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► To cite this version:

Widad Zernadji, Sihem Jebri, Faten Rahmani, Ismail Amri, Dorra Aissaoui, et al.. Effect of Gamma Irradiation on Pathogenic Staphylococcus aureus in Packaged Ready-to-Eat Salads Treated with Biological Extracts. Journal of Food Protection, 2024, 87 (3), pp.100232. <10.1016/j.jfp.2024.100232>. <pasteur-04973220>

HAL Id: pasteur-04973220

<https://riip.hal.science/pasteur-04973220v1>

Submitted on 2 Mar 2025

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Research Paper

Effect of Gamma Irradiation on Pathogenic *Staphylococcus aureus* in Packaged Ready-to-Eat Salads Treated with Biological Extracts

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ARTICLE INFO

Keywords:

Cytotoxicity
Decontamination
Food pathogens
Natural antimicrobials
Packaged Ready-to-eat salads
 γ -irradiation

ABSTRACT

Providing pathogen-free ready-to-eat (RTE) salads is critical for all consumers, especially individuals with weakened immunity. In this study, the efficacy of γ -irradiation on *Staphylococcus aureus* (*S. aureus*) in freshly packaged salads (4.24 log CFU/g) treated with essential oil (EO) and myrtle juice during 10 days of storage and their impact on organoleptic properties were investigated. EO was extracted by hydrodistillation and the chemical composition was analyzed by gas chromatography with Flame Ionization Detector (GC/FID) and gas chromatography/mass spectrometry (GC/MS). Myrtle juice was prepared from fresh fruits. The cytotoxic effects of *Thymus capitatus* (*T. capitatus*) EO against a normal human umbilical vein endothelial cell (HUVEC) were assessed. GC/FID and GC-MS analysis of the thyme EO revealed the presence of 13 compounds, including carvacrol (79.55%) and *p*-cymene (7.93%) as major components. The EO was found to be noncytotoxic, with concentrations lower than 0.16 μ L/mL. A reduction of more than 3 log CFU/g and a total inactivation of *S. aureus* were achieved with the combination of gamma irradiation at 0.5 kGy with myrtle juice at 6 μ L/mL and EO at 0.08 μ L/mL, respectively. The treatment of fresh RTE salads with thyme and myrtle juice was evaluated as acceptable by the sensory panel. The combined effect showed a synergistic potential on the inactivation of *S. aureus*.

Fresh and minimally processed foods are increasingly consumed due to consumers' awareness of the importance of eating fresh products. However, minimally processed fresh vegetables are more susceptible to spoilage because of their high water and nutrient content. Typical fresh produce processing does not provide an effective kill-step against foodborne pathogens. In fact, these products have been associated with foodborne outbreaks (Gurler et al., 2015; Yousef et al., 2020) and various pathogens are causing foodborne illness. Remarkably, *S. aureus* is recognized as one of the most important foodborne pathogens that cause food poisoning worldwide (Fetsch & Jöhler, 2018). Many foods could be contaminated by *S. aureus*, especially fresh-cut vegetables, due to manual handling or contaminated raw materials (Olaimat et al., 2021; Rahmani et al., 2021). *S. aureus* produces staphylococcal enterotoxins (SEs) causing severe symptoms such as violent vomiting, abdominal cramping, nausea, and diarrhea

(Lin et al., 2019; Olaimat et al., 2021). Furthermore, the antibiotic resistance of pathogenic *S. aureus* poses a great human health risk and a public safety concern (Lin et al., 2019; Shahraz et al., 2012). Ensuring the security and the quality of food is particularly necessary for people with weakened immunity, because foodborne illnesses are a major contributing factor of morbidity and mortality in immunosuppressed patients. Hence, to prevent foodborne illnesses, these patients must avoid consumption of high-risk foods, such as fresh produce, which restricts healthy fruits and vegetable choices from their diets (Feliciano et al., 2014; Feliciano et al., 2017; Lee et al., 2012).

RTE salads are raw consumed that do not undergo a kill-step before consumption in order to maintain fresh-like quality and nutritional value (Olaimat et al., 2021). Decontamination with disinfectants such as chlorine and citric acid is widely used by the majority of the fresh-cut industry to improve safety and shelf life profiles, but these

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<https://doi.org/10.1016/j.jfp.2024.100232>

Received 11 May 2023; Accepted 21 January 2024

Available online 24 January 2024

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processes have presented some limitations and disadvantages. It has been shown that the concentrations of chlorine (up to 200 ppm free chlorine) usually used to decontaminate freshly cut vegetables are not effective in reducing pathogens load (de Medeiros Barbosa et al., 2016) with the possible formation of carcinogenic chlorinated compounds (Gündüz et al., 2009, 2010; Gutierrez et al., 2009; Rahmani et al., 2021). Therefore, the development of alternative treatments and natural nontoxic preservatives to improve the safety of fresh-cut vegetables is a topic of great interest (Gündüz et al., 2010; Gutierrez et al., 2009; Rahmani et al., 2021).

In recent years, the use of natural antimicrobial agents for food preservation against foodborne pathogens constitutes a good alternative to chemicals since they are effective and safe for human consumption (Tawema et al., 2016). Numerous natural antimicrobials have been investigated for their potential use as food preservatives. Among natural antimicrobial agents, EOs are volatile secondary metabolites extracted from aromatic plants (Ndoti-Nembe, Vu, Doucet, et al., 2015). Many EOs are considered to be “generally recognized as safe” (GRAS) for natural food preservatives (Gündüz et al., 2009). They have been used effectively to control food spoilage and foodborne pathogenic bacteria associated with RTE vegetables (Gutierrez et al., 2009; Ndoti-Nembe, Vu, Han, et al., 2015; Tawema et al., 2016). Herbs such as rosemary, thyme, and fennel seeds are known for their content of EOs and phenolic compounds, which confer biological activity (Hanana et al., 2017; Khammassi et al., 2018). However, high volatility, low water solubility and effects on organoleptic quality limit their use in food systems (He et al., 2021; Maisanaba et al., 2017). Furthermore, the inappropriate use of EOs has potentially toxic effects on human health. Indeed, there is a demand for a thorough assessment of their safety, which is becoming increasingly complicated as the use of EOs is not subject to regulatory control in many countries (Sinha et al., 2014).

Myrtle (*Myrtus communis* L.) is a medicinal and aromatic plant that belongs to the family *Myrtaceae*, widely distributed in the Mediterranean region (Cheikh et al., 2020). In Tunisia, myrtle extract has been used traditionally for the treatment of gastrointestinal disorders. These extracts have also demonstrated antimicrobial activity against various foodborne pathogens since they are rich in bioactive molecules such as phenolic compounds (Jabri et al., 2016).

Innovative food preservation technologies have been developed using hurdle treatments to improve food safety and preserve the nutritional and organoleptic qualities of foods. In addition, these treatments have shown synergistic effects when used in combination to inactivate the targeted microorganisms (Ben-Fadhel et al., 2021; Khan et al., 2017).

Among food preservation techniques, irradiation is recognized as an effective and safe intervention, applied at appropriate dose to ensure food safety and to extend the shelf-life without affecting the organoleptic and nutritional qualities of foods. It is a promising technique and can be applied as postfood processing treatment (on the end-products such as packaged fresh products) (Ben-Fadhel et al., 2021; Ndoti-Nembe, Vu, Doucet, et al., 2015; Oduke et al., 2016; Tawema et al., 2016). Irradiation has been recognized as safe and wholesome by the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the International Atomic Energy Agency (IAEA) (Shankar et al., 2020). Food irradiation is currently approved in over 60 countries worldwide (Ben-Fadhel et al., 2021).

