Effect of oregano and thyme essential oils on the microbiological and chemical quality of refrigerated (4ºC) ready-to-eat squid rings

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SUMMARY

The efficacy of oregano and thyme essential oils (OEO and TEO, respectively) in the quality retention of a refrigerated (4ºC) squid (*Loligo vulgaris*) ring ready-to-eat (RTE) product was studied. Essential oils were added at different concentrations to the coating medium during processing. An inhibitory ($p<0.05$) effect of OEO on the microbial activity (aerobes, anaerobes, Enterobacteriaceae, psychrotrophs) of the squid rings was observed, with a more pronounced effect as OEO concentration increased. The addition of OEO also led to an inhibitory ($p<0.05$) effect on lipid oxidation, as determined by peroxide, thiobarbituric acid-reactive substance and interaction compound formation; however, no effect ($p>0.05$) of the OEO concentrations on lipid oxidation development was detected. The addition of TEO did not lead to an inhibitory effect ($p>0.05$) on the microbial activity of the refrigerated RTE squid, although a slight inhibitory ($p<0.05$) effect on lipid oxidation was observed in the batches including the higher TEO concentrations.

Running Head: Essential oils and refrigerated squid rings.

Keywords: Squid ring, oregano, thyme, ready-to-eat, microbial activity, lipid oxidation, quality.

INTRODUCTION

Marine species provide highly perishable products whose quality and freshness rapidly decline post-mortem due to different microbial and biochemical degradation mechanisms (Whittle *et al*., 1990; Olafsdóttir *et al*., 1997). A wide variety of preservation strategies have been satisfactorily tested to delay the spoilage of marine food while retaining its nutritional value and guaranteeing its safety. These strategies include chemical and/or physical treatments (Toledo-Flores & Zall, 1992; Ashie *et al.*, 1996; Sanjuás-Rey *et al*., 2011) and packaging employment (Ozen & Floros, 2001; Sivertsvik *et al*., 2002; Corbo *et al*., 2009).

With respect to the preservation techniques based on chemical methods, recent efforts have focused on the replacement of synthetic antioxidants by natural antioxidants. In this sense, essential oils have been reported to provide natural antimicrobial and antioxidant components with the potential to extend the shelf life of certain food products (Dziezak, 1989). Among the essential oils extracted from aromatic plants, oregano (*Origanum vulgare*) (Goulas & Kontominas, 2007; Giatrakou *et al*., 2008; Mexis *et al*., 2009) and thyme (*Thymus vulgaris*) (Del Nobile *et al*., 2009; Kykkidou *et al*., 2009; Erkan, 2010) oils have gained a great technological interest. The preservation effects found in both essential oils have been explained on the basis of the presence of different compounds such as carvacrol, thymol, p-cymene and γ-terpinene (Burt, 2004; Yanishlieva *et al*., 2006).

A recent strategy to increase marine product distribution according to new trends in food consumption and lifestyle is represented by the increasing commercialisation of ready-to-eat (RTE) food products, which include a myriad of refrigerated, frozen, cured and canned seafood products (Manrique & Jensen, 2001). Thus, novel and attractive RTE products are increasingly available today in the market and restorer sectors, all of

which require strict safety controls and attempt to satisfy the consumer's expectations for taste, flavour and healthiness (Gilbert *et al*., 2000; Gopinath *et al*., 2007).

Cephalopod catches have increased gradually in the last decade because of a growing market demand and the expansion of fisheries into new fishing grounds and deeper waters (FAO, 2007a). Among these cephalopods, squid species represent a major portion of the catches in many countries and are mainly exported as chilled or frozen products (FAO, 2007b).

The present study focused on an RTE product consisting of pre-fried squid rings maintained under modified atmosphere packaging (MAP) conditions. The main objective of this work was to investigate the effects of the addition of oregano and thyme essential oils (OEO and TEO, respectively) on the quality and shelf life of such food products. For it, different concentrations of the essential oils were added to the coating mixture of the pre-fried squid rings. The microbial growth and lipid oxidation events in squid rings during the refrigerated storage (4ºC) were observed and compared with those of control batches prepared in the absence of these essential oils.

