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Changes in quality parameters of ripened sausages added with olive leaf extract during refrigerated storage under modified atmosphere

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ABSTRACT

This study evaluated the influence of Olive Leaf Extract (OLE) on the quality parameters of sliced ripened sausages during 80 days of refrigerated storage in Modified Atmosphere Packaging (MAP). Shelf-life extension poses significant challenges to the meat industry. Lipid oxidation, microbial growth and color changes can induce undesirable alterations in sensory and nutritional traits during the storage of ripened sausages. OLE was used alone (F1, OLE = 1000 mg/kg) or in combination with nitrate and nitrite (F2, OLE = 500 mg/kg; NO_2-NO_3 = 35-35 mg/kg) compared to a control containing nitrate and nitrite alone (CTR). Microbiological analysis showed no microbial growth in any of the tested samples. Furthermore, the addition of OLE resulted in a lower b^* value and delayed lipid oxidation compared with CTR ($p < 0.05$). Notably, CTR reached the rancidity threshold (1 mg malondialdehyde/kg) between 60 and 80 days, while F1 and F2 remained below this limit throughout the storage period. The findings indicate the positive influence of OLE in keeping the quality of ripened sausages during storage, contributing significantly to microbial stability, color preservation, and the reduction of lipid oxidation.

1. Introduction

Lipid oxidation and microbial growth are the main phenomena related to the spoilage of meat and meat products during storage (Dominguez et al., 2019). Especially in ripened meat products, they lead to undesired changes in sensory attributes (such as color, texture and flavor) and nutritional traits, resulting in a decrease of consumer acceptability (Fernandes et al., 2018). For these reasons, it is essential to slow down these degradation phenomena to extend the shelf-life of meat products (Ameer et al., 2022; Zareian et al., 2018).

To this aim, several preservation technologies (e.g., refrigeration, vacuum packaging, modified atmosphere packaging) and synthetic food additives (e.g., nitrate and nitrite) are commonly utilized by food companies (Arokiyaraj, Dinakarkumar, & Shin, 2024). Regarding these latter, nitrate and nitrite are widely utilized in ripened meat products as common additives due to their effectiveness in stabilizing the characteristic reddish-pink color of meat (Flores & Toldrá, 2021). As reported by Zhang et al. (2023), they can extend the shelf-life of these products by inhibiting the growth of pathogenic and spoilage bacteria, such as *Clostridium botulinum* (and its toxin production) and *Listeria monocytogenes*. Additionally, nitrite acts against oxidation phenomena (e.g., lipid and protein oxidation) via several mechanisms, thereby enhancing oxidative stability (Perea-Sanz et al., 2018). Despite their useful functions, the addition of these additives to meat products has raised health concerns due to potential toxicity and carcinogenicity. In fact, nitrate and nitrite addition to ripened meat products may form N-nitroso compounds, especially N-nitrosamines (NAs) (Ozaki et al., 2021). NAs pose a risk to human health because of their toxic, mutagenic and teratogenic effects as well as the carcinogenic effects. NAs are generally formed by the reaction between nitrosating agents, derived from nitrite, and secondary amines, derived from protein degradation (Ozaki et al., 2021; Perea-Sanz et al., 2018). In response to these concerns, the European Commission has defined new limits for nitrosamines in meat products to reduce consumer exposure to these compounds while maintaining a protective effect against pathogenic bacteria. For ripened meat products, the maximum allowed amount that can be added during production, has been reduced for nitrite from 150 to 80 mg/kg, and for nitrate from 150 to 90 mg/kg (EU Commission, 2023).

As a result, natural ingredients from plant matrices, such as wastes and by-products from the agricultural and food industry, have been used

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as viable alternatives in ripened meat products (Difonzo, Totaro et al., 2022; Totaro et al., 2024; Zhang et al., 2023). Thus, in our previous studies we showed the effectiveness of the OLE (Olive Leaf Extract) in reducing nitrate and nitrite in ripened pork sausages produced both on lab-scale (Difonzo, Totaro, et al., 2022) and industrial scale (Totaro et al., 2024). OLE, rich in antioxidant, antimicrobial, and anti-inflammatory properties, contains bioactive compounds such as oleuropein, making it a promising natural alternative to synthetic preservatives. Olive leaves are a by-product of olive cultivation and a key resource from the olive tree, which is one of the most widespread and economically important plants in the Mediterranean region. OLE production adds value to agricultural waste (Difonzo, Crescenzi, et al., 2022), ligning with sustainability and the circular economy and supporting the growing trend toward incorporating natural and environmentally-friendly ingredients into food processing. At the end of ripening period, OLE exhibited notable antioxidant properties, reducing lipid and protein oxidation, as well as significantly lowering N-nitrosamine content, with no changes in the sensory quality of the sausages (Totaro et al., 2024). However, sausages with OLE had higher moisture content and water activity than those with nitrate and nitrite, which could potentially influence their shelf-life (Totaro et al., 2024). Similarly, Pateiro et al. (2015) and Martínez-Zamora et al. (2021) demonstrated that the inclusion of grape seed/chestnut extracts and citrus/leafy green vegetable extracts in dry-cured Spanish "chorizo" was more effective than synthetic additives in reducing lipid oxidation. Moreover, Ozaki et al. (2021) evaluated the positive effect of radish powder as a replacement for nitrite in dry-fermented sausages, showing that it effectively delayed lipid oxidation for 60 days during vacuum storage at 5 °C. In addition to its antioxidant effects, OLE also plays a role in inhibiting microbial growth (Difonzo, Totaro, et al., 2022; Totaro et al., 2024). This is in line with other studies that have investigated the use of natural additives for their ability to control microbial activity. For example, Riazi et al. (2016) found that incorporating dry red grape pomace as a substitute for sodium nitrite in dry-cured sausages was highly effective in inhibiting microbial growth during refrigerated storage. Additionally, Pateiro et al. (2015) and Martínez-Zamora et al. (2021) also reported similar antimicrobial effects, highlighting the role of natural extracts in controlling microbial growth during storage.

To the best of our knowledge, no studies have been published regarding the evolution of key quality parameters affecting the shelf-life of ripened pork sausages when OLE is used as a replacement for nitrate and nitrite. To this aim ripened sausages prepared with OLE, as unique additive or in combination to nitrate and nitrite, were stored for 80 days at 4 °C in MAP (70/30 N_2/CO_2) and analysed in comparison with control sausages prepared with nitrate and nitrite alone. Modified atmosphere packaging (MAP) was chosen as the packaging technology due to its increasing use for processed meat products in recent years (Ameer et al., 2022).