Previous studies reported that the addition of various natural antimicrobial compounds into vegetables before applying γ -irradiation could lead to an increase in the radiosensitization of foodborne pathogens (Ndoti-Nembe, Vu, Han, et al., 2015; Tawema et al., 2016; Turgis et al., 2012). This treatment enables the reduction of irradiation doses as well as the concentration of the antimicrobial compound required to inactivate pathogenic microorganisms (Ben-Fadhel et al., 2021; Caillet et al., 2006; Tawema et al., 2016).

In this study, we aimed to evaluate the antimicrobial activities of *T. capitatus* Hoffmanns. & Link, *Rosmarinus officinalis* L. (*R. officinalis*) and *Foeniculum vulgare* Mill (*F. vulgare*) EOs and myrtle juice, as well as determine the chemical composition of the selected EOs by GC/MS. We also aimed to evaluate the cytotoxicity of *T. capitatus* using the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) and the combined effect of natural antimicrobials and low dose γ -irradiation against *S. aureus*. A sensory analysis of the RTE salads treated with thyme EO and myrtle juice was also conducted.

Materials and methods

Plant material. Leaves of *T. capitatus* and *R. officinalis* were collected from Djebel Mansour, Zaghuan (North Tunisia) during the months of July and August 2020, while seeds of *F. vulgare* were harvested from Thibar, a region located in Beja in the Northwest of Tunisia, during the month of November 2020. Plant identification was insured by the Laboratory of Forest Ecology in the National Research Institute of Rural Engineering, Water and Forests (INRGREF), and voucher specimens (1TCZ20-7, RO20-8, FV20-1) were submitted to the herbarium section of the INRGREF. Myrtle berries (*Myrtus communis* L.) were collected from the region of Tabarka located in the Northwest of Tunisia.

Essential oil extraction and myrtle juice preparation. EOs were extracted by hydrodistillation of 200 g of dried samples with 2 liters of distilled water, using a Clevenger-type apparatus for 3 hours according to the European Pharmacopoeia (2004). The extracts were dried over anhydrous sodium sulfate and stored at 4°C until analysis. The extraction yield was calculated based on the volume of oil (mL) per dried leaves/seeds weight (g) multiplied by 100.

Myrtle juice was prepared after washing and grinding steps of the fruits. The juice was then filtered (filter size: 0.45 μ m) and preserved at -80°C until use.

GC/FID analysis. In this study, the EOs were analyzed using a Hewlett-Packard 5890 equipped with a FID and HP-5 MS capillary column (5% phenyl/95% dimethyl polysiloxane: 30 m \times 0.25 mm id, film thickness 0.25 μ m). The injector and the temperature detector were set at 250°C and 280°C, respectively. Oven temperature was kept at 50°C for 1 min; then, it was gradually raised to 250°C with an average of 5°C/min and held isothermal for 4 min. Helium, used as a carrier gas, was kept at a flow rate of 1.2 mL/min. Samples were diluted in hexane (1/100, v/v); then, 1.0 μ L of each was injected manually in splitless injection mode.

GC/MS analysis. For GC/MS detection, an electron ionization system, with an energy of 70 eV, a scan time of 1.5 s and a mass range of 40–300 amu. Helium was the carrier gas at a flow rate of 1.2 mL/min. Injector and transfer line temperatures were set at 250°C and 280°C, respectively. The oven temperature program was the same used for GC/FID analysis. 1.0 μ L of each diluted sample (1/10 in hexane, v/v) was injected manually in the splitless injection mode. Oil component identification was based on their Kovats retention indices and their comparison with those described in literature (Adams, 2007; Davies, 1990; Jennings & Shibamoto, 1980). The mass spectra were compared with those available in the NIST 02 and Wiley 275 mass spectra libraries.

Microbial culture and inoculum preparation. The bacterial strain used in this study was isolated from RTE salad samples. Isolated *S. aureus* strains were subjected to biochemical tests (Gram staining, production of catalase, coagulase test) and API Staph® tests (BioMérieux SA, France) for confirmation.

An inoculum of *S. aureus* was prepared using a loop of cells previously grown on Tryptic Soy Agar (Biokar diagnostics, France) in 20 mL of Tryptic Soy Broth (Biokar diagnostics, France). The turbidity of the cell suspension was measured at 600 nm and adjusted to the required

concentration of 10^7 CFU/mL for minimum inhibitory concentrations (MIC) determination and 10^4 CFU/mL for in situ tests.

Agar-well diffusion method. The antimicrobial activity of thyme, fennel, rosemary EOs, and the myrtle juice against *S. aureus* was tested using the agar well diffusion assay as described by Abdollahzadeh et al. (2014). Briefly, 100 μ L of bacteria suspension (10^7 CFU /mL) was spread on to Mueller–Hinton Agar (Biokar diagnostics, France). Afterward, wells (6 mm diameter each) were pierced in the agar and filled each with 5 μ L of EOs or 50 μ L of myrtle juice. Ampicillin was used as a positive control.

Inhibition zone diameters (clear zone) were measured after 24 h of incubation at 37°C. The scale of measurement was the following (disk diameter included): low activity (inhibition zone \leq 12 mm diameter), moderate activity (12 mm < inhibition zone < 20 mm), and strong activity (inhibition zone \geq 20 mm diameter) (Rota et al., 2008). Tests were performed in triplicate.

Determination of minimal inhibitory concentration. The natural antimicrobials that previously showed antimicrobial activity (\geq 12 mm zone of inhibition diameter) were screened for determination of MICs. The MIC of thyme EO and myrtle juice was determined using the broth macrodilution procedure adapted by Lv et al. (2011). Firstly, Serial twofold dilutions were prepared in Brain Heart Infusion Broth (Biokar diagnostics, France), with concentrations ranging between 0.016 μ L/mL and 5 μ L/mL for EO and 3.12 μ L/mL to 100 μ L/mL for juice. Then, a 500 μ L bacterial inoculum (10^7 CFU/mL) was added to each tube and incubated at 37°C for 18–24 h. MIC was defined as the lowest concentration of an antimicrobial agent required to prevent visible bacterial growth. A negative control without the antimicrobial agent was tested similarly.

Cell lines and cytotoxicity assay. HUVEC cell line was provided by Pasteur Institute, Tunis, Tunisia. RPMI 1640 (Capricorn) supplemented with 10% fetal calf serum and 1% (w/v) antibiotic (Ampicillin - Streptomycin) was used for cell culture. HUVEC cells were grown and maintained at 37°C in a 5% CO₂ humidified atmosphere.

MTT test. The viability of HUVEC cells after treatment with EO was determined by MTT assay as described by Shakeri et al. (2014). Briefly, HUVEC cells were plated at a concentration of 10^4 cells/well (100 μ L per well) into 96-well plates and incubated over night at 37°C in 5% CO₂ to attach. Then, EO was solubilized in dimethyl sulfoxide (DMSO) and diluted in culture medium. The maximum concentration of DMSO in each well was kept below 0.5%. *T. capitatus* EO was added to the wells in triplicate at concentrations ranging from 0.02 μ L/mL to 0.63 μ L/mL and was exposed for 24 h. At the end of the incubation time, the medium was discarded and MTT was added to each well at a final concentration of 0.5 mg/mL. The plates were incubated for 4 h to allow mitochondrial reduction in MTT into formazan applied to viable cells. Then, 100 μ L of DMSO was added and mixed thoroughly to dissolve the dark blue crystals of formazan. Two negative controls were used: untreated cells and cells with DMSO. Tests were repeated 3 times separately, and each condition in each assay was done in triplicate ($n = 3 \times 3$).