MATERIALS AND METHODS

Raw material, processing and sampling

Frozen individual peeled squid (*Loligo vulgaris*) mantle rings (weight: 19.0±1.0 g each squid ring) were acquired from a local seafood industry specialising in the commercialisation of RTE seafood products. Squid rings had been prepared from fresh squid and then kept frozen (-40ºC) for 1 month. Once in our laboratory, two different experiments were conducted to assess the effect of the presence of OEO and TEO independently. In both experiments, the thawing step was performed by overnight storage at 4ºC.

The first experiment concerned OEO (origanum oil, FCC grade, from *Thymus capitatus*; density: 0.939 g ml^{-1} at 25ºC; boiling point: 239ºC; Sigma Aldrich, Madrid, Spain) and included squid rings (n = 306) that were randomly assigned into 4 batches (72 rings in each). Three of the batches were processed with a coating mixture supplemented with OEO at the following concentrations: 0.010% (O-1 batch), 0.025% (O-2 batch) or 0.050% (O-3 batch) (w/w, OEO/squid ring). The fourth batch was prepared without oregano oil (control batch, C-O). The remaining rings (n = 18) were considered as the initial sample of the oregano experiment; they were distributed among 3 groups (6 rings in each) that were analysed independently.

The coating formulation consisted of wheat flour and cold water in a 3/2 ratio, and the mixture was homogenised for 5 min in an Ultraturrax (Janke & Kunkel, Ultraturrax T25, Manasquan, NJ, USA) mixer. After mixing, OEO was added, and the mixture was homogenised for 1 min. Then, the squid rings were immersed into each coating solution for 2 min and deep-fried with refined sunflower oil at 180±2ºC for 20 s in a Fritaurus Professional 4 domestic electric fryer (Taurus, Barcelona, Spain). After frying, the squid ring products were placed on polystyrene trays (20 cm x 12 cm), which were packed in retractile multiplayer co-extruded film bags. A mixture of gases consisting of 70% N_2, 25% CO_2 and 5% O_2 was injected into each sample at a ring/atmosphere ratio of 1/2 (w/v). The bags were immediately sealed to obtain the final commercial RTE product. Each tray contained 3 squid rings, so 24 trays were prepared for each treatment. The packed samples were stored in a refrigerated room at 4ºC. The samples were taken for analysis on days 3, 7, 10 and 14 of the refrigerated storage.

The second experiment concerned TEO (thyme oil white, FCC grade, from *Thymus vulgaris* and/or *Thymus zygis*; density: 0.917 g ml^{-1} at 25ºC; boiling point: 195ºC; Sigma Aldrich) and included squid rings (n = 234) that were randomly assigned into 4

batches (54 rings in each). Three of the batches were processed with a coating mixture supplemented with TEO at the following concentrations: 0.010% (T-1 batch), 0.025% (T-2 batch) or 0.050% (T-3 batch) (w/w, TEO/squid ring). A control batch (C-T) without thyme oil was also prepared. The remaining rings (n = 18) were considered as the initial sample of the thyme experiment; they were distributed among 3 groups (6 rings in each) that were analysed independently. The coating process, frying, packaging and storage temperature were the same as in the OEO experiment. Each tray contained 3 squid rings, so 18 trays were prepared for each treatment. Samples were taken for analysis on days 5, 8 and 12 of refrigerated storage.

In both the oregano and the thyme experiments, 6 trays corresponding to each treatment were analysed at each refrigeration time. For this analysis, the trays were distributed into 3 groups (2 trays per group), and each group was analysed separately.

Previous preliminary trials were performed to establish the maximum concentration of each essential oil that could be employed without modifying the product odour. Analysis of squid ring odour was conducted by a sensory panel of five experienced judges. For it, three-digital-code samples were presented to the panellists and qualified as “modified”, “not modified” or “doubtful”. Under the present experimental conditions, it was concluded that if the essential oils were added at a 0.075% (w/w; essential oil/squid ring) concentration or higher, the presence of the essential oil odour was noticed in the squid ring RTE product. Accordingly, concentrations tested in the present research are below this value.