2. Materials and methods

2.1. OLE characterization

A commercial OLE with an oleuropein content of 40% (Olive Leaf Extract, Hepatica, Germany), commonly marketed as dietary supplement, was used for the experiment. The oleuropein content was verified by HPLC-DAD (Agilent Technologies, Santa Clara, USA) while the total phenolic content (TPC) was determined using the Folin-Ciocalteu method following the methods reported in Difonzo, Totaro, et al. (2022). Antioxidant activity was assessed through ABTS and DPPH assays, following the procedures described in the same paper. All analyses were conducted on three different commercial batches.

2.2. Ripened sausages manufacture

Ripened sausages were produced at Salumi Martina Franca S.r.l. (Martina Franca, Taranto, Italy) following the company's standard industrial procedures. Fresh lean pork meat (shoulder pork and lean trimmings) and fatty tissue (pork belly) (85/15, *w/w*) were trimmed and minced with an industrial grinder equipped with a pre-mixer (TCA-10, Omet Foodtech, Poggibonsi, Italy). The resulting minced mixture was then divided into three equal batches to prepare three sausage formulations. These formulations varied in the proportions of OLE, nitrate (potassium nitrate or E252; SolMar, Taranto, Italy), and nitrite (sodium nitrite or E250; SolMar, Taranto, Italy) as follows: CTR, OLE: 0 mg/kg; NO_2-NO_3: 75-75 mg/kg; F1, OLE:1000 mg/kg; NO_2-NO_3: 0 mg/kg; F2, OLE: 500 mg/kg; NO_2-NO_3: 35-35 mg/kg. During the mechanical kneading process (Model K-400, Omet Foodtech, Poggibonsi, Italy), salt (20 g/kg) and pepper (1 g/kg) were incorporated. The resulting mixture was mechanically filled using an F20 stuffer (Omet Foodtech, Poggibonsi, Italy) into natural pork casings with a diameter of 40 mm. Subsequently, the sausages underwent a series of processing steps, including stewing (23 °C, RH 95%, 24 h), followed by drying (17–20 °C, RH 60–75%, 96 h) and finally ripening (15–18 °C, RH 80%). Ten sausages per formulation were produced to allow for replicated analyses. At the end of 30-day ripening period, 15 sausages (five for each batch) were sampled, three of which were mechanically sliced and immediately analysed (T0). The remaining 12 were sliced, placed in sterile plastic trays (95 × 10 mm), and packed in MAP (VGP 25n, Orved, Italy) consisting of 70:30 N_2/CO_2 (Rivoira S.p.A., Modugno, Italy) with a plastic film composed by orientated polyamide/polypropylene (OPA/PP) (Orvedo, Italy). The gas permeability of the packaging film (15 μm thickness) was $\leq 27 \pm 0.70$ $cm^3/m^2 \times 24\ h \times bar$ for O_2 and $\leq 120 \pm 1$ $cm^3/m^2 \times 24\ h \times bar$ for CO_2.

All packs were stored at 4 °C for 80 days, and key parameters such as microbial growth, sensory properties, and oxidative degradation - critical factors affecting the shelf-life of ripened sausages - were measured. Microbial growth was assessed only at T0 and T80 to evaluate the overall trends in microbial growth during long-term refrigerated storage under MAP. In contrast, the other parameters were evaluated at T0, 10 days (T10), 20 days (T20), 40 days (T40), 60 days (T60), and 80 days (T80) of storage.

2.3. Analytical determinations

2.3.1. Physico-chemical analyses

pH was determined using a pH-meter (HANNA instruments, Woonsocket, RI, USA) equipped with a glass probe for penetration.

The concentrations of gas in the packaging were measured at each sampling time before the opening. Headspace gas samples were withdrawn with a gas-tight syringe and the concentrations of O_2 and CO_2 were monitored with a gas analyser (CheckMate 3, DanSensor, Ametek, Milan, Italy).

Colorimetric parameters were determined using a colorimeter CM-600d (Konica Minolta, Tokyo, Japan) equipped with the software SpectraMagic NX (Konica Minolta, Tokyo, Japan). Lightness (L^*), redness (a^*) and yellowness (b^*) were determined as color coordinates. The measurements were taken on three different points on both surfaces of each slice contained in each tray.

Lipid oxidation level during storage was determined by thiobarbituric acid reactive substances (TBARs) as described by Rosmini et al. (1996), with minor modifications. Briefly, 10 mL of 10% trichloroacetic acid (*v/v*) (Sigma-Aldrich, Steinheim, Germany) and 5 mL of 0.02 M 2-thiobarbituric acid solution (Sigma-Aldrich, Steinheim, Germany) were added to 4 g of ground sausage. After vortexing (Heidolph Instruments, Schwabach, Germany) for 5 min and centrifuging (Thermo Fisher Scientific, Osterode am Harz, Germany) at 3500 rpm for 5 min, the supernatant was recovered. The samples were then incubated in a bath at 100 °C for 30 min and cooled to room temperature for 20 min. The absorbance of the resulting solution was measured at 532 nm using a Cary 60 spectrophotometer (Agilent Technologies, Milan, Italy), and the lipid oxidation level of the samples was expressed in mg malondialdehyde (MDA) per kg of ripened sausages, using a calibration curve obtained using 0.01 M 1,1,3,3-Tetraethoxypropane (TEP) as standard (Sigma-Aldrich, Steinheim, Germany) at different concentrations (0.22 mg/kg - 2.2 mg/kg).

2.3.2. *Microbiological analyses*

Microbiological parameters evaluated included Total Viable Counts (TVC), mesophilic lactic bacteria, Enterobacteriaceae, moulds and yeasts, determined according to ISO 4833 (2013), ISO 15214 (1998), ISO 21528 (2017), and ISO 21527 (2008) methods, respectively. The measurement of these parameters in ripened sausages is essential for monitoring the overall hygiene, fermentation process, and potential contamination, thereby guiding producers in maintaining high standards of food safety and quality. Indeed, TVC is widely recognized as an important indicator of overall microbial load, essential for evaluating food hygiene and potential spoilage. Monitoring TVC provides a broad perspective on microbial dynamics during storage (Patsias et al., 2006). Mesophilic lactic bacteria are crucial for fermentation, flavor development, and preservation by producing antimicrobial compounds like lactic acid and bacteriocins. However, their excessive growth during extended storage could contribute to spoilage, making their monitoring essential (Fadda, López, & Vignolo, 2010). Enterobacteriaceae includes both spoilage microorganisms and potential pathogens, whose presence often reflects contamination or poor hygiene during processing and storage. Thus, the determination is critical for ensuring product safety and evaluating the effectiveness of preservation strategies (Garritani et al., 2008). Finally, moulds and yeasts can act as potential spoilage microorganisms in processed meat causing food safety issues (Fung, 2014). Results were expressed as the log of colony-forming units (cfu) per gram of sample. The absence of *Listeria monocytogenes*, as well as *Clostridium* sulphite reducers and spores, coliforms, *Escherichia coli* beta-glucuronidase positive, and coagulase-positive *Staphylococci* in all samples at T0 was confirmed using the methodologies outlined in Difonzo, Totaro, et al. (2022). These determinations, conducted and highlighted in our previous study (Totaro et al., 2024), are also crucial for ensuring both the microbiological safety and regulatory compliance of the product, as required by EU Commission (2007).