The absorbance was measured at 570 nm using a microplate reader (Thermo Scientific Multiskan®), and the percentage of viability was determined using the formula: % viability = ((absorbance of untreated cells – absorbance of treated cells)/absorbance of untreated cells) \times 100.

The morphological changes of HUVEC cells treated with the EO of *T. capitatus* for 24 h were assessed using a digital high-definition color Leica-MC 170 HD camera (SG) coupled with a Leica Microsystems optical microscope (Wetzlar, Germany) Ltd. The images were treated by Leica Application Suite (version 4.9.0).

Collection of salad samples and inoculation procedure. RTE salads were purchased from local supermarket in Tunisia. Commercialized salads were composed of lettuce, cherry tomato, red cabbage, and julienne carrots; they are packaged in plastic boxes. The shelf life indicated in salads was 5 to 6 days. A total of 15 RTE salads samples were

subjected to E-beam irradiation at a dose of 4 kGy at the radiotreatment unit of National Center for Nuclear Sciences and technologies, Tunisia (CNSTN) to eliminate the potential background of *S. aureus*.

For inoculation, 200 μ L of the inoculum were spotted into the surface of 25 g of packaged salad. The artificially contaminated fresh produce samples were dried for 2 h at 22°C and held for 22 h at 4°C (Lang et al., 2004).

Decontamination using E-beam process. Decontamination was carried out using electron-beam accelerator (CIRCE 3, SGN, France) with an energy level of 7.5 MeV, an average dose rate of 40 kGy/min and an average current of 0.3 mA at room temperature. Regarding dose verification systems, B3 DoseStix Dosimeters - B300XDS (GEX B3 Film Dosimeter Products) was used.

Treatment with natural antimicrobials. Concerning EO treatment, in the light of the cytotoxicity assay results, an antimicrobial solution (0.08 μ L/mL) was prepared in sterile distilled water in the presence of 0.5% (w/v) prefiltered DMSO (0.20 μ m) as an emulsifying agent. Afterward, the salads were submerged in the antimicrobial treatment solutions for 5 minutes with gentle manual shaking.

For juice treatment, the antimicrobial solution (6 μ L) was uniformly sprayed (0.3 mL per 25 g of sample) under sterile conditions on RTE salad samples. All samples were then allowed to dry for 30 min under a laminar flow hood. After drying, packaged salads were stored at 4°C overnight prior to irradiation treatment. Untreated samples were used as controls.

Gamma irradiation treatment. Packaged RTE salad samples were irradiated at a dose of 0.5 kGy using Cobalt 60 source (Manufacturer: FRANCE/CANADA) with a dose rate of 19.59 Gy/min and a homogenization index of 1.03. The dose rate is determined through irradiation of a punnet of salad (dose mapping) and determination of the zone where the dose is minimal. Dose rate was determined as the ratio between this minimum dose and irradiation time. The irradiator is calibrated using a defined point located at a defined distance and height from the source center, the dose rate at a specific point should be measured at the beginning of irradiation process, allowing to calculate the irradiation time needed to obtain the required dose. The dosimetry type used to perform irradiation is HARWELL Perspex (Red, Amber). The temperature during irradiation was \leq 22°C (room temperature), which is maintained by a cold air conditioning system installed on the roof.

The microbial analysis was performed immediately (day 0) and after 6, 8, and 10 days of storage period at 4°C. The experimental design included 4 groups of treatments: (1) Untreated salads (control); (2) irradiated salads at 0.5 kGy; (3) treated salads (EO and irradiation at 0.5 kGy); (4) treated salads (juice and irradiation at 0.5 kGy).

Sensory evaluation. An acceptance test was performed to evaluate the sensory attributes of salad with or without EO or juice. A nine-point structured scale was used to indicate a range from 1 (I like it very much) to 9 (I extremely dislike it). The sensory parameters were evaluated for odor, color, flavor, appearance, and overall acceptability by a panel of 30 untrained judges (Gutierrez et al., 2009).

Statistical analysis. The data were analyzed with the SPSS software (IBM SPSS Statistics), and the mean comparison among treatments was based on Duncan's test ($p \leq 0.05$). TIBCO® STATISTICA Data Science was used for graphics generation.

Results and discussion

Chemical composition of the Thyme, Fennel, and Rosemary essential oils. Chemical compositions of EOs of thyme, rosemary, and fennel were analyzed by GC/MS technique. The oils components, Kovats retention indices, area percentages, and chemical classes of tested EOs are shown in Table 1.

GC–MS analyses of the oil samples showed the presence of a total of 32 components distributed in four terpene classes. All oils showed a

Table 1
Essential oils composition of *T. capitatus*, *R. officinalis*, and *F. vulgare*

Peak	Component	Formula	Chemical class	RI ^a	<i>T. capitatus</i>	<i>R. officinalis</i>	<i>F. vulgare</i>
1	tricyclene	C ₁₀ H ₁₆	MH ^b	926	–	0.07	–
2	α-pinene	C ₁₀ H ₁₆	MH ^b	939	0.31	8.15	1.99
3	camphene	C ₁₀ H ₁₆	MH ^b	954	–	4.13	0.07
4	sabinene	C ₁₀ H ₁₆	MH ^b	975	–	–	0.41
5	β-pinene	C ₁₀ H ₁₆	MH ^b	979	–	3.26	–
6	β-myrcene	C ₁₀ H ₁₆	MH ^b	990	1.02	1.08	0.38
7	α-phellandrene	C ₁₀ H ₁₆	MH ^b	1002	–	0.09	0.20
8	δ-3-carene	C ₁₀ H ₁₆	MH ^b	1011	–	0.20	–
9	α-terpinene	C ₁₀ H ₁₆	MH ^b	1017	0.86	0.21	–
10	<i>p</i> -cymene	C ₁₀ H ₁₄	MH ^b	1024	7.93	–	–
11	limonene	C ₁₀ H ₁₆	MH ^b	1029	–	–	8.00
12	1,8-cineole	C ₁₀ H ₁₈ O	OM ^c	1031	–	38.69	–
13	γ-terpinene	C ₁₀ H ₁₆	MH ^b	1059	2.57	0.62	0.74
14	fenchone	C ₁₀ H ₁₆ O	OM ^c	1086	–	–	7.69
15	α-terpinolene	C ₁₀ H ₁₆	MH ^b	1088	–	0.34	–
16	linalool	C ₁₀ H ₁₈ O	OM ^c	1096	–	1.34	–
17	β-linalool	C ₁₀ H ₁₈ O	OM ^c	1096	1.55	–	–
18	fenchol	C ₁₀ H ₁₈ O	OM ^c	1116	–	0.13	0.13
19	camphor	C ₁₀ H ₁₆ O	OM ^c	1146	–	16.61	0.28
20	borneol	C ₁₀ H ₁₈ O	OM ^c	1169	0.23	7.01	–
21	terpinen-4-ol	C ₁₀ H ₁₈ O	OM ^c	1177	0.83	1.27	0.07
22	α-terpineol	C ₁₀ H ₁₈ O	OM ^c	1188	–	4.93	–
23	(<i>Z</i>)-anethole	C ₁₀ H ₁₂ O	OM ^c	1252	–	–	79.25
24	thymol	C ₁₀ H ₁₄ O	OM ^c	1290	0.72	–	–
25	carvacrol	C ₁₀ H ₁₄ O	OM ^c	1299	79.55	–	–
26	carvacryl acetate	C ₁₂ H ₁₆ O ₂	OM ^c	1372	0.15	–	–
27	α-bornyl acetate	C ₁₂ H ₂₀ O ₂	OM ^c	1418	–	0.89	–
28	β-caryophyllene	C ₁₅ H ₂₄	SH ^d	1419	2.09	1.57	–
29	β-selinene	C ₁₅ H ₂₄	SH ^d	1490	–	0.20	–
30	δ-cadinene	C ₁₅ H ₂₄	SH ^d	1523	–	0.12	–
31	spathulenol	C ₁₅ H ₂₄ O	OS ^e	1590	–	0.46	–
32	caryophyllene oxide	C ₁₅ H ₂₄ O	OS ^e	1582	0.93	1.05	–
Total identification (%)						98.74	99.21
Monoterpene hydrocarbons (%)						12.69	11.79
Oxygenated monoterpenes (%)						83.03	87.42
Sesquiterpene hydrocarbons (%)						2.09	–
Oxygenated sesquiterpenes (%)						0.93	1.51

