Evaluation of quality and safety of beef hamburgers fortified with Ozonated Extra Virgin Olive Oil

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ABSTRACT

This study investigates the impact of Ozonated Extra Virgin Olive Oil (OEVO) on the shelf life and quality of beef hamburgers during a 15-day storage period. Firstly, the Extra Virgin Olive Oil (EVO) was ozonated, and its oxidative stability and Fatty Acid Profile (FAP) were evaluated before and after ozonation. The oxidative stability of EVO decreased insignificantly ($p > 0.05$) after ozonation with a 3.54% Reduction in Induction Period (RIP). However, ozonation significantly increased ($p < 0.05$) the oleic acid and lignoceric acid contents. Then, the EVO and the produced OEVO were used to formulate two hamburgers. A conventional hamburger was also prepared as the control sample. The quality and safety of the prepared hamburgers were analyzed using TBARS, colorimetric, and microbiological assays. The TBARS index was extremely low, ranging from 0.049 to 0.270 mg MDA/kg hamburgers. There was no significant difference ($p > 0.05$) between the redness (a* value) of hamburgers on different days, excluding days 7, 13, and 15. The combined properties of EVO and ozone had a prominent antimicrobial effect, increasing the shelf life of the beef hamburgers by reducing the number of Total Viable Count (TVC), Lactic Acid Bacteria (LAB), Enterobacteriaceae (EB), and Coliforms (CF).

1. Introduction

Nowadays, meat-based products are consumed more than in the past, owing to their quick preparation (Godfray et al., 2018). These products are considered fundamental components in the human diet as they have a high quantity of vitamins, proteins, and minerals. Among these products, hamburgers are more popular due to their desirable sensory attributes (Borella et al., 2019). However, meat is an ideal substrate for pathogenic bacteria proliferation, and it is susceptible to oxidative degradation, decreasing shelf life and causing unpleasant smells, flavors, and colors over time (Cullere et al., 2018; da Silva et al., 2021). Moreover, the denaturation of myoglobin in ground beef can cause premature browning before cooking, making the meat's color undesirable (Sepe et al., 2005). Therefore, the search for preservation technologies to maintain meat safety and quality is an open question (Zhou et al., 2010).

Adding antioxidant-rich ingredients to meat products is highly used to extend their shelf life, preserve positive sensory characterization, and prevent the damage caused by lipid oxidation (Homayounpour et al.,

2021; Ribeiro et al., 2019). From a clean label perspective in food science, since synthetic antioxidants are identified as disadvantageous substances, significant interest has emerged in natural antioxidants (Barbieri et al., 2021). In this scenario, EVO has a potent antioxidant activity, resulting from a high quantity of polyphenols (e.g., hydroxytyrosol and tyrosol) (Deflaoui et al., 2021). Polyphenols could have a significant antimicrobial effect and impede the oxidative degradation of food products (Ebrahimi & Lante, 2021, 2022). Hence, as a polyphenol-rich oil, EVO could be used as a high-effect antibacterial and antioxidative agent in different food products (Fei et al., 2018; Ramírez-Anaya et al., 2015). Moreover, it shows high oxidative stability without any foreign smells or changes in the structure for a long time (Radzimierska-Kaźmierczak et al., 2021). EVO can enhance the sensory attributes of hamburgers and has positive effects on human health, owing to its nutritional properties (Hur et al., 2008). In fried meat products, it can decrease the formation of heterocyclic amines, which are the etiology of cancer (J. Lee et al., 2011). Therefore, it could be a potential choice to use in the formulation of hamburgers.

As a GRAS-certified and green technology, ozone could positively impact the safety of beef using its antimicrobial effects (Khanashyam et al., 2022; Lyu et al., 2016). This has been studied over many microbes (e.g., Gram-positive, Gram-negative bacteria, fungi, yeast, and spores) since ozone is an emerging antimicrobe against pathogens, widely used in post-harvest treatments, storage, and processing of foods, including meat, fruits, vegetables, etc. (Niveditha et al., 2021). Ozone can quickly auto-decompose into molecular oxygen, leaving no hazardous halogenated substances in the food. Although the low concentration of ozone causes positive effects, its high concentrations can negatively impact food quality by decreasing the bioactive compounds and deteriorating the organoleptic properties (Pandiselvam et al., 2019). As reported by several authors, a 5-min exposure time to ozone is an optimum condition for obtaining antimicrobial properties (Choudhury et al., 2018; Epelle et al., 2022).

From a practical point of view, ozone can be used either as a gas (mixture of ozone and oxygen) or liquid (ozonated water/oil) (Nardi et al., 2020). Ozonated olive oil has noticeable antibacterial, antifungal, antiviral, and antiparasitic properties (Bouzid et al., 2021). To the best of our knowledge, there is no research investigating the effect of ozonated vegetable oil on the quality and safety of ground meat.

This study is intended to investigate the impact of OEVO on the shelf life of beef hamburgers. In this regard, several parameters, including color, lipid oxidation, FAP, TVC, and the growth of LAB, EB, and CF were evaluated during a 15-day storage period.

2. Material and methods

2.1. Materials and reagents

EVO (La Masseria, Italy) and beef were purchased from a local market in Padova, Italy. All chemicals and reagents used in the present paper were of analytical grade. Ringer tablets were purchased from Merck KGaA (Darmstadt, Germany) to prepare Ringer's solution. All the microbiology culture media (MRS, PCA, MCC) were bought from Biolife Italiana (Milan, Italy). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The deionized distilled water was employed for all the trials.