2.4. *Sensory analysis*

Sensory assessment was performed by a panel of 14 semi-trained members (7 males; 7 females; age range 35–52) recruited, based on their previous experience in the sensory evaluation of meat-based product, among the workers of the producing company (Salumi Martina Franca S.r.l., Martina Franca, Italy), the technicians and the researchers of the Department of Plant, Soil, and Food Sciences of the University of Bari, Italy. All panellists were regular consumers of meat-based products and had no allergies or intolerances. Pre-test sessions were carried out: (i) to define the list of descriptors to be evaluated in the samples object of the study; (ii) to define the intensity range of each descriptor; (iii) to fix the scale anchors of each descriptor; (iv) to verify reliability, consistency, and discriminating ability of panellists when testing meat-based products. The study protocol followed the ethical guidelines of the laboratory. Panellists were given information about study aims, and individually written informed consent was obtained from each participant. Samples cut into slices of approximately 5 mm thick were coded with three-digit number and presented in small plates to each participant. Panellists were asked to evaluate the following sensory attributes using a structured scale from 0 to 9: color intensity of ripened sausages (0 = extremely pale, 9 = extremely bright color), rancid odour (0 = absent; 9 = very strong), intensity of the typical odour of ripened sausages (0 = absent, 9 = very strong) and bitterness (0 = absence of perception; 9 = extremely intense perception). The sensory sessions were carried out at 0, 10, 20, 40, 60 and 80 days of storage. In each session, two whole slices from each sausage were presented in coded plastic plates to all panel members at room temperature, in an air-conditioned room free of external aromas. All samples were presented randomly to avoid any order effect.

2.5. *Statistical analysis*

Results were reported as means ± standard deviations. Data were analysed using One-way ANOVA and Tukey's test, considering storage time and sausage formulation as independent variables ($p < 0.05$). With the exception of microbial analysis, Two-way ANOVA was also performed for to evaluate first-order variable interactions. All data were processed using Minitab Statistical Software (Minitab Inc., State College, PA, USA).

3. Results and discussion

3.1. *Characterisation of olive leaf extract (OLE)*

According to Folin-Ciocalteau, DPPH and ABTS methods, the total phenolic content and antioxidant activity of the commercial OLE showed, respectively, the following results: 191.61 ± 1.22 mg gallic acid equivalents (GAE)/g, 1611.88 ± 10.06 μmol Trolox equivalents (TE)/g of and 1078.00 ± 11.72 μmol TE/g of extract. The most abundant phenolic compound was oleuropein, detected by HPLC-DAD, which was found in a concentration of 406.01 ± 3.99 mg/g, in agreement with the declared minimum content of the extract equal to 40%.

3.2. *Gas concentration*

To avoid the presence of O_2, which is one of the promoters of both lipid oxidation and growth of aerobic spoilage organisms (Gurunathan et al., 2022), a gas mixture consisting of 70% N_2 and 30% CO_2 was used. Carbon dioxide has antimicrobial and antioxidant effect and prolongs the storage of meat and meat products (Ozturk, Yilmaz, & Gunes, 2010). Nitrogen, instead, is an inert gas used in packaging atmospheres as filler gas, as well as to prevent pack collapse that could be caused by CO_2 (Gurunathan et al., 2022). At the beginning of the storage period the concentration of O_2 in the trays was close to 0% and increased to a maximum level of 1% after 80 days of storage (data not shown), while the concentration of CO_2 decreased to 25%

3.3. *pH measurement*

Table 1 shows the pH values of the ripened sausages during storage. This parameter was significantly affected by both "formulation" (F) and "storage time" (T) variables ($p < 0.001$). Significant was also the first order interaction between the variables ($p < 0.001$) indicating a

Table 1

Mean value, standard deviation and results of the statistical analysis of pH of ripened sausages during 80 days of storage in modified atmosphere packaging at 4 °C ($n = 3$).

	CTR	F1	F2	*p*-value
T0	6.19 ± 0.01 ^{A,a}	6.04 ± 0.02 ^{A,b}	6.02 ± 0.01 ^{A,b}	0.011
T10	5.92 ± 0.03 ^{B,a}	5.55 ± 0.02 ^{B,b}	5.56 ± 0.03 ^{B,b}	<0.001
T20	5.88 ± 0.02 ^{B,a}	5.53 ± 0.04 ^{B,b}	5.51 ± 0.03 ^{BC,b}	<0.001
T40	5.88 ± 0.01 ^{B,a}	5.52 ± 0.02 ^{B,b}	5.50 ± 0.02 ^{BC,b}	<0.001
T60	5.80 ± 0.01 ^{C,a}	5.38 ± 0.01 ^{C,c}	5.42 ± 0.01 ^{C,b}	<0.001
T80	5.78 ± 0.02 ^{C,a}	5.37 ± 0.02 ^{C,b}	5.39 ± 0.01 ^{D,b}	<0.001
p-value	<0.001	<0.001	<0.001	F*T, <0.001

T: storage time. F: formulation. CTR = OLE: 0 mg/kg; NO_2-NO_3: 75-75 mg/kg; F1 = OLE:1000 mg/kg; NO_2-NO_3: 0 mg/kg; F2 = OLE: 500 mg/kg; NO_2-NO_3: 35-35 mg/kg. OLE: olive leaf extract; NO_2-NO_3: nitrite/nitrate. Different uppercase letters ($^{A-E}$) in the same column (same formulation at different time) denote significant differences at $p < 0.05$. Different lowercase letters ($^{a-c}$) in the same row (same time for a different formulation) denote significant differences at $p < 0.05$. F*T denotes the first order variables interaction at $p < 0.05$.

different evolution of the pH during storage as a function of the formulation.

