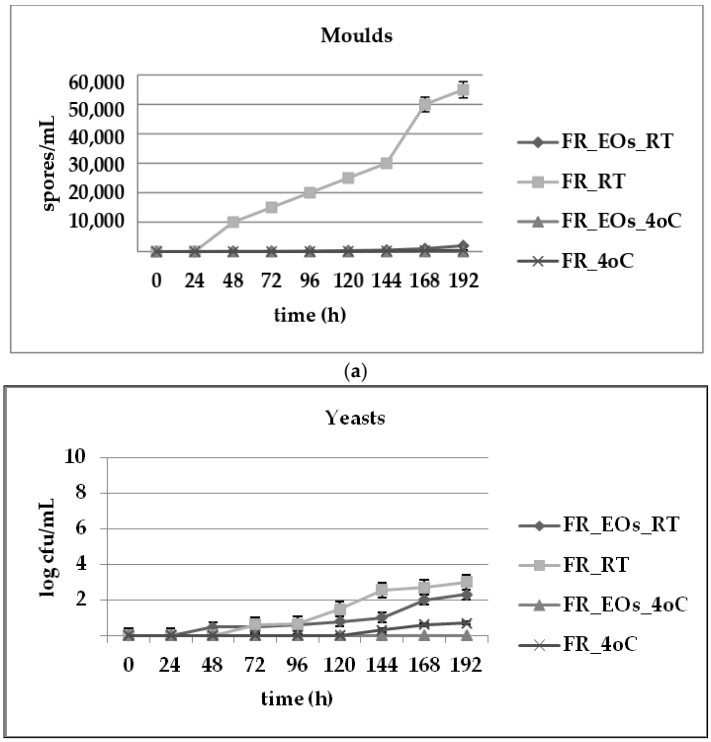


Figure 2. Spoilage of fruit juices (FR) supplemented with *P. lentiscus* and *F. margarita* EOs (**a**) from molds, (**b**) yeasts, during storage at room temperature (RT) and at 4 °C. Fruit juices with no EOs served as controls.

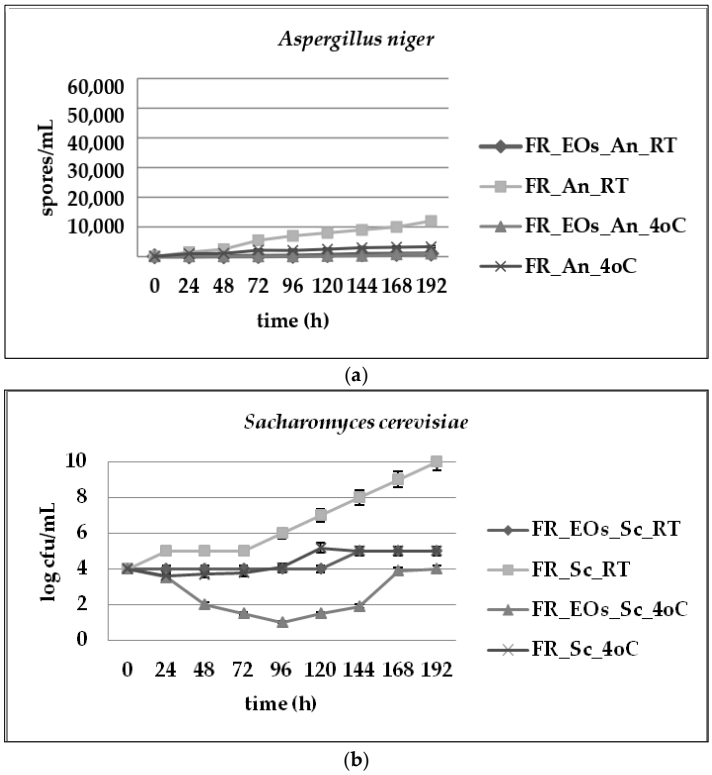


Figure 3. Counts of (**a**) *A. niger*, (**b**) *S. cerevisiae*, in spiked samples of fruit juice (FR) supplemented with *P. lentiscus* and *F. margarita* EOs during storage at room temperature (RT) and at 4 °C. Spiked samples of fruit juice with no EOs served as controls.

4. Discussion

In the literature, there are several studies concerning the antimicrobial activity of various EOs. Many efforts have focused on the incorporation of EOs into foods, in which they are mainly used as antimicrobials, with the aim of drastically reducing chemical preservatives.