Microbiological analyses

Squid rings were dissected aseptically from the coating medium in the refrigerated products; the initial squid rings were directly analysed. For this analysis, 10 g of squid

ring was mixed with 90 ml of 0.1% peptone water and homogenised in a stomacher (AES, Combourg, France) as previously described (Ben-Gigirey *et al*., 1998, 1999). In all cases, serial dilutions of the microbial extracts were prepared in 0.1% peptone water. The total aerobe counts were determined in plate count agar (PCA, Oxoid Ltd., London, UK) after incubation at 30ºC for 48 h as previously described (Ben-Gigirey *et al*., 1998, 1999). The anaerobe counts were also determined in PCA at 30ºC, but the plates were introduced inside an anaerobiosis jar. The psychrotroph counts were also determined in PCA under aerobic conditions, but the incubation was carried out at 7-8ºC for 7 days. The Enterobacteriaceae counts were determined by pour plating on Violet Red Bile Glucose Agar (VRBG) and subsequently incubated at 30ºC for 24-48 h.

In all cases, the bacterial counts were converted into log CFU g^{-1} squid ring before undergoing statistical analysis. All analyses were performed in triplicate.

Chemical analyses

The squid rings were separated from the coating medium, minced, homogenised and employed for the different chemical analyses: pH, total lipids, peroxide value (PV), thiobarbituric acid index (TBA-i) and fluorescence ratio (FR). The initial squid rings were analysed after mincing and homogenising.

The evaluation of the pH values in the squid rings throughout the storage time was performed via a 6-mm diameter insertion electrode (Crison, Barcelona, Spain).

The lipids were extracted from the squid rings using the Bligh & Dyer (1959) method, which employs a single-phase solubilisation step of the lipids using a chloroform/methanol (1/1) mixture. The quantification of results was expressed as g lipid kg^{-1} squid ring.

The PV was determined in the lipid extract by peroxide reduction with ferric thiocyanate according to the method of Chapman & McKay (1949). The results were expressed as meq active oxygen kg^{-1} lipids.

The TBA-i was determined according to the method of Vyncke (1970). This method is based on the reaction between a trichloracetic acid extract of the squid ring and thiobarbituric acid. The concentration of thiobarbituric acid reactive substances (TBARS) was spectrophotometrically measured at 532 nm, and the results were expressed as mg malondialdehyde kg^{-1} squid ring.

The formation of fluorescent compounds was determined by measurements at 393/463 nm and 327/415 nm as described by Aubourg & Gallardo (1997). The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission maximum, and F_{st} is the fluorescence intensity of a quinine sulphate solution (1 µg ml^{-1} in 0.05 M H_2SO_4) at the corresponding wavelength pair. The FR was calculated as the ratio between the two RF values: $FR = RF_{393/463\ nm} / RF_{327/415\ nm}$. Results on fluorescent compound formation are expressed as FR values and were determined in the lipid fraction resulting from the squid ring extraction (Bligh & Dyer, 1959).

All analyses were performed in triplicate.

Statistical analyses

The data from the different analyses were subjected to analysis of variance (ANOVA). PASW Statistics 18 (SPSS, Chicago, IL, USA) was used to explore the statistical significance of the differences due to the presence of the essential oil and the refrigeration time. Differences were considered significant for a confidence interval at the 95% level ($p<0.05$) in all cases.

RESULTS AND DISCUSSION

Microbiological stability

The comparative evolution of aerobes, anaerobes, Enterobacteriaceae and psychrotrophs during the refrigerated storage of RTE squid rings treated with OEO is shown in Table 1. Significantly ($p<0.05$) lower aerobic mesophile counts were measured in the squid rings treated with any of the three oregano concentrations relative to the control batch. Remarkably, aerobe counts below 3 log units were determined after 14 days of storage, whereas the control batch exhibited concentrations of approximately 6 log units on day 10. With respect to the development of anaerobic bacteria, lower counts at advanced storage times (10-14 days) were determined in the squid rings treated with any of the oregano concentrations tested than in the control batch. This inhibitory effect was found to be higher at day 10 for the two highest oregano concentrations (O-2 and O-3 conditions). It should be noted that the final anaerobe concentrations remained below 2 log units in the OEO batches, whereas these values were above 5 log units in the control batch on day 10.