A decline in pH during the storage of sausages under CO_2-enriched MAP condition has been observed by other researchers, probably due to CO_2 solubilization in the water fraction of the meat, leading to the formation of carbonic acid (Summo et al., 2016). In our experimental trials, a more pronounced pH decline was observed during the initial storage period in the samples containing OLE (F1 and F2) compared to the control (CTR), which was formulated with only nitrate and nitrite. This significant difference in pH evolution may be attributed to different moisture levels at T0 (29.55% ± 0.24, 26.98% ± 0.69, and 22.36% ± 0.28 for F1, F2, and CTR, respectively), which can affect the rate of CO_2 solubilization in meat products (Robertson, 2012). Additionally, the presence of fermentable carbohydrates in the OLE, as demonstrated in our previous study (Totaro et al., 2024), may provide an extra substrate for microorganisms, enhancing lactic acid production (Djeri & Williams, 2014). After T10, the pH evolution became comparable for all formulations under investigation. However, at the end of the storage period, the control group (CTR) showed significantly higher pH values than F1 and F2, where OLE had been added. This result is consistent with findings from other studies that utilized vegetable extracts as substitutes for nitrate and nitrite (Pateiro et al., 2015; Zeraat Pisheh et al., 2023).

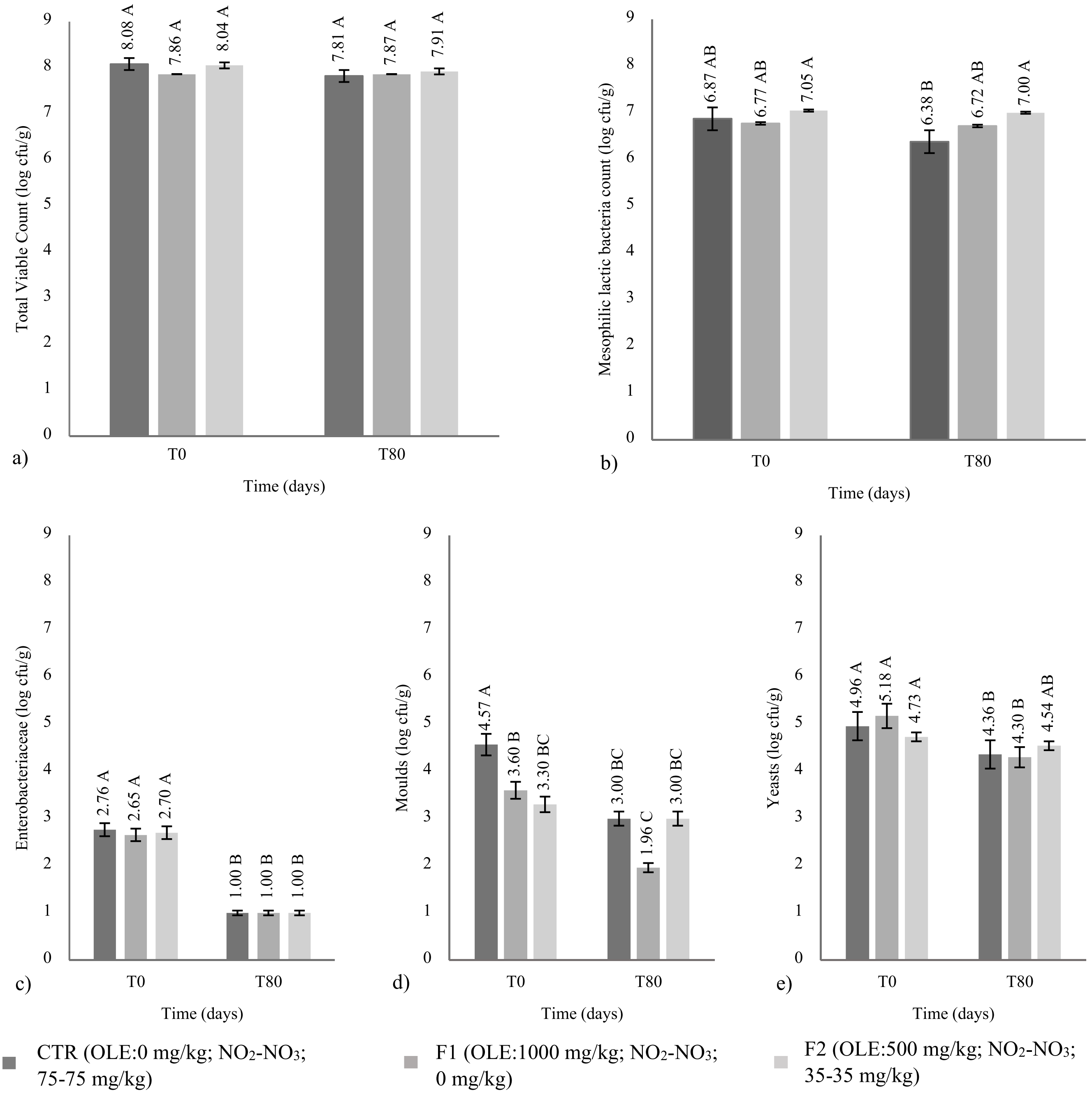

Fig. 1. Evolution of the Total Viable Counts (TVC) (a), mesophilic lactic bacteria (b), Enterobacteriaceae (c), moulds (d) and yeasts (e) growth (log cfu/g) in ripened sausages at the beginning (T0) and after 80 days (T80) of storage in modified atmosphere packaging at 4 °C. Different letters denote significant differences at $p < 0.05$.

3.4. Microbiological analyses

The mean values and the results of the statistical analysis of the microbial indicators (TVC, mesophilic lactic bacteria, Enterobacteriaceae, moulds, and yeasts) assessed in ripened sausages at the beginning and the end of storage period are summarised in Fig. 1a–e. At T0 the mould content was significantly lower in the sausages containing OLE (F1 and F2) than in CTR (which contained only nitrate and nitrite). The antimicrobial effect of OLE on mould growth has previously been demonstrated in other fermented foods, such as table olives and olive-based pâté (Caponio et al., 2019; Difonzo et al., 2019). This effect is attributed to oleuropein, which constitutes 40% of the OLE extract used in this study. Kurt and Ceylan (2017) observed no effect of OLE, used at the dose of 125, 250 and 500 mg/kg, on mould growth in dry fermented Turkish beef sausages (Sucuk). As highlighted in a recent study (Al-Rimawi et al., 2024), the effect of oleuropein and OLE on mould growth is dose-dependent. At low concentrations (0.2%), oleuropein exhibited no antimicrobial activity; however, at higher concentrations (0.4%, as applied in our study), its antifungal effect was confirmed. No significant differences among the other microbial indicators were found at T0 among the different formulations. This result is important from a technological point of view. In fact, considering the important role of nitrate and nitrite in microbiological safety and shelf-life extension of meat products (Zhang et al., 2023), it suggests that OLE alone, or in combination with low amounts of nitrate and nitrite, provides microbial stability comparable to formulations in which nitrate/nitrite are used at the maximum level allowed by current regulations (EU Commission, 2023).