2.2. Sample preparation

Preparing OEVO was carried out on the same day as the hamburgers. The ozone was generated using an ozone generator (Hercules, Poland) from medical oxygen (O_2) with an ozone yield of 1000 mg/h. The ozone generator was operated at 230 V and power of $\leq$40 W. The ozonation process was done at 20 °C for 5 min by flowing ozone in 150 mL of the oil through a porous dispenser (Fig. S1, Supplementary Material). The produced OEVO was subjected to FAP and oxidative stability tests immediately after preparation and was transferred to a hamburger production company in dark glass bottles. The hamburgers were prepared with the help of a food company in Padova, Italy, following the Good Manufacturing Practices (GMPs) and Good Hygienic Practices (GHPs). An initial batch of meat mixture and other ingredients were used to develop three different formulations of hamburger samples, as shown in Table 1. The prepared hamburgers weighed 100 g and were

Table 1
Formulation of different hamburger samples.

Samples	Ingredients' proportion (%)					
	Ground beef	Vegetable fiber	Salt	Water	EVO	OEVO
A	96	2	1	1	–	–
B	94	2	1	1	2	–
C	94	2	1	1	–	2

*A: Control sample, B: Hamburgers with 2% EVO, C: Hamburgers with 2% OEVO.

stored at 4 °C until the subsequent trials on days 1, 2, 5, 7, 9, 12, 13, and 15 of the storage period. The OEVO and the hamburgers were produced in triplicates.

2.3. Analysis of EVO and OEVO

2.3.1. Oxidative stability

The oxidative stability of the OEVO and EVO was measured using the Rancimat method, as described by Tinello et al. (2020). Firstly, 3 g of these oils were weighed and loaded into the Rancimat instrument (Metrohm, model 743, Herisau, Switzerland) to evaluate the oxidative stability over time, which measures the formation of volatile acids produced by the free radical chain reaction. The temperature and airflow set for the oil analysis were 120 °C and 20 L/h, respectively. The oxidative stability is reported as the Induction Period (IP), which corresponds to the time (h) at which the water conductivity (μS/min) starts to increase as a result of the production of compounds involved in the lipid oxidation. The RIP was calculated by Equation (1) (Lante et al., 2011).

$$\%RIP = \frac{IP \text{ of untreated oil} - IP \text{ of ozonated oil}}{IP \text{ of untreated oil}} \times 100 \tag{1}$$

2.3.2. FAP analysis

The content of saturated and unsaturated fatty acids in the OEVO and EVO was characterized using a two-dimensional gas chromatographic analysis (GC × GC), according to the method described by Amare et al. (2021). A gas chromatography Agilent 7890A GC system (Agilent Technologies, Cernusco sul Naviglio, Milano, Italy) equipped with an Agilent 7683 autosampler, a Flame Ionization Detector (FID), and Agilent CFT modulator for GC × GC analysis were used. First, 40 mg OEVO/EVO was mixed with 1 mL of CH_3NaO in methanol (0.5 M) and 1 mL of 0.6 mg/mL internal standard (C13:1 in n-heptane) in a small glass test tube and incubated at 50 °C for 15 min. After cooling for 10 min at room temperature, 1.5 mL of methanol containing 5% HCl was added. The obtained mixture was incubated at 80 °C for 15 min and cooled at room temperature for 10 min. Then, 1 mL of n-heptane and 2.5 mL of 6% K_2CO_3 were added to the cooled mixture. The obtained solution was vortexed for 30 s and centrifuged at 4000×g at 4 °C for 5 min. The supernatant containing fatty acid methyl ester was injected into a Gas Chromatograph (GC). The GC program was adjusted as follows: initial oven temperature 50 °C, isotherm (hold time) 2.0 min, heating rate 50 °C/min up to 150 °C, isotherm (Hold time) 25 min, temperature increase by 2 °C/min up to 240 °C, isotherm 20 min, injection port temperature 270 °C, and detector port temperature 270 °C. The injected volume was 1 μL in a split mode (160:1). Hydrogen was used as the carrier gas. As the primary column, a Supelco SP-2560 (75 m × 0.18 mm × 0.14 μm film thickness) with a flow rate from 0.22 mL/min to 0.34 mL/min at a rate of 0.002 mL/min was used, and as the second column, an Agilent J&W HP-5ms (3.8 m × 0.25 mm × 0.25 μm film thickness) with a flow rate from 20 mL/min, increased to 25 mL/min at a rate of 0.18 mL/min, was adopted. The valves were set to a modulation delay of 1 min, a modulation period of 2.9 s, and a sampling time of 2.76 s. One microliter sample was injected in the pulsed split mode at a pressure of 25 psi for 0.3 min and a split ratio of 160:1. The split-splitless inlet was run at a temperature of 270 °C. The resulting two-dimensional chromatograms were analyzed with comprehensive GCxGC software (GC Image R 2.2 GC × GC: Zoex Corp., Houston, TX, USA). The fatty acid composition was calculated using Equation (2).

$$Total \text{ fatty acids } (mg/g \text{ of sample}) = \frac{(V_{TP} - V_{IS}) \times mg \text{ of internal standard}}{(V_{IS} \times sample \text{ weight } (g))} \tag{2}$$

where V_{TP} is the volume of total peaks and V_{IS} is the volume of internal standard.