–, not detected.

^a RI, retention indices on HP-5MS column.

^b MH, monoterpene hydrocarbons.

^c OM, oxygenated monoterpenes.

^d SH, sesquiterpene hydrocarbons.

^e OS, oxygenated sesquiterpenes.

specific richness in oxygenated monoterpenes (70.87–87.42%) and monoterpenes hydrocarbons (11.79–18.15%). In thyme EO contents and chemical composition, 13 constituents were identified; carvacrol (79.55%) and *p*-cymene (7.93%) are the main components of this oil. It was worth noting that the composition contents of thyme EO in this work are similar to those stated in literature for the same species, yet with slight differences in percentage values. A previous study showed that carvacrol (61.6%) and *p*-cymene (13.7%) were the main components of the EO of *T. capitatus* collected from three different regions in Tunisia (Bounatirou et al., 2007). A high content of carvacrol (73.03%) and a low content of thymol (0.31%) was also identified in *T. capitatus* cultivated in Italy (Granata et al., 2018).

In rosemary EO, 23 components were identified. The major components were 1, 8-cineole (38.69%) followed by camphor (16.61%) and α-pinene (8.15%). Chemical composition of *R. officinalis* EO was previously reported in Tunisia by Hanana et al. (2017), showing the richness of rosemary EO in 1, 8-cineole (41%) and α-pinene (24%). These results are in agreement with those reported in the current work. However, the EO from Turkish *R. officinalis* leaves was rich with *p*-cymene (44.02%), linalool (20.5%), and γ-terpinene (16.62%) (Özcan & Chalchat, 2008).

In the case of fennel EO, 8 components were identified. *Z*-anethole (79.25%), limonene (8.00%), and fenchone (7.79%) were the main volatile constituents. The chemical composition of fennel EO in this

study was different from those reported elsewhere. Estragole (66.09–85.23%), fenchone (5.18–23.09%), and limonene (4.3–10.25%) are the major components of the 16 populations of *F. vulgare* Mill in another study conducted in Tunisia (Khammassi et al., 2018).

In fact, the differences in the chemical compositions observed in analyzed samples could be attributed to differences in geographic origin, the season at the time of the collection, the stage of development, plant material (fresh or dry), or the extraction method. The occurrence of chemotypes can also explain this variability (Khammassi et al., 2018; Lv et al., 2011).

Antimicrobial activity. Antimicrobial inhibition zones and MIC values for EOs and myrtle juice against *S. aureus* are shown in Table 2.

The EOs displayed a variable degree of antimicrobial activity against pathogenic *S. aureus* isolated from RTE salad. The thyme EO showed the highest activity, with an inhibition zone greater than 34 mm. Meanwhile, *S. aureus* exhibited a low susceptibility to rosemary, with 9 mm in diameter inhibition halo. However, *S. aureus* was highly resistant to EO of fennel. Myrtle juice had an antimicrobial activity against *S. aureus* with an inhibition zone of 17.33 mm.

Several studies investigated the effects of *T. capitatus* EO on *S. aureus* (Bounatirou et al., 2007; El-Jalel et al., 2018; Moumni et al., 2020). The results showed that thyme oils had strong antimicrobial activity against the tested strain. The high antimicrobial activity of *T. capitatus*

Table 2Antimicrobial activity of investigated essential oils and myrtle juice against *S. aureus* isolated from RTE salad

Antimicrobial agents	Inhibition zone diameter(mm)	MIC ($\mu\text{L}/\text{mL}$)
EOs		
<i>T. capitatus</i>	35.33 \pm 1.15	0.63
<i>R. officinalis</i>	9 \pm 0.0	ND ^a
<i>F. vulgare</i>	–	ND ^a
Myrtle juice	17.33 \pm 0.57	6

Values are reported as mean \pm standard deviation of three replicates.

–, indicates no zone of inhibition.

^a ND, not determined.

EO investigated in this work could be explained by the presence of a significant quantity of monoterpenoid phenol carvacrol (79.55%). In fact, carvacrol can affect the bacterial membrane permeability, causing structural and functional damages to cytoplasmic membrane, leading to cell death (Bounatirou et al., 2007; Granata et al., 2018; Moumni et al., 2020).

The myrtle juice was demonstrated to be active against *S. aureus*. Similarly, myrtle (*Myrtus communis* L.) seed extract from Tunisia showed a significant reduction in the growth of all tested bacterial strains, including *S. aureus* (Jabri et al., 2016). The antibacterial activity of the aqueous extract could be attributed to its richness in phenolic compounds, especially flavonoids and anthocyanins (Harich et al., 2017).

The MIC values confirmed the results obtained by the agar disk diffusion method (Table 2). Thyme EO had a lower MIC (0.63 $\mu\text{L}/\text{mL}$) for the tested microorganism, while myrtle juice exhibited a MIC value of 6 $\mu\text{L}/\text{mL}$. A similar MIC value (0.73 $\mu\text{L}/\text{mL}$) of *T. capitatus* against *S. aureus* was reported by Moumni et al. (2020).

In our study, *R. officinalis* EO showed relatively low antimicrobial activity compared to thyme EO. Similar result was reported by Lopez et al. (2005), showing that rosemary oil had low inhibitory effects against *S. aureus* compared to other oils. This low inhibitory activity of *R. officinalis* EO might be explained by its richness in 1, 8-cineole, camphor, and α -pinene, which are known to have a lower antimicrobial effects than phenolic and alcoholic compounds (Soulaïmani et al., 2021).

No noticeable inhibitory effect of fennel oil was observed in this study. Similarly, *F. vulgare* Mill EO was shown to be mildly effective against *B. subtilis*, *P. aeruginosa*, and *S. aureus* (Diao et al., 2014). Meanwhile, several studies have shown that fennel EO was effective against foodborne pathogenic bacteria and fungi (Dadaloğlu & Evrendilek, 2004; Ghasemian et al., 2020). The difference in antimicrobial activity of fennel oils may be related to strain susceptibility as well as the composition of the EO, which lacks components associated with higher antimicrobial activity, such as carvacrol and thymol (Burt, 2004).