After 80 days of storage under MAP in refrigerated conditions, no samples showed significant microbial growth, supporting the efficacy of OLE in controlling microbial activity. Our results are also consistent with Kurt and Ceylan (2017), who reported no growth of total aerobic mesophilic bacteria and lactic acid bacteria in ripened beef sausages containing OLE after 60 days of vacuum storage at 4 °C. A more detailed analysis of the evolution of individual microbial indicators during storage revealed a decrease in Enterobacteriaceae, moulds, and yeasts in all formulations, compared to T0. The decrease in Enterobacteriaceae was not influenced by the formulation and is likely attributed to the antimicrobial effect of CO_2 in the packaging atmosphere, which is further supported by the low storage temperature and the moisture levels of the samples. Both of the latter two factors, in fact, ensure high solubility of CO_2 in the sausages, a necessary condition to exert its antimicrobial activity. The decrease in moulds and yeasts, was significant for CTR (nitrate and nitrite only) and F1 (OLE only) formulations, but not for F2 samples. This decrease could be attributed to the antimicrobial activities of CO_2 on moulds and yeasts, as confirmed by other studies (Gonzalez-Fandos, Vazquez de Castro, Martinez-Laorden, & Perez-Arnedo, 2021; Schirmer et al., 2020), combined with the effect of OLE in F1 and F2 samples.

The differences observed at T80 between F1 (1000 mg/kg of OLE) and F2 (500 mg/kg of OLE) may be linked to the different amounts of OLE in the samples, confirming the dose-dependent effect of OLE on mould controlling. Significant differences were found among samples, at T80, for the lactic bacteria, detected in low amount in CTR than the formulation with OLE added. This result could be related both to the presence of fermentable carbohydrates in the OLE and the low effect of OLE against the lactic bacteria, as already verified in other studies (Kurt and Ceylan, 2017; Natrella et al., 2020).

3.5. Color parameters

Table 2 presents the lightness (L^*), redness (a^*), and yellowness (b^*) of the ripened sausages during storage. Both the formulation and storage time significantly influenced the redness index, a^*. This is particularly important in meat products, as the red intensity of the meat is valued by consumers and associated with higher quality.

Table 2
Mean value, standard deviation and results of the statistical analysis of the colour parameters (lightness, redness, yellowness) of ripened sausages during 80 days of storage in modified atmosphere packaging at 4 °C (n = 3).

	Lightness (L^*)				Redness (a^*)				Yellowness (b^*)			
	CTR	F1	F2	p-value	CTR	F1	F2	p-value	CTR	F1	F2	p-value
T0	$33.59 \pm 0.29^{A,b}$	$36.61 \pm 0.24^{A,a}$	$34.68 \pm 0.09^{A,ab}$	<0.001	$8.78 \pm 1.39^{AB,a}$	$6.43 \pm 0.70^{AB,b}$	$6.87 \pm 1.05^{ABC,ab}$	0.043	$7.65 \pm 0.64^{B,a}$	$6.85 \pm 0.42^{B,b}$	$6.63 \pm 0.81^{B,b}$	0.009
T10	$30.22 \pm 1.47^{B,b}$	$32.12 \pm 0.54^{B,b}$	$34.35 \pm 0.15^{B,a}$	<0.001	$9.32 \pm 1.74^{A,a}$	$6.83 \pm 0.18^{A,b}$	$8.72 \pm 1.70^{A,a}$	<0.001	$7.70 \pm 1.38^{B,a}$	$6.93 \pm 2.37^{AB,b}$	$6.47 \pm 0.03^{B,b}$	<0.001
T20	$31.88 \pm 1.54^{AB,a}$	$31.60 \pm 0.30^{BC,a}$	$32.50 \pm 0.47^{C,a}$	0.552	$9.01 \pm 0.93^{AB,a}$	$6.23 \pm 1.08^{AB,b}$	$8.50 \pm 0.75^{A,a}$	0.024	$7.94 \pm 1.00^{AB,a}$	$7.39 \pm 0.54^{AB,a}$	$7.37 \pm 0.55^{AB,a}$	0.090
T40	$30.91 \pm 0.87^{BC,b}$	$31.20 \pm 0.30^{C,ab}$	$32.48 \pm 0.21^{C,a}$	0.010	$7.08 \pm 0.63^{ABC,a}$	$5.28 \pm 0.18^{B,b}$	$8.09 \pm 0.65^{AB,a}$	0.002	$8.18 \pm 0.10^{A,a}$	$7.51 \pm 0.80^{A,b}$	$7.58 \pm 1.21^{A,b}$	0.010
T60	$28.89 \pm 0.54^{CD,a}$	$29.15 \pm 0.07^{D,a}$	$29.05 \pm 0.76^{D,a}$	0.847	$6.28 \pm 0.21^{BC,a}$	$5.20 \pm 0.02^{B,c}$	$5.86 \pm 0.14^{BC,b}$	0.006	$8.30 \pm 1.28^{A,a}$	$7.87 \pm 1.53^{A,b}$	$7.63 \pm 0.48^{A,b}$	0.005
T80	$28.36 \pm 0.14^{D,a}$	$28.30 \pm 0.08^{D,a}$	$28.15 \pm 0.70^{D,a}$	0.117	$5.06 \pm 0.44^{C,b}$	$4.96 \pm 0.13^{C,b}$	$5.30 \pm 0.63^{C,a}$	0.007	$8.67 \pm 0.12^{A,a}$	$8.23 \pm 0.69^{A,b}$	$8.18 \pm 1.50^{A,b}$	0.028
p-value	<0.001	<0.001	<0.001	F*T, <0.001	0.001	0.005	0.003	F*T, = 0.059	0.008	0.005	0.015	F*T, = 1.000

T: storage time (days). F: formulation CTR = OLE: 0 mg/kg; NO_2-NO_3: 75-75 mg/kg; F1 = OLE:1000 mg/kg; NO_2-NO_3: 0 mg/kg; F2 = OLE: 500 mg/kg; NO_2-NO_3: 35-35 mg/kg. OLE: olive leaf extract; NO_2-NO_3: nitrite/nitrate. Different uppercase letters ($^{A–D}$) in the same column (same formulation at different time) denote significant differences at $p < 0.05$. Different lowercase letters ($^{a-b}$) in the same row (same time for a different formulation) denote significant differences at $p < 0.05$. F*T denotes the first order variables interaction at $p < 0.05$.