P. lentiscus EO acted protectively against foodborne pathogens in ice cream, as it significantly suppressed the survival of *E. coli*, *L. monocytogenes*, and *P. fragi* in spiked samples, while the mixture of *P. lentiscus* and *F. margarita* EOs inhibited the growth of *A. niger* and *S. cerevisiae* in spiked fruit juice.

Inhibitory efficacy of *P. lentiscus* EO was also observed by Gkogka et al. [37] against *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella* Typhimurium, *Staphylococcus aureus*, two species of yeasts (*Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*) and three species of fungi (*Penicillium roquefortii*, *Aspergillus flavus* and *Eurotium amstelodami*). The most susceptible to the EO microorganisms were found to be *C. perfringens*, followed by *S. cerevisiae*, *Z. bailii*, and *C. jejuni*. MIC values for *S. aureus* and *B. cereus* were 0.75% (*v*/*v*), while *S.* Typhimurium strains were both more resistant [MIC of 1% (*v*/*v*)]. *E. coli* and *S.* Typhimurium were the most resistant among all tested bacteria and the MIC for yeasts was determined at 2% (*v*/*v*). In contrast, fungi were resistant (MIC > 4% *v*/*v*). Tabanca et al. [38], investigated the antimicrobial activity of *P. lentiscus* EO from different region plants in Turkey (MGEO-1, MGEO-2, MGEO-3) against 6 microorganisms, including 3 Gram-negative (*E. coli*, *S. typhimurium*, and *K. pneumoniae*), 2 Gram-positive bacteria (*S. aureus* and *L. monocytogenes*) and the yeast C. *albicans*. MGEO 1 resulted in weak inhibitory effects with MIC values ranging from 5–10 mg/mL against all the bacterial pathogens tested except for *E. coli*. MGEO-2 and 3 demonstrated selective antibacterial activity against *K. pneumoniae* (MIC 5 mg/mL) and *S.* Typhimurium (MIC 5 mg/mL). As an antifungal agent, MGEO-1 was twice as effective against*C. albicans* than MGEOs-2 and 3. MGEOs resulted in higher MICs compared to the positive controls, such as chloramphenicol, amoxicillin, and amphotericin B.

P. lentiscus EO was incorporated into ice creams and fruit juices at concentrations over the MIC values of all bacterial strains except for *E. coli*. However, *E. coli* counts in the ice cream were also significantly decreased. According to Leja et al. [39], sub-inhibitory concentrations of EOs (lower than MIC) do not cause cell death, but affect, among others, the intracellular activity of bacteria. Thus, they may cause an increase in the amount of unsaturated fatty acids responsible for the fluidity of the cell membrane, which results in structural changes in the membrane, loss of ability to move, which is probably due to the change in the shape of the cell under the influence of oils, and flagella damage. *Pistachia* EO has been also tested as a biopreservative in various food systems. Hassanzadazar et al. [40] assessed the quality and shelf life of trout (*Oncorhynchus mykiss*) minced meat impregnated with EO isolated from *P. atlantica* ssp. *kurdica* (Bene) fruits at concentrations of 0.125, 0.25, and 0.5% *v*/*w* and stored at 4 °C for 12 days. The bacterial load increased proportionally in all fish samples and was significantly lower in treated fish samples than in control samples. The results of the microbial evaluation and sensory analysis showed that cooked treated fish samples remained acceptable for consumption during 8 days of storage. Similarly, Ellahi et al. [41] investigated the antimicrobial effect of gum EO derived from *P. atlantica* (wild pistachio) tree (GEO) and designed a new film based on polypropylene polymer coated with silica nanoparticles and GEO incorporated in milk. The antimicrobial activity of the packaging film was evaluated with or without milk on *S. aureus*, *S. enterica*, *E. coli*, and *L. monocytogenes* for 35 days. The results showed that GEO EO had significant antibacterial properties and it was most effective against *S. enterica*, while its effect on *L. monocytogenes* was the weakest. Likewise, Krichen et al. [42] proposed the EO from pistachio by-product as an effective food preservative to inhibit bacterial growth in minced beef meat. In another study, Elhadef et al. [43] evaluated the effect of aqueous pistachio hull extract (PHE) at concentrations of 0.156% (PHE1), 0.312% (PHE2), and 0.625% (PHE3) on the quality of raw minced beef kept at 4°C for 14 days. At the end of storage, mesophilic total viable plate, psychotropic, and *Enterobacteriaceae* counts in PHE samples were significantly lower.