2.4. Analysis of hamburgers

2.4.1. Evaluation of lipid oxidation: Thiobarbituric acid reactive substances (TBARS)

TBARS index was measured using the method described by Botsoglou et al. (1994) with some modifications. In brief, 2 g of hamburger samples were transferred into a centrifuge tube, and a stock solution containing 8 mL of 5% Trichloroacetic Acid (TCA) and 5 mL of 0.8% Butylated Hydroxytoluene (BHT) in hexane was added. The obtained solution was Ultra-Turraxed using a T-25 high-speed homogenizer (Janke and Kunkel, Germany) for 1 min at low speed and centrifuged for 10 min at 5000×*g* (Hettich Zentrifugen, MOD: Universal 320R, Tuttlingen, Germany). The supernatant was poured into a 10 mL flask after the filtration with 0.45 μm cellulose acetate filters. The filtrate was diluted with 5% TCA, and 2.5 mL of the obtained solution was added to a 10 mL tube containing 1.5 mL of 0.8% TBA. The blank was prepared in another tube containing 2.5 mL of 5% TCA and 1.5 mL of 0.8% TBA. The tubes were incubated in a water bath (JULABO GmbH, Seelbach, Germany) at 70 °C for 30 min to favor the reaction between malondialdehyde (MDA) and TBA with the formation of the chromophore complex. The final product was cooled, and the absorbance values at 532 nm were measured using a Varian Cary 50 Bio UV/Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). TBARS values were calculated from a standard curve ($R^2 = 0.99$) prepared using 1,1,3,3-tetramethoxypropane (MDA) and reported as mg MDA/kg hamburgers.

2.4.2. Colorimetric analysis

The colorimetric analysis was carried out using a CM-600d Spectrophotometer (Konica-Minolta, Milan, Italy), according to a previous study (Lante & Zocca, 2010). Before the trials, the apparatus was calibrated on a standard white tile (L* = 97.70, a* = 0.36, b* = 0.76). The CIELAB parameters (a*, b*, L*, C*, and h*) were evaluated. The whole visible spectrum (400–700 nm) was recorded ($\Delta\lambda = 2$ nm), and the illuminant was "natural light'' D65/10°. Results are expressed as L* (100: lightness, 0: darkness), a* (+a*: redness, −a*: greenness), b* (+b*: yellowness, −b*: blueness), respectively, on the Hunter scale. Chroma (C*) and hue (h*) parameters were defined as $C^*_{ab} = [(a^*)^2 + (b^*)^2]^{1/2}$ and $h_{ab} = \tan^{-1} b^*/a^*$, respectively. For the evaluation of h_{ab}, the angles of 0°, 90°, 180°, and 270° were assigned to semi-axis +a* (redness), semi-axis + b* (greenness), semi-axis -a*, and semi-axis -b* (blueness), respectively.

2.4.3. Microbiological assays

The microbiological trials were carried out according to the method used by Lyu et al. (2016) with some modifications. Briefly, 20 g of the hamburger samples were added into separate stomacher bags (Seward Medical, UK) containing 180 mL of sterile Buffered Peptone Water (BPW) (0.9%). Then, the samples were homogenized in a stomacher (Lab Blender 400, Seward Medical, UK) for 45 s at 230 RPM. Afterward, suitable serial decimal dilutions were prepared for each sample in BPW (0.9%). The amount of 0.1 mL of these serial dilutions of beef homogenates was spread on the surface of agar media. The EB and CF were determined using MacConkey Agar (MCC). Plate Count Agar (PCA), and de Man, Rogosa and Sharpe (MRS) agar were employed in determining TVC and LAB, respectively. The plates for TVC were incubated at 30 °C for 48 h. The LAB count, EB, and CF plates were incubated at 37 °C for 48 h. Microbial counts were expressed as $\log_{10}$ CFU/g.

2.5. Statistical analysis

All the analyses on hamburgers and oil samples were carried out in triplicate. The experimental design of this project is shown in Fig. 1. The data obtained from the trials were processed using IBM SPSS Statistics (Version 20.0, SPSS Inc, Chicago, IL, USA). The obtained data were subjected to Analysis of Variance (ANOVA), and the comparisons were made using Tukey's test with a significance level and confidence level of 0.05 and 95%, respectively. For statistical comparisons in FAP and oxidative stability, Independent T-test was used.

3. Results and discussion

3.1. Oxidative stability of EVO and OEVO

The oxidative stability is impacted by several factors, including the content of saturated and unsaturated fatty acids, phenolic compounds present in the oil, and storage conditions (Tinello et al., 2018). The double bonds of carbon in unsaturated fatty acids are frequently used for the chemical modification of the oil. Since ozone reacts only with carbon-carbon double bonds, ozonation could be considered a principle modification method, yielding peroxides, aldehydes, and ozonides, which are responsible for enhancing the biological activity of oil (Criegee, 1975; Radzimierska-Kaźmierczak et al., 2021). The reaction between these double bonds and ozone was recently described by several studies (Cetraro et al., 2019; Cox et al., 2020; Jiang et al., 2012; Siese et al., 2001).

Ozonation may considerably impact the oxidative stability of the oil, reducing its oxidative stability by the pro-oxidant effect of ozone, especially on polyunsaturated fatty acids, as observed by several authors (Criegee, 1975; Sadowska et al., 2008). However, the efficacy of ozone treatment is correlated with exposure time and system temperature. The extreme increase in ozonation time may have a destructive effect on the quality of the final product. Understanding the degree of ozonation with the ability to balance both microbial reduction and quality retention is very substantial (Khanashyam et al., 2022). Since ozone can promote the
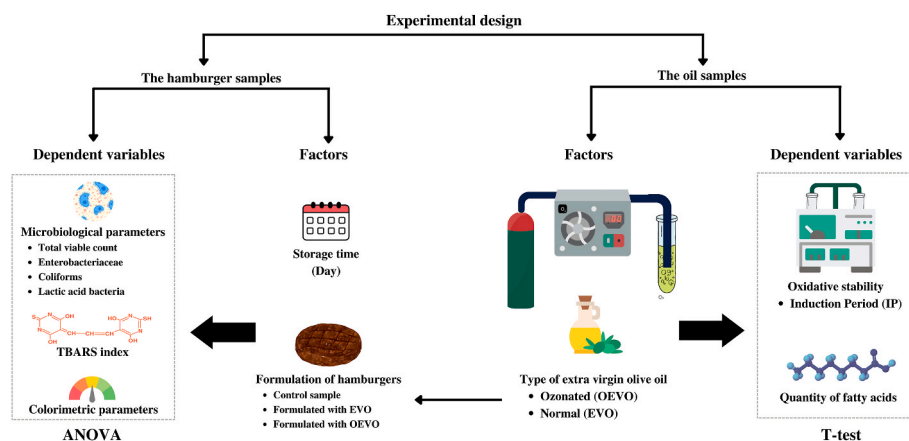