However, the lack of a significant interaction between these variables ($p = 0.059$) suggests that the evolution of a^* during storage was independent of the formulation; indeed, for all the formulations an increase was observed during the first ten days of storage, followed by a decrease. A similar trend was observed by Efenberger-Szmechtyk et al. (2021) using various leaf extracts as natural preservatives in pork sausages. This trend may be attributed to the initial formation of carboxymyoglobin (CoMb), which imparts attractive and preferred bright cherry-red color, similar to that of oxymyoglobin (OxyMb) (Ramanathan et al., 2020), due to the bond between myoglobin and carbon monoxide (CO) generated during the solubilization of carbon dioxide (Sakowska et al., 2016). Conversely, the subsequent decrease observed until the end of the storage period could be related to the formation of metmyoglobin (MetMb). Factors such as lipid oxidation may influence the conversion of CoMb to MetMb, leading to discoloration of the ripened sausages (Ameer et al., 2022). Significant differences in redness were observed among the formulations at each storage time, with F1 (OLE alone) showing a significantly lower a^* than F2 and CTR. This result, which was already observed at the end of the ripening period (T0), is likely related to the absence of nitroso-myoglobin synthesis. Nitroso-myoglobin, typically induced by nitrate and nitrite, plays a crucial role in maintaining the stable red color of meat products (Nowak et al., 2016). Nevertheless, consumers may be more inclined to accept a less vibrant color if the reasoning behind it is clearly communicated and linked to the product's health benefits (Hung et al., 2016). Strategic and transparent communication emphasizing the absence of synthetic additives could greatly enhance consumer acceptance of the color variation (Guedes-Oliveira, Brad Kim & Conte-Junior, 2021; Hung et al., 2016). Regarding yellowness (b^*), the control sample, which contained only nitrate and nitrite, exhibited significantly higher values compared to the samples containing OLE. The lowest yellowness values were observed in F2, although no significant differences were observed compared to F1. The lower b* values observed in F1 and F2 compared to the CTR can be attributed to the antioxidant compounds present in OLE (Manafi Dizajyekan et al., 2021), which act as effective radical scavengers and metal chelators, thereby mitigating oxidative processes that cause color changes. However, the possibility of a combined antioxidant effect of OLE and nitrite cannot be excluded. This agrees with the findings of Jeong et al. (2010), who observed that the combination of red beet and sodium nitrite contributes to the color stability of low-fat sausages during storage. Moreover, b^* increased during storage reaching, for all the samples, significantly higher values at T80 than at T0. Kim et al. (2014) demonstrated that the increase in yellowness of MAP-packed meat products is related to oxidation of red pigments. These results confirm that sausages suffered discoloration during storage as a result of pigment oxidation (Ameer et al., 2022). In terms of lightness, samples F1 and F2, both containing OLE, exhibited generally higher values compared to the control sample (CTR). This increase in lightness may be attributed to the higher moisture content of these samples, which likely enhances light scattering on the surface of the slices (Summo et al., 2016). L^* decreased at the end of storage (T80), reaching significantly ($p < 0.001$) lower values than T0 for all the samples. This finding is consistent with a previous study of Ameer et al. (2022) on sausages stored in nitrogen and carbon dioxide-based modified atmosphere packaging (MAP).

3.6. TBA-test

Lipid oxidation is one of the most important phenomena determining the quality of meat and meat products. Although it plays a crucial role in the development of the characteristic aroma of fermented meat products, it is also responsible for off-flavors, undesirable tastes, discoloration, loss of nutritional value, formation of toxic compounds, and other changes that can affect consumer perception and overall acceptance of these products (Efenberger-Szmechtyk et al., 2021). The evolution of the lipid oxidation of ripened sausages and the effect of the OLE addition is showed in Fig. 2a. CTR sample, containing only nitrate and nitrite, exhibited a significantly higher level of lipid oxidation at all sampling times. In contrast, F2 (partial replacement of nitrite and nitrate by OLE) consistently demonstrated significantly lower malondialdehyde (MDA) contents over time when compared to the other two samples.

Specifically, lipid oxidation levels in the CTR increased rapidly, reaching the limit of rancidity equal to 1 mg MDA/kg - which is considered the maximum acceptable level of lipid oxidation for ripened sausages (Kurt & Ceylan, 2017; Sarıçoban & Unal, 2022) - even before the end of the storage period, particularly between 60 and 80 days. In contrast, F1 and F2 remained below this limit during all the storage periods. The properties of OLE are related to its polyphenols able to chelate metal and scavenge free radicals, inhibiting lipid oxidation (Difonzo, Crescenzi, et al., 2022). Similar results were found by Pateiro et al. (2015) when they evaluated the inclusion of tea, chestnut, grape seed and beer extracts in the formulation of Spanish dry-cured sausage "Chorizo" during storage. On the contrary, Sucu and Turp (2018) stated the necessity of adding nitrite to sausages, since the MDA content of samples with freeze-dried leek powder used as a nitrate replacer were higher than those of control sausages with nitrite. Therefore, it is essential to assess the efficacy of these natural extracts on a case-by-case basis, as the results of various studies, while generally consistent, depend on the specific composition of each extract, the doses used, and the type of meat product being examined. Kinetic models are a useful tool for monitoring and predicting quality changes in foods. They are used to describe the formation of undesirable compounds, textural alterations and inactivation of enzymes and microorganisms (Manzocco et al., 2016). Therefore, to predict the time needed for F1 and F2 to reach the rancidity limit, which had not been reached in 80 days, kinetic models were defined. The first step was the identification of a suitable oxidation marker linked to quality loss, followed by the definition of the acceptability limit. As regards ripened sausages, TBA-test is considered one of the main analytical indices used to monitor the evolution of oxidation during storage (Manzocco et al., 2016), while the acceptability limit is equal to 1 mg MDA/kg (Kurt & Ceylan, 2017; Sarıçoban & Unal, 2022). In the next step, the reaction order of quality index was estimated based on R^2 obtained from the changes in MDA levels for F1 ($R^2 = 0.994$) and F2 ($R^2 = 0.976$), as a function of the storage time (Fig. 1b). These results showed that the chosen quality index fitted with the zero-order reaction model. Finally, data describing the evolution of the oxidation marker as a function of time were submitted to modelling according to equation (1) reported in Manzocco et al. (2016):

$$SL = \frac{I_{lim} - I_0}{k} \tag{1}$$

where SL is shelf-life; I_{lim} is the oxidation value corresponding to the previously defined acceptability limit; I_0 is the value of the oxidation marker just after sausages production; k is the rate constant. Based on the kinetic models, the acceptability limit defined for lipid oxidation would be reached F1 and F2 after 99 and 157 days of storage, respectively. As it was demonstrated for other meat products (Alirezalu et al., 2017) the results obtained confirm the effectiveness of using OLE also for improving and prolonging the shelf-life of ripened sausages.