Cytotoxicity assay. In recent decades, there has been increasing interest in natural alternatives to chemically synthesized antimicrobials for fresh vegetable preservation, with particular attention to EOs as promising natural compounds for their diverse biological activities (de Medeiros Barbosa et al., 2016). But, the inappropriate use of EOs may cause risks to consumer's health (Sinha et al., 2014). In fact, EOs can be acutely toxic at low concentrations due to their variable chemical composition (Lanzerstorfer et al., 2021). They have been used traditionally for a long time, but most of the researches have recently focused on their toxicity (Prakash et al., 2018). To the best of our knowledge, no data was reported concerning the cytotoxicity of EOs or the morphological alterations they cause in human cell lines before their use as preservatives in fresh vegetables. Only MIC was determined in literature (Table 3). Carvacrol and thymol were the main components in EOs used to control pathogenic bacteria in fresh vegetables (De Azeredo et al., 2011; de Medeiros Barbosa et al.,

2016; Gutierrez et al., 2008). These compounds have shown cytotoxic activity against normal human cells (Džamić et al., 2015; Ranjitkar et al., 2021). Hence, further investigation of the EO toxicity is required to ensure its safe use as a preservative in RTE vegetables.

To investigate the biosafety of the *T. capitatus* EO on human health, normal human cell lines were treated with various concentrations of EO for 24 h and then submitted to the MTT test. Thyme EO has growth inhibition effects on normal human cells in a dose-dependent manner as shown in Figures 1 and 2. At a concentration of 0.63 $\mu\text{L}/\text{mL}$, the thyme EO exhibited a cell viability of 10.42%. Based on these data, we calculated the IC₅₀, which represents the concentration of thyme EO required to reduce cell viability by 50%. The IC₅₀ value was estimated at 0.16 $\mu\text{L}/\text{mL}$. This is consistent with the finding of a previous study performed by Džamić et al. (2015), showing a cytotoxic effect of *T. capitatus* EO on normal lung fibroblasts MRC-5 (IC₅₀ = 0.15 $\mu\text{L}/\text{mL}$). The cytotoxicity of *T. capitatus* EO on a human keratinocyte cell line was also observed by Kapustová et al. (2021) with an IC₅₀ of 0.176 $\mu\text{L}/\text{mL}$, which is extremely in accordance with our results. Cytotoxicity of thyme EO might be attributed to its natural lipophilic molecules that accumulate in the cell membranes; this induces an increase in cell permeability, a subsequent leakage of enzymes, and metabolites leading to cell death (Shakeri et al., 2014). EOs demonstrated increased antimicrobial activities and were found to be promising for food preservation. Therefore, specific ISO standards are needed to establish legal aspects to give general usage guidelines and authorize maximum permissible limits (Prakash et al., 2018).

Antimicrobial effect of combined preservation technologies against *S. aureus* during storage of RTE salads. The antimicrobial inactivation by gamma irradiation combination technologies was evaluated against *S. aureus* during storage at 4°C of RTE salads. The first combination strategy was carried out using EO, and the second using myrtle juice.

The concentration of *S. aureus* in untreated salads was 4.24 log CFU/g at Day 0. In the control sample (Unirradiated salads without natural antimicrobials treatment), the significant increase in *S. aureus* concentration was observed after day 6 (5.39 log CFU/g) compared to day 0 of storage. In the irradiated packaged salad samples at 0.5 kGy, a reduction of 2.44 log at day 0 and a reduction of 1.48 log at day 6 compared to the control ($p \leq 0.05$) at the same day of treatment were observed, respectively. However, with this treatment, there was an increase in bacterial concentration from day 6 to day 10 compared to day 0 of treatment (Figs. 3 and 4). In fact, a low dose of gamma irradiation has been reported to be effective against pathogenic bacteria on fresh vegetables. According to Rahmani et al. (2021), irradiation treatment at 0.5 kGy reduced the initial concentration of *S. aureus* by 2.48 log in freshly packaged carrot salads. These results are in agreement with those obtained in this present study. A reduction in the level of *Listeria* inoculated in RTE carrots by 1.31 log at a dose of 0.5 kGy was also reported by Turgis et al. (2012).

The treatment of RTE salad samples with a subinhibitory concentration (noncytotoxic concentration) of *T. capitatus* EO, combined with gamma irradiation at a dose of 0.5 kGy, reduced significantly ($p \leq 0.05$) the growth of *S. aureus* below detectable levels, compared to an initial count of 4.24 log CFU/g in untreated salads. Moreover, *S. aureus* was undetectable up to 10 days of storage (Fig. 3).

Several combinations of natural antimicrobials and gamma irradiation are widely used to inhibit the growth of microbial pathogens. The irradiation of mini-carrots at 0.5 kGy with mountain savory EO induces a reduction in the concentration of *Salmonella typhimurium* by 3.8 log at day 1 and was under the detection limit at day 9 (Ndoti-Nembe, Vu, Han, et al., 2015). Furthermore, the antimicrobial effect of mountain savory EO and low-dose γ -irradiation was studied to eliminate *L. monocytogenes* during refrigerated storage of RTE carrots. This combination effectively eliminated *L. monocytogenes* in carrot samples (Ndoti-Nembe, Vu, Doucet, et al., 2015). Another study focusing on the combination of low doses gamma irradiation with natural

Table 3
Application of EOs in fresh and minimally processed vegetable products

Essential oil	Product	MIC or applied concentration	Cytotoxicity assays performed	References
Rosemary	Fresh leafy vegetables	10 $\mu\text{L}/\text{mL}$	NP ^a	de Medeiros Barbosa et al., (2016).
Thyme	Minimally processed lettuce	0.25 $\mu\text{L}/\text{mL}$	NP ^a	Siroli et al., (2015).
Oregano	Minimally processed vegetables	5 $\mu\text{L}/\text{mL}$	NP ^a	De Azeredo et al., (2011).
Rosemary	Minimally processed vegetables	40 $\mu\text{L}/\text{mL}$	NP ^a	De Azeredo et al., (2011).
Oregano	Fresh-cut iceberg lettuce	0.025, 0.04 and 0.075 $\mu\text{L}/\text{mL}$	NP ^a	Gündüz et al., (2010).
Myrtle	Iceberg lettuce and tomatoes	0.5, 0.75 and 1 $\mu\text{L}/\text{mL}$	NP ^a	Gündüz et al., (2009).
Combined mixture of oregano and thyme	Ready-to-eat lettuce and carrots	0.125 $\mu\text{L}/\text{mL}$ (Oregano) 0.25 $\mu\text{L}/\text{mL}$ (Thyme)	NP ^a	Gutierrez et al., (2009).
Thyme	Minimally processed lettuce and carrots	0.5 $\mu\text{g}/\text{mL}$	NP ^a	Gutierrez et al., (2008).
Thyme	Romaine lettuce and baby carrots	0.1–10 $\mu\text{L}/\text{mL}$	NP ^a	Singh et al., (2002).

^a NP, not performed.