Silver nanoparticles synthesized by *F. margarita* fruit juice were effective against *E. cloacae*, *E. coli*, *P. mirabilis*, *Bacillus* sp., *S. aureus*, *Streptococcus* spp., *K. pneumonia* and *P. aeruginosa* and their activity was concentration dependent [44]. Wang et al. [45] evaluated the antimicrobial activity of the EO isolated by *F. crassifolia* peel against *E. coli*, *S. typhimurium*, *S. aureus*, and *B. cereus.* The MIC values ranged from 50–70 μg/mL and 37.5–67.5 μg/mL for Gram-negative and Gram-positive bacteria, respectively. These findings are contradictory to our results, as no antibacterial activity was observed for *F. margarita* EO, but it inhibited *A. niger* and *S. cerevisiae* growth. Similar activity of *F. margarita* EO was previously witnessed by Fitsiou et al. [19]. The concentration of *F. margarita* EO in ice cream and in fruit juices ranged in values significantly lower than the MICs for *A. niger* and *S. cerevisiae*. Thus, suppression of *A. niger* and *S. cerevisiae* growth was probably due to *P. lentiscus* EO, although a synergistic effect cannot be excluded. Likewise, Wang et al. [44], investigated the effect of *F. crassifolia* EO (concentration at MIC levels) on *E. coli*, *B. subtilis,* and *B. cereus* survival in a food model medium (meat-based model media) during storage at 37 °C. Bacteria counts showed a steep decline during the first 1 h and then remained stable until the end of the assay.

Although ice cream is considered a safe product since it is stored in freezing temperatures, contamination from environmental pathogens is an issue of public health concern yet. According to an assignment conducted by the FDA [21] to inspect ice cream production facilities across the USA in 2016 and 2017, in order to determine the prevalence of *Salmonella* species and *L. monocytogenes* in the final products and in environmental samples following 16 recalls of ice cream products, due to the presence of pathogens in the prior three years and an outbreak of listeriosis linked to an ice cream maker in 2015 that involved three deaths, a serious risk still exists, underscoring the need for the development and implementation of a food safety plan. In this vein, the use of EOs to suppress the survival of harmful bacteria constitutes a tempting alternative. Noticeably, it is the first time that EOs have been applied in the industrial production of ice cream, to the best of the authors' knowledge.

Likewise, although the use of EOs to protect juices from spoilage is thoroughly described in the literature, the industrial application is still under consideration, mainly due to their volatile nature and sensory effects, often incompatibility with food taste, reduced solubility in water, etc. [46]. Boukhatem et al. [47] studied *Eucalyptus globulus* EO as a natural preservative against *Candida albicans*, *C. parapsilosis*, and *Saccharomyces cerevisiae*. The combination of heat treatment (70 °C for 2 min) and EO at different concentrations (0.8 to 4 mg/mL) was more effective in reducing *S. cerevisiae* populations in orange juice, in relation to juice with chemical preservatives. Helal et al. [48] observed that fortification of orange, guava, or banana juices with *Cymbopogon citratus*, *Ocimum basilicum*, or *Origanum majorana* EOs, separately, resulted in a significant reduction in the levels of *A. niger*, *A. flavus* or *Ceccharoma* in spiked samples during storage at room temperature. The effect of the incorporation of clove EO (4500 and 9000 mg/L) on the total mesophilic flora in watermelon juice during 7 days of storage at 37 °C was also studied. At the end of the incubation period, clove EO at a concentration of 9000 mg/L reduced the total mesophilic flora by 6 logcfu/mL, whereas when the EO concentration was 4500 mg/L, the reduction was about 4 logcfu/mL [49]. The incorporation of lemon EO (0.08%, 0.12%, and 0.16% *v*/*v*) in concentrated lemon juice caused total inhibition of germination and spore growth of *Alicyclobacillus acidoterrestris* after freezing for 11 days [50].

Ice cream and fruit juice markets are among the fastest growing sectors in the food industry. The composition of ice cream and juice products along with their physicochemical properties define the microbiological safety and overall quality throughout their self-life. The thermal process (pasteurization) applied to milk and juices partially reduce the existing microbial flora and has a significant effect on the nutritional value, while the use of chemical preservatives is not desirable by consumers. For these reasons, the use of non-thermal technologies, such as application of natural antimicrobial compounds in combination with low temperature storage represent an intriguing alternative [51–56].

5. Conclusions

The results revealed that *P. lentiscus* EO in ice creams and the combination of *P. lentiscus* and *F. margarita* EOs in fruit juices may be used as biopreservatives against spoilage and as a protective shield against foodborne pathogens, since they resulted in significant suppression of *E. coli*, *L. monocytogenes*, *P. fragi*, *A. niger*, and *S. cerevisiae*. However, more research is still required to fully understand their mode of action, in order to elucidate the differences observed regarding their efficiency in various microbial species.

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