Fig. 1. The experimental design of preparation of the oils (EVO and OEVO), and the hamburgers.

autoxidation of oil, the oxidative stability of OEVO and EVO was tested (Table 2). According to the *t*-test, there is no significant difference (p > 0.05) between the IP of OEVO and EVO for a 5-min exposure time which is an optimum exposure time for obtaining antimicrobial properties (Choudhury et al., 2018; Epelle et al., 2022).

The percentage of RIP shows a decrease of 3.54% in IP. Although the results explain that adding ozone to the oil causes oxidation earlier, the advantage of low ozone concentrations as an anti-microbial agent outweighs the disadvantages stemming from oxidation (Pandiselvam et al., 2019). Moreover, adding ozone in the form of ozonated oil using EVO brings the benefit of having natural antioxidants. Many authors researched the effect of adding phenolic-rich ingredients to beef hamburgers. Barbieri et al. (2021) studied the lipid oxidation of beef hamburgers enriched with the phenolic extract of olive vegetation water. They reported that adding phenolic-rich compounds to the formulation of beef hamburgers can decrease lipid oxidation during the storage period. Furthermore, Pereira et al. (2022) added grape pomace as a phenolic-rich ingredient to beef hamburgers to increase their overall quality. Therefore, the increase in the oxidation rate of oil is negligible, as adding phenolic-rich ingredients has a lot of positive effects on beef hamburgers.

3.2. FAP of EVO and OEVO

The FAP of oils is known as the most assertive parameter influencing the oxidation stability of oils (Tinello et al., 2018). The ozonation effect could be impacted by many parameters such as organic content, temperature, the physical state of ozone, initial microbial load, etc. (Khanashyam et al., 2022). The results of the FAP analysis of EVO and OEVO are shown in Table 3. The results show some significant changes (p < 0.05) in the quantity of saturated and unsaturated fatty acids. Lignoceric acid content increased significantly (p < 0.05) after the ozonation. The changes in the content of unsaturated fatty acids (i.e., α-Linolenic acid, Gondoic acid, etc.) result from the attachment of oxygen atoms, the disruption of fatty acid molecules at the site of double bonds, the breakdown of these unstable intermediates, and the formation of oxygen derivatives of organic compounds (Radzimierska-Kaźmierczak et al., 2021). Furthermore, this result is plausible considering the high redox potential of ozone (2.07 V) and the considerable sensitivity of the fatty acids (M'Arimi et al., 2020).

Most of the olive oil fatty acids are monounsaturated, with oleic acid as predominant (65–85%) (Radzimierska-Kaźmierczak et al., 2021). Oleic and palmitic acid had the highest quantity in the EVO and OEVO. This is similar to the results obtained by Khandouzi et al. (2021) and Moretto et al. (2020). Monounsaturated fatty acids, such as oleic and palmitoleic acid, can decrease the level of Low-Density Lipoproteins (LDL) and keep the level of High-Density Lipoproteins (HDL) constant, which is necessary to control blood levels of cholesterol in the plasma. Therefore, adding olive oil to hamburgers can make them healthier food (Moretto et al., 2020). The research conducted by Teixeira et al. (2021) confirms that olive oil can increase the oleic acid content of hamburgers.

Moreover, olive oil has relatively high amounts of phenolic compounds, owning approximately 36 types of them with a total phenolic content (TPC) of 500–700 mg GA/kg (Khandouzi et al., 2021). Therefore, formulating hamburgers with olive oil (EVO and OEVO) could cause health benefits for consumers.

Table 2
Induction period of extra virgin olive oil, before and after ozonation process.

Extra virgin olive oil	IP (h)	%RIP
Untreated (EVO)	10.74 ± 0.41[a]	–
Ozonated for 5 min (OEVO)	10.36 ± 0.42[a]	3.54

*Data is reported as mean ± SD of triplicate samples (n = 3)** Same letters (a) on the same column means there is no statistically different values (p > 0.05).

Table 3
Fatty acid profile of EVO and OEVO used in the formulation of hamburgers.

Fatty Acids	Quantity (mg/g extra virgin olive oil)	
	Before ozonation (EVO)	After ozonation (OEVO)
Palmitic acid (C16:0)	15.60 ± 0.01[a]	14.41 ± 0.02[b]
Stearic acid (C18:0)	2.70 ± 0.03[a]	2.75 ± 0.03[a]
Arachidic acid (C20:0)	0.46 ± 0.01[a]	0.41 ± 0.02[b]
Behenic acid (C22:0)	0.16 ± 0.01[a]	0.15 ± 0.03[a]
Lignoceric acid (C24:0)	0.05 ± 0.03[a]	0.33 ± 0.05[b]
Palmitoleic acid (C16:1, *cis*-9)	1.25 ± 0.07[a]	1.47 ± 0.08[b]
Oleic acid (C18:1, *cis*-9)	68.19 ± 0.20[a]	69.30 ± 0.15[b]
Vaccenic acid (C18:1, *cis*-11)	2.10 ± 0.05[a]	1.99 ± 0.09[a]
Linoleic acid (C18:2, *cis, cis*-9,12)	6.48 ± 0.10[a]	6.54 ± 0.04[a]
α-Linolenic acid (C18:3, all *cis*-9,12,15)	0.79 ± 0.09[a]	0.01 ± 0.01[b]
Gondoic acid (C20:1, *cis*-11)	0.34 ± 0.02[a]	0.28 ± 0.03[b]
Pentadecanoic acid (C15:1, *trans*-10)	0.01 ± 0.01[a]	0.04 ± 0.01[b]

*Data is reported as mean ± SD of triplicate samples (n = 3)**Different letters (a and b) on the same row means there are statistically different values (p < 0.05).