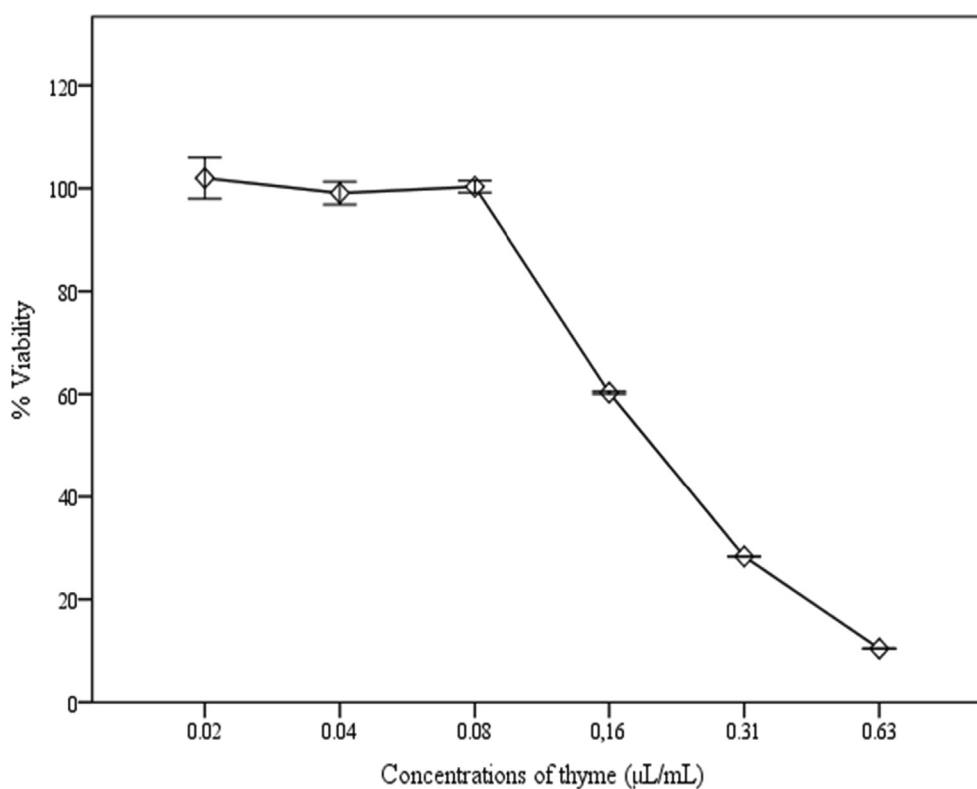


Figure 1. Cytotoxic activity of *Thymus capitatus* essential oil against HUVEC cells. Tests were repeated 3 times separately, and each condition in each assay was done in triplicate ($n = 3 \times 3$).

antimicrobial formulations showed that this process was effective against *L. monocytogenes* and *E. coli* populations. In fact, bacterial concentrations were reduced below the detection levels during the storage period of freshly cut cauliflower (Tawema et al., 2016).

Despite the demonstrated antimicrobial potential of *T. capitatus* EO, its use as a preservative in RTE salads has been limited due to its toxicity, high volatility, low water solubility, strong sensory properties, and the high concentrations required to achieve wide use antimicrobial efficacy (He et al., 2021; Maisanaba et al., 2017; Prakash et al., 2018). In this context, myrtle juice could be considered as a natural potential alternative to ensure microbiological safety and extend the shelf-life of fresh-cut salads without the risk of toxicity.

A reduction of 3.14 log at day 0 compared to the control was observed ($p \leq 0.05$) in salads treated with myrtle juice and irradiation at 0.5 kGy (Fig. 4). Additionally, cell concentration did not signifi-

cantly change at day 6 of storage ($p = 0.958$). After 10 days of storage period, the concentration of *S. aureus* in untreated sample increased to reach 6.17 Log CFU/g and about 3.69 Log CFU/g in treated samples. The reduction of more than 3 log shows the effectiveness of this combined preservation technique which, to the best of our knowledge, was not previously reported in the literature.

The combinations of natural antimicrobials with nonthermal treatments represent a potentially important approach to improve efficacy and reducing the energy or dose required to inactivate foodborne pathogens and spoilage micro-organisms while maintaining or improving product quality (Shankar et al., 2020). According to previous studies, microorganisms that survive after γ -ray treatment will be likely more sensitive to adverse environmental conditions, such as the presence of antimicrobial substances (Caillet et al., 2006; Ndoti-Nembe et al., 2015; Shankar et al., 2020). Indeed, irradiation inactivates