With respect to Enterobacteriaceae, significant differences were found on day 7 between the three batches treated with oregano and the control batch. On day 14, significant differences between the control batch and the two most concentrated oregano batches (O-2 and O-3) were also observed. As in the case of aerobes and anaerobes, the Enterobacteriaceae assessment of oregano-treated squid samples exhibited a decreasing ($p<0.05$) value after processing and storing for 3 days. Since raw samples were analysed before the pre-frying step, the decrease in the microbial numbers determined for all batches on day 3 can be easily explained as a result of such frying step, which is required for the industrial elaboration of the RTE food product. However, when the pre-fried batches were compared (3-14-day period), the significant differences found among

OEO batches and controls for all microbial groups evidenced the inhibitory effect derived from the presence of such essential oil.

Differences in psychrotroph counts were also observed after 10 and 14 days of storage. Thus, on day 10, lower counts were obtained for the OEO batches than for the control batch. On day 10, differences between the control batch and the O-1, O-2 and O-3 batches, respectively, were observed.

In conclusion, OEO in the coating medium of squid rings had a marked inhibitory effect on microbial development. Our study included a MAP strategy and refrigerated storage, which were combined with the OEO treatment. Our results are in agreement with those previous studies where OEO resulted in an enhancement of quality when combined with other preservation technologies. For example, an inhibitory effect of OEO on microbial activity development (total volatile base-nitrogen and trimethylamine-nitrogen assessment; TVB-N and TMA-N, respectively) was reported for the combination of OEO-MAP in salted sea bream (*Sparus aurata*) that were stored under refrigerated conditions (4±0.5ºC) (Goulas & Kontominas, 2007). Likewise, Giatrakou *et al*. (2008) reported positive results in microbial count and volatile amine assessment for the combination of OEO-MAP in swordfish (*Xiphias glaudius*) fillets stored at 4ºC. More recently, Pyrgotou *et al*. (2010) reported an inhibitory effect of the combination of OEO and MAP on the microbial activity (determined via the microbial counts and volatile amine formation) of salted rainbow trout (*Oncorhynchus mykiss*) fillets stored at 4ºC. In a different type of combination (an OEO-package provided with an oxygen absorber), Mexis *et al*. (2009) found that microbial activity was partially inhibited (microbial count and volatile amine assessment) during the storage of rainbow trout (*O. mykiss*) fillets at 4ºC.

The assessment of microbial growth on the RTE squid rings from the TEO experiment is shown in Table 2. The comparative evolution of aerobic mesophiles did not significantly ($p>0.05$) differ with the addition of TEO to the coating medium during processing. A marked decrease ($p<0.05$) was observed in all cases after processing (namely, frying) and storage for 5 days. Then (5-12-day period), an increase in aerobe counts with storage time was observed for all types of squid batches; a marked effect of MAP conditions can be involved since aerobe numbers observed at the end of the experiment could be considered relatively low (counts under 3.50 log units) in all cases. Only slight differences in the number of anaerobes among batches were detected; thus, the presence of thyme in the coat did not have an effect ($p>0.05$) on anaerobe growth. Contrary to the case of aerobes, a decreasing mean number of anaerobes with storage time was observed in most batches, and these decreases were significant ($p<0.05$) in the case of the T-3 batch after processing and storage for 5 days and on day 8 for the remaining sample types (Table 2). As in the case of the aerobe numbers, the anaerobe numbers in all batches could be considered relatively low (scores under 3.20) and be the probable effect derived from the application of MAP conditions, especially of the presence of CO_2 in the packaging atmosphere.

The Enterobacteriaceae counts were markedly decreased ($p<0.05$) after processing and storage for 5 days in all batches. Such a decrease can be mostly explained as a result of the pre-frying process, as stated above. Subsequently (5-8-day period), no significant ($p>0.05$) differences due to storage time were observed, except for the control batch. In addition, no effect ($p>0.05$) of the thyme addition at any concentration to the coating medium on the Enterobacteriaceae counts was observed (Table 2). As for Enterobacteriaceae, psychrotroph counts showed a marked decrease ($p<0.05$) after processing and storage for 5 days; then, no significant differences ($p>0.05$) were

observed for this microbial group, a result that may also be explained as an effect of MAP conditions on all kinds of samples.