3.3. TBARS value of hamburgers

Malondialdehyde or MDA (1,3-propanedial) is one of the most prominent aldehydes generated during the secondary lipid oxidation of polyunsaturated fatty acids (Domínguez et al., 2019). TBARS assay determines secondary oxidation products, showing the degree of lipid oxidation in food products. The increase in TBARS index may change the color and sensorial characteristics of meat products (Hemmatkhah et al., 2020). Fig. 2 illustrates the TBARS index trend for hamburgers during the storage period.

The initial MDA concentration on day 1 in all the samples is significantly different from each other (p < 0.05). The same phenomenon is seen on day 5 since sample C had a value of 0.190 mg MDA/kg, significantly lower than samples A and B with 0.270 and 0.246 mg MDA/kg, respectively. The TBARS index peaked on day 5 for all the samples. The reason for this significant increase could be the decomposition of primary oxidation products (hydroperoxide) into the secondary products of oxidation (aldehydes and ketones), which resulted in high MDA and increased TBARS index during the storage period (Boghori et al., 2020). Indeed, hydroperoxides are the most prominent primary oxidation products, decomposing rapidly due to their transitory nature giving rise to secondary oxidation products (Domínguez et al., 2019). Moreover, as Borella et al. (2019) reported in a study on the
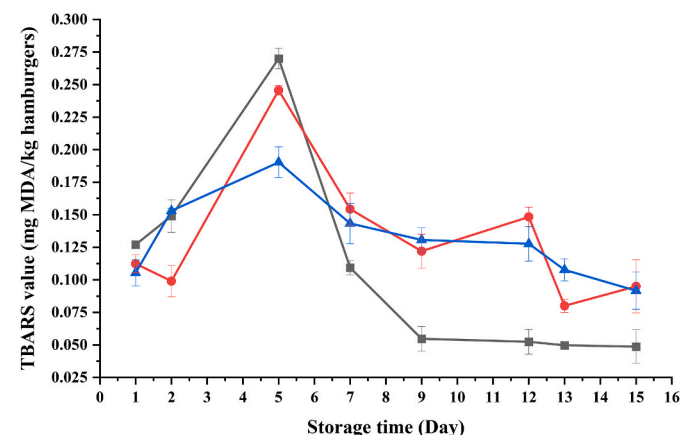

Fig. 2. Trend of the TBARS values (mg MDA/kg) of the hamburgers during the storage period at 4 °C.
*■: Sample A (Control sample), ●:Sample B (Hamburgers with 2% EVO), ▲: Sample C (Hamburgers with 2% OEVO). **The points of each curve represent the mean ± SD of triplicate samples (n = 3).

effect of rosemary in the formulation of hamburgers, this kind of increase is normal due to the formation of oxidative compounds throughout the storage time. However, samples B and C had lower TBARS indexes on this day, which could result from the polyphenol content of the oil (Bahmanyar et al., 2021). The peak on day 5 was subsequently followed by a decline until the end of the storage period for all the samples. The reason for this reduction may be the decomposition or interaction of TBARS with other compounds when it reaches the threshold values, thus resulting in lower values (Maqsood et al., 2016). Moreover, the decrease in MDA values could be due to the reaction of the MDA with the proteins existing in the substrate, which generates insoluble components not detectable by the TBARS assay (Dallabona et al., 2013).

Sample A, differing insignificantly (p > 0.05), showed lower TBARS values from day 9–15 compared to samples B and C, while samples B and C have significantly different means (p > 0.05) in the same period, excluding days 9, and 12 for sample C. The same general trend of an initial increase and a subsequent decline over time was reported by Hautrive et al. (2019), who analyzed the effect of chitosan and golden flaxseed flour on hamburgers. Moreover, Tang et al. (2006) reported the same general trend for the TBARS values of beef meatballs packaged in a modified atmosphere. After day 5, the TBARS values for samples B and C were higher than for the untreated sample. The reason for this could be the reaction of ozone with the polyunsaturated fatty acids of the phospholipids in the cell membrane of the bacteria present in the hamburger, resulting in a peroxidation process. This reaction results in the formation of ozonide, which can decompose into lipid peroxides (Khanashyam et al., 2022). The research conducted by Lyu et al. (2016) confirms that ozone in beef meat results in higher TBARS values. Also, the lower concentration of MDA for sample A in the mentioned period could be derived from microbial development, affecting the TBARS analysis by preventing the determination of MDA values, as reported by Vargas et al. (2011), who analyzed the effect of sunflower oil on pork hamburgers. Numerous factors may influence the evolution of TBARS values, including microbial development and the method of packaging (Sallam & Samejima, 2004). Additionally, meat is a complex food matrix that naturally induces the formation of artifacts impacting the obtained results. There are also some limitations to the TBARS assay, as the reaction with TBA is not specific to MDA because other aldehydes and oxidation products may react with TBA (Domínguez et al., 2019).