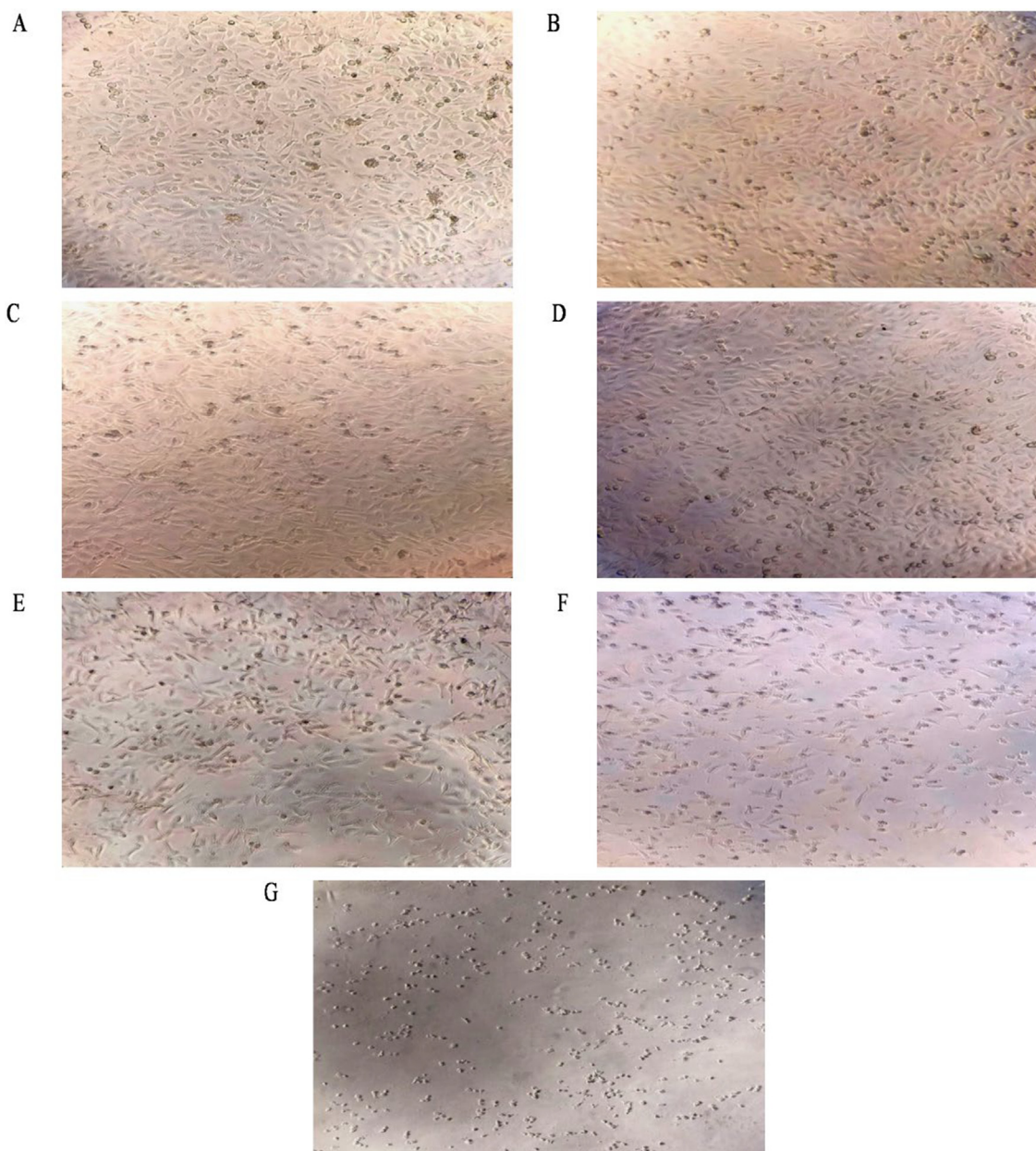


Figure 2. Morphological changes in HUVEC cells after treatment with *Thymus capitatus* essential oil at different concentrations (10X magnification). (A) Control (untreated cells); (B) treated with 0.02 $\mu\text{L}/\text{mL}$; (C) treated with 0.04 $\mu\text{L}/\text{mL}$; (D) treated with 0.08 $\mu\text{L}/\text{mL}$; (E) treated with 0.16 $\mu\text{L}/\text{mL}$; (F) treated with 0.31 $\mu\text{L}/\text{mL}$; (G) treated with 0.63 $\mu\text{L}/\text{mL}$.

microorganisms by breaking chemical bonds within the DNA molecules and alteration of membrane permeability (Lopez-Gonzalez et al., 1999; Tawema et al., 2016), which can facilitate the contact between antimicrobial compounds and cell membranes and consequently enhance their inhibitory effects (Caillet et al., 2006). In the present study, the increased radiosensitization of *S. aureus* was related to the chemical composition of *T. capitatus* which is highly concentrated in phenolic compounds, particularly carvacrol (79.55%). These observations are in agreement with those obtained previously by many researchers (Caillet et al., 2006; Chiasson et al., 2005; Shankar et al., 2020). Regarding myrtle juice, its efficiency is associated with its richness in phenolic compounds (Jabri et al., 2016). Similarly, Harich et al. (2017) showed that the antimicrobial effect of cranberry juice was mainly related to its high content of phenolic compounds. These antimicrobial compounds have the capacity to damage both the cell

wall and the cell membrane and induce cell lysis which facilitates the leakage of cell contents. They also react with bacterial DNA, resulting in cell death (Wu et al., 2008).

Sensory analysis. The sensory attributes evaluated in the RTE salad with or without *T. capitatus* EO and myrtle juice were odor, taste, color, and overall acceptability.

The statistical analyses showed significant differences ($p \leq 0.05$) regarding odor and taste between control and treated samples with *T. capitatus* EO. However, the color and overall acceptability of the control salads were not significantly different ($p > 0.05$) from those of the treated salads (Fig. 5). Our results showed that salads treated with *T. capitatus* EO were evaluated as “like it very much” for aroma and overall acceptability (mean scores 8.04 and 8.08, respectively) and “like it slightly” for taste and color (mean scores 6.24 and 6.80, respectively).

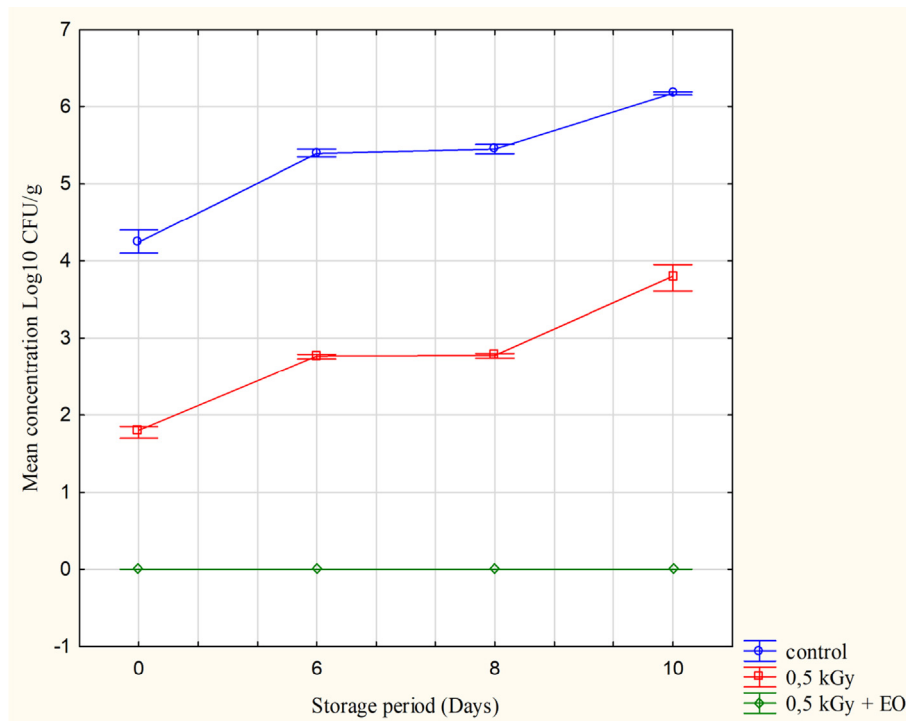


Figure 3. Combined effect of *Thymus capitatus* essential oil and γ -irradiation treatments against *Staphylococcus aureus* inoculated in freshly packaged salads during 10 days of storage at 4°C. Error bars show standard deviations.

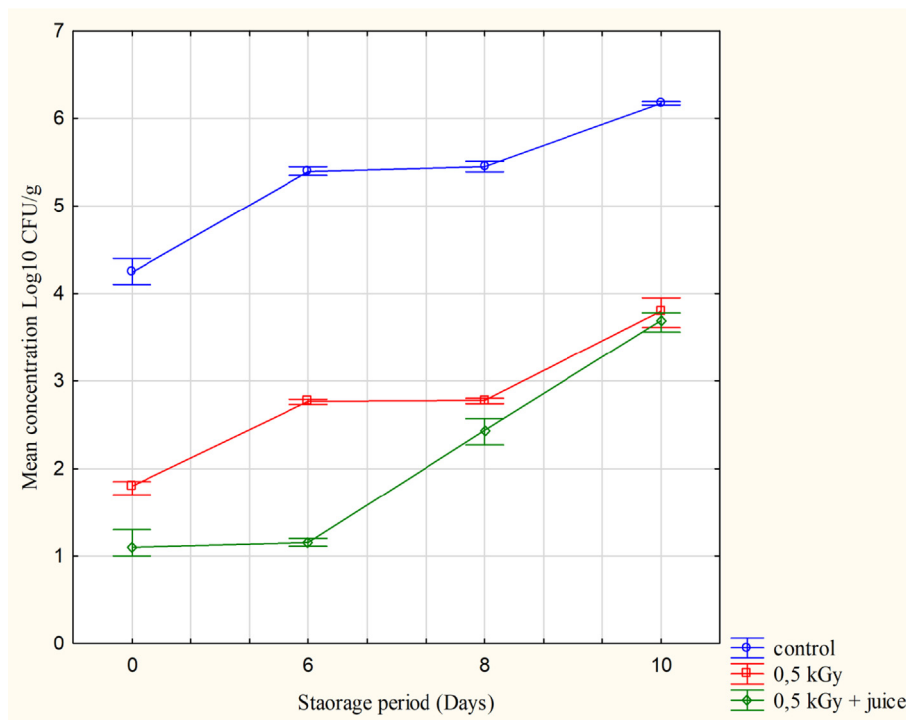


Figure 4. Combined effect of myrtle juice and γ -irradiation treatments against *Staphylococcus aureus* inoculated in freshly packaged salads during 10 days of storage at 4°C. Error bars show standard deviations.