3.7. Sensory analysis

The results of the sensory analysis carried out on the ripened sausages during storage are shown in Table 3. Regarding the perception of color intensity, a significant and expected reduction in this attribute was observed during storage. Consistent with the instrumental determination (section 3.4), the F1 sample, added only with OLE, exhibited the most pronounced decrease in color intensity, particularly during the first period of storage (F*T, $p < 0.001$). Ghafouri-Oskuei et al. (2020) observed a similar decline in color hue during storage of sausages added of flaxseed and tomato powder. Throughout the entire storage period,

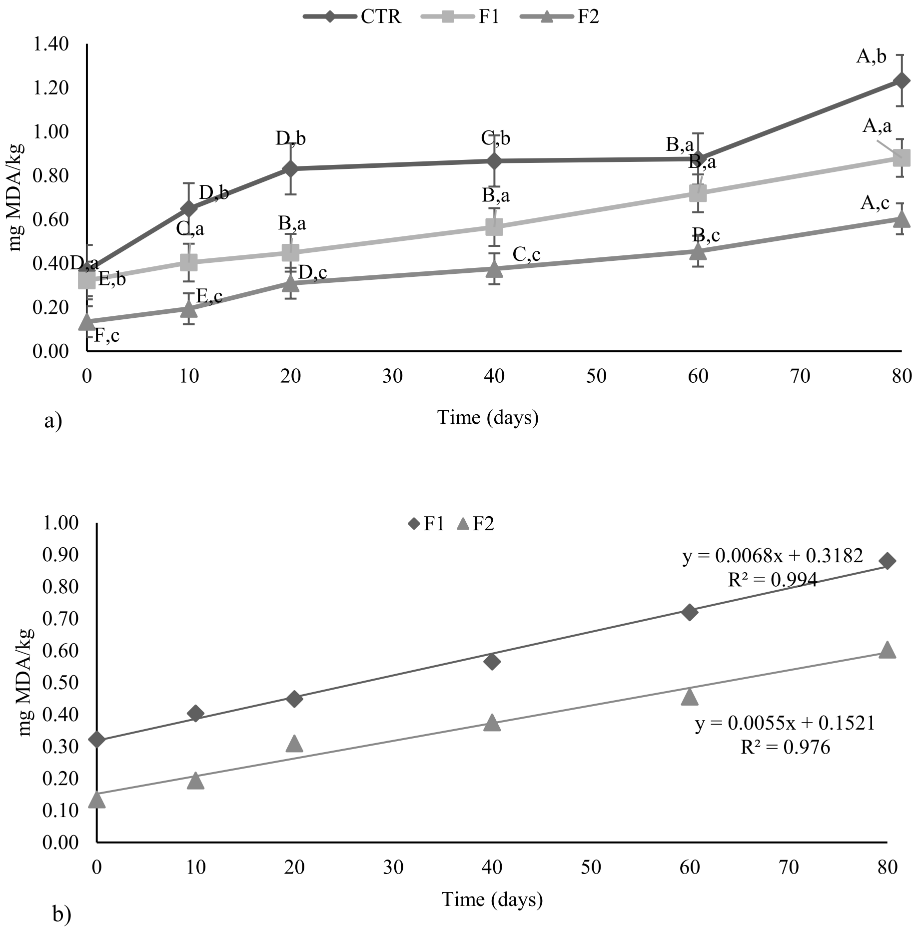

Fig. 2. (a) represents the evolution of TBARs (mg MDA/kg) of ripened sausages where different uppercase ($^{A-D}$) and lowercase ($^{a-c}$) letters denote significant differences at $p < 0.05$ in same formulation at different time and in same time at different formulation, respectively; (b) represents the kinetic chart of ripened sausages during MAP storage at 4 °C. F: formulation. CTR = OLE: 0 mg/kg; NO_2-NO_3: 75-75 mg/kg; F1 = OLE:1000 mg/kg; NO_2-NO_3: 0 mg/kg; F2 = OLE: 500 mg/kg; NO_2-NO_3: 35-35 mg/kg. OLE: olive leaf extract; NO_2-NO_3: nitrite/nitrate.

significant differences in color intensity were observed by the panellists among the different formulations, with sample F1 characterized by a significantly lower score. Interestingly, at T0 (the beginning of storage), no significant differences between formulations were detected by the panellists, in contrast with the findings from the instrumental color measurements. In F1, the absence of nitrate and nitrite, which are known to 'fix' the characteristic color associated with cured meat products (Braghieri et al., 2016), may explain the observed results. It is important to note that the scores recorded for all formulations throughout the storage period were acceptable, except for sample F1, which received a score lower than 6.0 at T80. The typical odour of sausages was not affected by the OLE addition at the beginning of ripening period, in fact no significant differences among the samples were observed at T0. A gradual decrease in this parameter was observed as the storage time increased, occurring similarly for all samples (F*T, $p > 0.05$). However, samples containing nitrate and nitrite exhibited a more pronounced typical sausage odour compared to F1, with significant differences at T20, T60, and T80. Also in this case, the presence of nitrate and nitrite could have contributed to the development and maintenance of the typical odour during storage (Perea-Sanz et al., 2018). Hence, the partial replacement of nitrate and nitrite by OLE (F2) did not induce perceptible differences in the sensory attributes of color and odour in comparison to the control added of nitrate and nitrite alone. This outcome is in line with the study of Sucu and Turp (2018), who observed that the sensory score of fermented beef sausages added of beetroot extracts, during storage, was comparable to that of control with nitrite alone. The perception of rancid odour increased during storage for all samples, consistent with TBARs results ($R^2 = 0.744$). Notably, the control sample exhibited significantly higher scores for this odour attribute compared to F1 and F2, which contained OLE. As mentioned in section 3.6, the observed results could be attributed to the antioxidant properties of OLE. Finally, regarding bitterness, as already reported in our previous study (Totaro et al., 2024), at the end of ripening period (T0) sample with only OLE (F1) showed the highest scores compared to other two formulations. This could be due to the presence of oleuropein in the OLE characterised by a clearly perceivable bitter taste. However, the combination of OLE with nitrite and nitrate could have masked this perception since no significant differences emerged by comparing the sample CTR with F2. Moreover, no significant differences were observed during storage for each formulation. The differences among the formulations observed at T0 were confirmed at each time of storage (data not shown).