Although there are no legal limitations for secondary oxidation products determining the definitive degradation of meat products, the off-flavors associated with lipid oxidation are readily detectable at TBARS values higher than 2 mg MDA/kg (Hashemi Gahruie et al., 2017). Moreover, it is reported that values up to 0.6 mg MDA/kg in meat products are acceptable (Coll Cárdenas et al., 2011). The TBARS values determined in the present work range from 0.049 mg MDA/kg to 0.270 mg MDA/kg. A general comparison with other publications shows that the TBARS values detected in this project are extremely low, indicating a low secondary oxidation level. Sallam and Samejima (2004) determined the lipid oxidation of minced beef with added sodium chloride, and the obtained values were in the range of 0.177–0.49 mg MDA/kg. Similar intervals were also observed by García-Lomillo et al. (2017), who studied beef meatballs enriched with pomace extract. Lee et al. (2014) evaluated the antioxidant effect of a lemon extract incorporated in hamburgers with 3% soybean oil, and the TBARS values in the first five days of storage ranged from 0.18 to 0.35 mg MDA/kg, which are consistent with the outcome of the present work.

3.4. Color of hamburgers

The color and appearance affect consumer acceptability of meat products noticeably (Hemmatkhah et al., 2020). The discoloration issue in hamburgers is controllable by combining ozone treatment with other existing methods or technologies (Khanashyam et al., 2022). The addition of antioxidants can retard this discoloration process by delaying oxymyoglobin deterioration and slowing down the formation of metmyoglobin (Moemeni & Yazdanpanah, 2020).

Table 4 shows the surface color properties of different samples during the storage period, including L*, a*, b*, C*, and h*. There is no significant difference in L* between different samples, excluding days 13 and 15, in which sample c had higher values, which means it had a lighter color. Furthermore, there are upward trends in the L* values of all samples during the storage period. These results are consistent with the result obtained by Hemmatkhah et al. (2020). Generally, the low L* values may be related to the smaller light reflection due to the large diameters of ground meat (Moretto et al., 2020).

The red color in meat products is related to oxymyoglobin concentration. Conversion of oxymyoglobin to metmyoglobin causes changes in color from bright red to brown and decreases in a* value (Moemeni & Yazdanpanah, 2020). There is no significant difference (p > 0.05) between the a* values of samples on different days, excluding days 7, 13, and 15. These significantly lower values of sample C on days 13 and 15 could be the result of ozonation on the meat, as ozone could affect the meat's color (Stivarius et al., 2002). In other words, degradation of a* value is due to the oxidation of myoglobin and oxymyoglobin to metmyoglobin, which causes lower redness as an impact of ozone. This emphasizes the importance of ozonation degree on the quality of the products (Khanashyam et al., 2022). This result is consistent with the results obtained by Coll Cárdenas et al., (2011), who analyzed the effect of ozone on the quality of beef.

As it is deducible from the data, adding EVO and OEVO to hamburgers increased the yellowness (b*) as olive oil has a color between dark green to golden yellow. However, there is no significant difference (p > 0.05) between samples B and C during the final days of the storage period.

In an overall assessment of the results of chroma, there is an upward trend for samples A and B, while sample C, which had the OEVO, has a downward trend. Moreover, the hue angle in sample C for all days except days 5 and 7 was higher than the others, with some significant differences mostly in the final days of the storage period.

3.5. Microbiological properties of hamburgers

The TVC and the charges of EB, CF, and LAB in the initial meat used for producing hamburgers were 2.78 ± 0.09, 2.09 ± 0.19, 1.80 ± 0.17, 2.42 ± 0.10 Log_{10} CFU/g, respectively. Although hamburgers are subjected to cooking, it is prominent to consider the impact of formulation on their microbiological quality, as they must be within the accepted microbiological limits (Moretto et al., 2020). Fig. 3 shows the changes in the number of TVC (a), EB (b), CF (c), and LAB (d) in the hamburgers during the storage period at 4 °C.

The initial number of TVC in the hamburgers ranged from 4.39 to 4.58 Log_{10} CFU/g, which is not differing significantly (p>0.05) between the samples. These amounts are lower than the results obtained by Hemmatkhah et al. (2020), who investigated the effect of encapsulated cumin seed essential oil on beef burgers. The obtained results in the present paper could be justified as GMPs and GHPs were applied in the production phase.

Sample A (Control) had the maximum level of TVC during the storage period. After day 7, TVC for sample C was significantly lower than other samples (p < 0.05), and it was noticeably lower than the maximum recommended TVC in minced meat (6.67 Log_{10} CFU/g) (European Union, 2005), confirming that OEVO increased the shelf life of the hamburgers. Moreover, the result of TVC for sample C is consistent with the results obtained by Lorenzo et al. (2018) when they added pitanga leaf extracts to pork hamburgers. Nonetheless, the TVC reported by Homayounpour et al. (2021), who added nano-encapsulated *Allium sativum* L. essential oil in beef hamburgers, was higher than our results. This finding shows the remarkable performance of OEVO as a potent antimicrobial agent compared to other strategies. Even a minimal ozone concentration is proven to be an antimicrobial agent during the

Table 4
Color properties of the hamburgers during the storage period at 4 °C.