The odor, taste, and overall acceptability of the control salads were not significantly different ($p > 0.05$) from those tested in samples treated with myrtle juice. On the other hand, the color of the treated salads was significantly different from that of the control salads ($p = 0.009$) (Fig. 6). The overall acceptability, taste, and aroma of the salads

containing myrtle juice were “like it very much” (mean average scores between 8.06 and 8.17) and “neither like it nor dislike it” for color (mean average score of 5.28). Siroli et al. (2015) tested the effect of EOs on the quality and sensory properties of minimally processed lettuce and concluded that the presence of thyme EO was not perceived

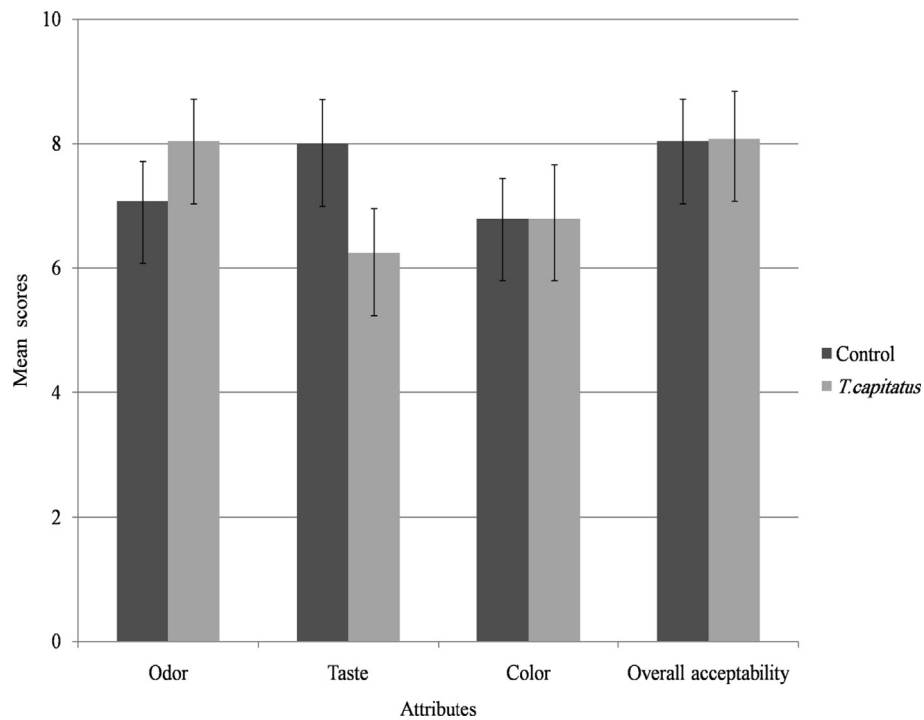


Figure 5. Sensory evaluation results of ready-to-eat salads treated with *Thymus capitatus* essential oil. Error bars show standard deviations.

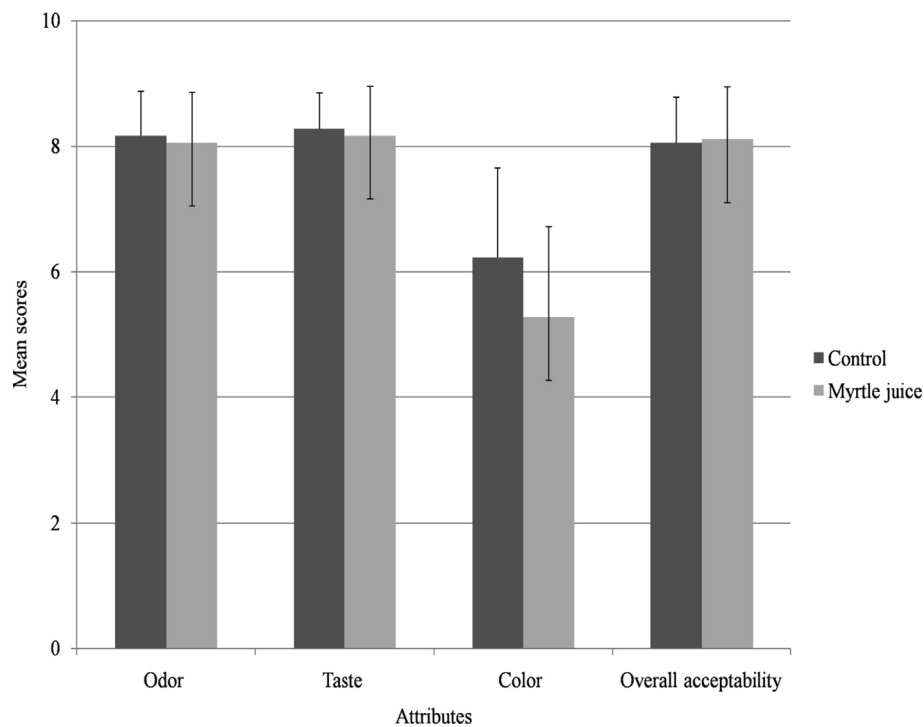


Figure 6. Sensory evaluation results of ready-to-eat salads treated with myrtle juice. Error bars show standard deviations.

as negative by consumers. RTE lettuce treated with oregano at 0.25 $\mu\text{L}/\text{mL}$ was also deemed acceptable by a sensory panel as they found no differences between treated lettuce and that washed with chlorinated water (Gutierrez et al., 2008). In literature, we found no reports about the effects of myrtle juice on the sensory properties of RTE salad.

To the best of our knowledge, the present study could be considered as the first work carried out to investigate the biosafety

of *T. capitatus* EO on human health before its use as a preservative agent on RTE salads. The combined effect of natural antimicrobials with low-dose gamma irradiation generated a synergistic effect to control pathogenic *S. aureus* and extend the shelf-life of RTE salad samples, without a noticeable negative effect on sensory attributes. Combination of gamma irradiation at 0.5 kGy with myrtle juice reduced *S. aureus* concentration by more than 3 logs at day 0. Irradiation at 0.5 kGy, combined with subinhibitory noncytotoxic

concentration of *T. capitatus* EO, can effectively inactivate *S. aureus* for up to 10 days of storage at 4°C. As a result, this innovative preservation process could be considered as an effective tool to provide safe and healthy RTE foods for diverse types of consumers such as immunosuppressed patients.

Author Contribution

Widad ZERNADJI: Carried out the experiments and data statistical analysis, and wrote the manuscript. **Sihem JEBRI:** Conceived the study and revised the manuscript. **Faten RAHMANI:** Aided in the investigation, the data statistical and interpreting analysis. **Ismail AMRI; Dorra AISSAOUI; Med Hedi TRABELSI:** Aided in the investigation and provided the study materials. **Mariem YAHYA:** Provided technical support and designed the experiments. **Islem AMRI:** Designed the experiments and provided the study material. **Fatma HMAIED:** Conceived the study, supervised the project, and edited the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Center for Nuclear Sciences and Technologies (CNSTN). We greatly appreciate the technical staff of the radio-treatment unit (CNSTN) and Pr Lamia HAM-ROUNI (INRGREF).

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