4. Conclusion

This study responds to the growing interest by researchers and the food industry in replacing or reducing nitrate and nitrite with natural extracts. It highlights the effectiveness of OLE in controlling microbial growth, maintaining color stability, and slowing lipid oxidation in ripened sausages, thereby enhancing overall quality and extending product shelf-life. Notably, at the end of storage period, the positive effect of OLE on product safety was underscored, with a microbial growth consistently below the allowed thresholds, even in the sample prepared with OLE alone. The TBA-test results and the rancid odour

Table 3
Mean value, standard deviation and results of the statistical analysis of sensorial analysis of ripened sausages during 80 days of storage in modified atmosphere packaging at 4 °C ($n = 3$).

	Colour intensity				Typical odour of ripened sausages				Rancid odour			
	CTR	F1	F2	*p*-value	CTR	F1	F2	*p*-value	CTR	F1	F2	*p*-value
T0	7.18 ± 0.08 ^{A,a}	6.83 ± 0.29 ^{A,a}	7.15 ± 0.02 ^{A,a}	0.756	7.58 ± 0.08 ^{A,a}	7.35 ± 0.13 ^{A,a}	7.55 ± 0.10 ^{A,a}	0.075	0.71 ± 0.07 ^{D,a}	0.58 ± 0.02 ^{E,b}	0.48 ± 0.03 ^{D,b}	0.001
T10	7.12 ± 0.12 ^{A,a}	6.42 ± 0.07 ^{B,b}	7.00 ± 0.05 ^{A,ab}	0.001	7.36 ± 0.26 ^{AB,a}	7.17 ± 0.07 ^{A,a}	7.33 ± 0.33 ^{AB,a}	0.091	0.81 ± 0.01 ^{C,a}	0.64 ± 0.04 ^{D,b}	0.58 ± 0.02 ^{CD,b}	<0.001
T20	6.98 ± 0.11 ^{A,a}	6.43 ± 0.08 ^{B,b}	6.78 ± 0.08 ^{B,ab}	0.001	7.40 ± 0.07 ^{A,a}	7.06 ± 0.06 ^{AB,b}	7.12 ± 0.12 ^{B,b}	0.007	1.65 ± 0.10 ^{B,a}	1.45 ± 0.05 ^{C,b}	1.42 ± 0.07 ^{B,b}	0.021
T40	6.67 ± 0.10 ^{B,a}	6.33 ± 0.08 ^{BC,b}	6.50 ± 0.08 ^{BC,ab}	0.001	6.87 ± 0.12 ^{BC,ab}	6.70 ± 0.18 ^{B,b}	7.10 ± 0.10 ^{B,a}	0.032	1.81 ± 0.01 ^{B,a}	1.50 ± 0.05 ^{C,b}	1.16 ± 0.16 ^{C,c}	0.001
T60	6.52 ± 0.02 ^{B,a}	6.05 ± 0.05 ^{C,b}	6.42 ± 0.09 ^{BC,a}	<0.001	6.94 ± 0.06 ^{B,a}	6.44 ± 0.11 ^{BC,b}	6.85 ± 0.15 ^{C,a}	0.005	2.80 ± 0.02 ^{A,a}	1.94 ± 0.05 ^{B,b}	1.37 ± 0.07 ^{B,c}	0.001
T80	6.22 ± 0.22 ^{C,a}	5.78 ± 0.12 ^{D,b}	6.08 ± 0.08 ^{C,ab}	0.043	6.87 ± 0.12 ^{C,a}	6.28 ± 0.28 ^{C,b}	6.68 ± 0.18 ^{C,a}	0.002	2.83 ± 0.17 ^{A,a}	2.09 ± 0.07 ^{A,b}	1.67 ± 0.33 ^{A,c}	0.032
p-value	<0.001	<0.001	<0.001	F*T, = 0.014	0.001	0.013	0.001	F*T, = 0.430	<0.001	<0.001	<0.001	F*T, <0.001

T: storage time. F: formulation. CTR = OLE: 0 mg/kg; NO_2-NO_3: 75-75 mg/kg; F1 = OLE:1000 mg/kg; NO_2-NO_3: 0 mg/kg; F2 = OLE: 500 mg/kg; NO_2-NO_3: 35-35 mg/kg. OLE: olive leaf extract; NO_2-NO_3: nitrite/nitrate. Different uppercase letters ($^{A-D}$) in the same column (same formulation at different time) denote significant differences at $p < 0.05$. Different lowercase letters ($^{a\text{-}c}$) in the same row (same time for different formulation) denote significant differences at $p < 0.05$. F*T denotes the first order variables interaction at $p < 0.05$.

attribute, which are associated with oxidative processes, were reduced by the addition of OLE, both when used alone (F1) and in combination with nitrate and nitrite (F2), in contrast to the control sample. Kinetic models further indicated a prolonged shelf-life for ripened sausages added of OLE (F1 up to 99 days; F2 up to 157 days), while the control had a shelf-life between 60 and 80 days. The inclusion of OLE in the formulation mitigated sensory changes related to color and odour, contributing to an overall improvement in product quality and potentially increasing consumer acceptance. This is particularly relevant as consumers are increasingly inclined to choose healthier products, particularly those with reduced or without nitrate and nitrite, which are commonly present in commercially available products. These findings demonstrate that OLE could serve as a valuable natural additive to reduce nitrate and nitrite, further enhancing the quality and the stability of ripened sausages during storage and providing a feasible and more sustainable strategy for product improvement in the meat industry. However, it is important to emphasize that OLE has not yet received official approval as a food additive, which limits its commercial application. Although the results of this study, together with many other studies that have tested the use of OLE in different food formulations, provide valuable data regarding product quality and safety, regulatory approval remains a barrier to its large-scale industrial adoption. Therefore, it is crucial that future research addresses these regulatory challenges and works toward obtaining the necessary approvals, ensuring that OLE can be safely and sustainably implemented as an alternative to synthetic preservatives in the food industry.

CRediT authorship contribution statement

Michela PiaTotaro: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Graziana Difonzo:** Methodology, Formal analysis, Conceptualization. **Gabriele Ventrella:** Writing – original draft. **Francesco Caponio:** Writing – review & editing. **Antonella Pasqualone:** Writing – review & editing. **Carmine Summo:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Data curation, Conceptualization.

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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.

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Data availability

Data will be made available on request.

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