L*	Storage time (Day)							
Samples	1	2	5	7	9	12	13	15
A	43.25 ± 2.00[a,A]	47.82 ± 2.26[b,A]	47.17 ± 3.1[ab,A]	48.85 ± 1.63[b,B]	44.84 ± 1.59[ab,A]	46.34 ± 2.81[ab,A]	47.42 ± 0.73[ab,A]	46.32 ± 1.32[ab,A]
B	44.49 ± 1.23[a,A]	47.93 ± 1.81[b,A]	48.56 ± 1.76[b,A]	47.98 ± 1.02[b,B]	46.41 ± 1.14[ab,A]	48.94 ± 1.43[b,A]	48.59 ± 0.58[b,AB]	48.42 ± 1.62[b,AB]
C	45.88 ± 2.16[ab,A]	48.18 ± 1.22[abcd,A]	48.98 ± 2.62[bcd,A]	44.76 ± 1.54[a,A]	46.40 ± 2.41[abc,A]	49.78 ± 1.67[cd,A]	49.21 ± 1.38[bcd,B]	50.55 ± 1.62[d,B]
a*	Days							
Samples	1	2	5	7	9	12	13	15
A	6.57 ± 1.05[ab,A]	5.67 ± 1.35[a,A]	6.76 ± 0.94[ab,A]	5.75 ± 0.56[a,A]	7.31 ± 0.52[ab,A]	7.52 ± 1.68[ab,A]	8.05 ± 0.89[b,B]	8.23 ± 1.14[b,B]
B	6.55 ± 0.54[ab,A]	5.66 ± 0.51[a,A]	5.87 ± 0.65[ab,A]	5.68 ± 0.72[a,A]	6.47 ± 1.09[ab,A]	6.82 ± 0.60[abc,A]	7.19 ± 0.40[bc,B]	8.21 ± 0.78[c,B]
C	6.01 ± 0.48[ab,A]	5.47 ± 1.05[a,A]	6.46 ± 1.08[ab,A]	7.13 ± 0.29[b,B]	6.56 ± 0.88[ab,A]	5.85 ± 0.52[ab,A]	5.68 ± 0.62[ab,A]	6.12 ± 0.70[ab,A]
b*	Days							
Samples	1	2	5	7	9	12	13	15
A	7.36 ± 1.37[a,A]	7.38 ± 1.20[a,A]	9.04 ± 1.10[ab,A]	7.93 ± 1.95[ab,A]	8.84 ± 1.50[ab,A]	9.59 ± 0.63[ab,A]	9.13 ± 0.56[ab,A]	9.83 ± 0.24[b,A]
B	10.33 ± 1.00[bcd,B]	8.14 ± 1.17[a,A]	10.69 ± 0.89[cd,B]	8.59 ± 1.19[ab,A]	8.96 ± 1.19[abc,A]	12.02 ± 1.03[d,B]	11.83.44 ± 0.50[d,B]	11.64 ± 0.89[d,B]
C	11.98 ± 1.19[b,B]	10.32 ± 0.69[ab,B]	10.67 ± 0.89[ab,B]	9.44 ± 1.21[a,A]	10.58 ± 1.27[ab,A]	11.54 ± 0.41[b,B]	11.21 ± 0.36[ab,B]	11.06 ± 0.38[ab,B]
C*	Days							
Samples	1	2	5	7	9	12	13	15
A	9.91 ± 1.31[a,A]	9.42 ± 0.80[a,A]	11.36 ± 0.36[ab,A]	9.81 ± 1.89[a,A]	11.51 ± 1.16[ab,A]	12.27 ± 0.77[b,A]	12.25 ± 1.03[b,A]	12.85 ± 0.62[b,A]
B	12.24 ± 0.95[bc,B]	9.95 ± 0.86[a,A]	12.21 ± 0.58[bc,AB]	10.30 ± 1.3[a,A]	11.12 ± 0.74[ab,A]	13.85 ± 0.69[cd,B]	13.81 ± 0.47[cd,B]	14.27 ± 0.77[d,B]
C	13.42 ± 1.01[b,B]	11.70 ± 0.93[a,B]	12.52 ± 0.72[ab,B]	11.84 ± 1.03[ab,A]	12.50 ± 0.82[ab,A]	12.94 ± 0.48[ab,AB]	12.32 ± 0.44[ab,A]	12.65 ± 0.56[ab,A]
h*	Days							
Samples	1	2	5	7	9	12	13	15
A	47.99 ± 6.57[a,A]	52.28 ± 9.77[a,A]	53.09 ± 7.07[a,A]	53.44 ± 4.29[a,A]	50.08 ± 5.54[a,A]	52.21 ± 7.42[a,A]	50.56 ± 0.78[a,A]	50.23 ± 4.37[a,A]
B	57.56 ± 2.90[ab,B]	54.88 ± 5.50[ab,A]	61.11 ± 4.37[b,A]	56.46 ± 1.94[ab,A]	54.01 ± 7.51[ab,A]	60.28 ± 4.00[ab,AB]	51.53 ± 2.56[a,A]	54.77 ± 3.62[ab,A]
C	63.21 ± 3.41[b,B]	62.21 ± 4.19[b,A]	58.82 ± 5.49[ab,A]	52.71 ± 3.34[a,A]	58.00 ± 5.97[ab,A]	63.14 ± 2.05[b,B]	61.11 ± 2.57[b,B]	61.11 ± 2.57[b,B]

*A: Control sample, B: Hamburgers with 2% EVO, C: Hamburgers with 2% OEVO.

**Data is reported as mean ± SD of triplicate samples (n = 3).

***Different capital letters in each column and small letters in each row indicate significant differences (p < 0.05).
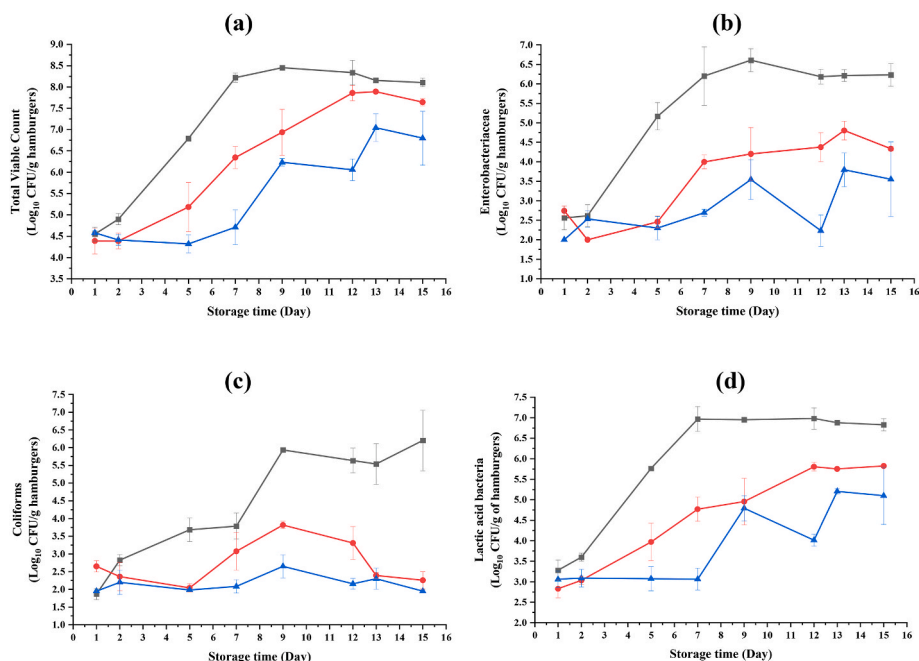

Fig. 3. TVC (a), and the growth of EB (b), CF (c), and LAB (d) in the hamburgers during the storage period at 4 °C.
*■: Sample A (Control sample), ●:Sample B (Hamburgers with 2% EVO), ▲: Sample C (Hamburgers with 2% OEVO).
** The points of each curve represent the mean ± SD of triplicate samples (n = 3).

processing and storage of food products (Pandiselvam et al., 2019). The antimicrobial effect of ozone can be explained by the presence of anti-microbial compounds, such as aldehydes and carboxylic acids (e.g., azelaic and pelargonic acids), formed by the ozonation process of vegetable oils (Ugazio et al., 2020). Also, it is reported that an increase in the antibacterial activity of ozonated oil happens when the peroxide number increases (Díaz et al., 2006).

The initial counts of CF were in the range of 1.87–2.65 Log_{10} CFU/g, which is lower than the CF acceptable limit for minced beef in USA (<3

Log_{10} CFU/g) (AMS, 2020). After the first day, sample C had the lowest CF count during the storage period, ranging from 1.95 to 2.65 Log_{10} CFU/g. This is lower than the results obtained by Babaoğlu et al. (2022) when they added blackberry, black chokeberry, blueberry, and red currant pomace extracts to beef patties. It has been previously reported that the ozonation process significantly affects the reduction of co-liforms in meat products (El Dahshan et al., 2013). At the end of the storage period, the CF count for sample C was under the acceptable limit of CF, which means that OEVO increased the shelf life of the

hamburgers.

The growth of EB for sample A significantly increased (p < 0.05) after day 2. Sample C had the lowest number of EB, ranging from 2 to 3.79 Log_{10} CFU/g, during the storage period, excluding day 2. There is no significant difference (p>0.05) in EB counts for sample B from day 7–15. Further, the EB in sample B ranged from 2 to 4.80 Log_{10} CFU/g, which is similar to the results obtained by Turan and Şimşek (2021) when they added freeze-dried black mulberry water extract to beef patties.

The LAB count for sample A was higher than the other samples during the storage period reaching a peak on day 12 with 6.98 Log_{10} CFU/g. LAB count for sample C was 5.10 Log_{10} CFU/g on day 15, showing the effectiveness of ozone on the decrease of LAB growth in hamburgers. There is no significant difference (p>0.05) in LAB counts for sample C from day 1–7. The LAB counts for the present paper are lower than those obtained by Turan and Şimşek (2021) for the beef patties fortified with freeze-dried black mulberry water extract. There is no information about the maximum permitted LAB and EB limit in meat preparations in the European Union Commission Regulation (European Union, 2005).

The better performance of sample B, with a bacterial growth lower than sample A, confirms the effect of EVO as an antibacterial agent (Serreli & Deiana, 2018), as previously proved by Moretto et al. (2020) by adding olive oil to buffalo burgers. As reported by Nazzaro et al. (2019), the antibacterial activity of EVO is related to its phenolic content.

Beef hamburgers typically have a maximum shelf life of 3 days at 4 °C due to their fast microbial growth (Parafati et al., 2019). The OEVO could increase the shelf life of beef hamburgers since sample C, during the 15-day storage period, had the lowest bacterial growth compared to samples A and B. This confirms that the combined effect of ozone and EVO plays an essential role in increasing the shelf life of hamburgers, in agreement with the antimicrobial effect of ozonated olive oil suggested by Radzimierska-Kaźmierczak et al. (2021). Therefore, the proposed method could be successfully used to improve the shelf life of beef hamburgers.

4. Conclusions

The results highlighted that combining a low concentration ozone with EVO acts as a highly biologically active and antimicrobial agent that prolongs the shelf life of the final product, and the oxidation of the oil triggered by the ozonation process is negligible when the ozone concentration is low.

The results of this study could be the starting point for further research projects since it is necessary to further examine the proposed formulations by performing sensory analysis, texture analysis, and testing other physicochemical properties of the hamburgers. However, the proposed method is simple and meets the need for a clean label nutritious food, and after sufficient studies, it could be used in the food industry to ensure the quality, safety, and organoleptic properties of meat products.

CRediT authorship contribution statement

Peyman Ebrahimi: Data curation, Formal analysis, Visualization, Investigation, Methodology, Writing – original draft. **Anna Lante:** Conceptualization, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Riccardo Miotti Scapin:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Stefania Zannoni:** Data curation, Formal analysis. **Barbara Contiero:** Data curation, Validation. **Paolo Catellani:** Data curation, Formal analysis, Methodology. **Valerio Giaccone:** Conceptualization, Methodology, Project administration, Resources, Supervision.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.114100.

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