Accordingly, an inhibitory effect of TEO in the coating medium on the microbiological quality of squid ring products subjected to MAP and refrigeration was not observed. Contrary to our results, the use of thyme extended the shelf-life of fresh fish, such as wild and farmed gilthead sea bream (*S. aurata*) fillets, by approximately 5 days as assessed by TVB-N, TMA-N, free amino acid formation and water-holding capacity assays (Attouchi & Sadok, 2010). The presence of TEO has also been reported to improve the microbial quality in terms of microbial counts, TMA-N and TVB-N assessment and the sensory quality of refrigerated (4.0±0.5ºC) sea bass (*Dicentrarchus labrax*) fillets when combined with MAP (Kostaki *et al*., 2009). This preservation combination (TEO-MAP) led to an improvement in the microbial quality in blue fish burgers stored at 4ºC for 28 days (Del Nobile *et al*., 2009) and inhibited the microbial activity (microbial count, TMA-N and TVB-N assessment) and sensory quality loss of swordfish (*X. gladius*) during 18 days of storage at 4ºC (Kykkidou *et al*., 2009). Additionally, the combination of TEO and vacuum packaging of hot smoked rainbow trout (*O. mykiss*) stored at 2ºC was reported to inhibit the microbial activity (in terms of microbial count and TVB-N and TMA-N assessments) (Erkan, 2010).

In previous research, Mejlholm & Dalgaard (2002) found that the addition of OEO and TEO reduced microbial growth in cod (*Gadus morhua*) fillets and extended the shelf life of these fillets during refrigerated storage (2ºC), and the antimicrobial activity of OEO was higher than that of TEO. However, Harpaz *et al*. (2003) showed that both OEO and TEO additions slowed the spoilage of Asian sea bass (*Lates calcarifer*) during storage at 0-2ºC without showing a differential effect between both types of oils.

On contrast to these reports, the lack of effect of TEO on the microbial development in pre-fried squid rings observed in our study may be explained as follows: (i) unlike other previous studies, the initial frying step considered in our study might have exerted a negative effect on TEO stability; (ii) unlike other previous studies that considered aerobic storage or vacuum-packaging of fish, the squid rings considered in our study were subjected to MAP. The particular atmosphere considered in our study may imply the selection of a different type of spoilage microflora as compared with previous reports, and this might be related to the lack of effect of TEO in our study; and (iii) while most of the previous reports have been focused on fish fillets, the final TEO concentrations in elaborated products such as squid rings might need to be modified due to the potential different level of microbial contamination in this type of products. In all cases, and although these causes may explain the results obtained, the lack of effect of TEO in our study will need an additional research effort.

Chemical stability

In the oregano experiment (Table 3), lower pH values were obtained at all storage times for the two batches treated with the higher OEO concentrations. The differences in the pH values were significant ($p<0.05$) on day 7 for the squid rings in the O-3 batch compared with those of the control batch. However, in the thyme experiment (Table 4), a marked tendency could not be discerned when comparing the different batches. With respect to the effect of the storage time, both experiments (Tables 3-4) showed a marked pH increase ($p<0.05$) after processing and storage until day 3 and day 5 for the oregano and thyme experiments, respectively. After longer storage times, no differences ($p>0.05$) were observed for each essential oil because of the refrigerated storage conditions. In agreement with our results on the pH in the oregano experiment, other

authors have previously reported lower pH values as a result of the OEO treatment of sea bream (*S. aurata*) fillets refrigerated at 4±0.5ºC (Goulas & Kontominas, 2007) and rainbow trout (*O. mykiss*) fillets refrigerated at 4ºC (Mexis *et al*., 2009). Likewise, previous research has reported no effect of TEO treatment on the pH value when compared with the control; this observation accounts for the preservation of blue fish burgers during 28 days of storage (Del Nobile *et al*., 2009) and of swordfish (*X. gladius*) fillets during storage at 4ºC (Kykkidou *et al*., 2009). However, other authors have reported lower pH values as a consequence of the application of TEO when compared with a control; such research refers to different fish species such as sea bass (*D. labrax*) during refrigerated storage at 4±0.5ºC (Kostaki *et al*., 2009) and wild and farmed gilthead sea bream (*S. aurata*) fillets stored in ice (Attouchi & Sadok, 2010).

The lipid content of the initial non-fried squid ring samples was 4.4±0.5 and 4.5±0.5 g kg^{-1} squid ring for the oregano and thyme experiments, respectively. The lipid content increased for all types of processed material such that the lipid contents were in the 8.0-11.0 and 8.5-11.5 g kg^{-1} ranges for the samples corresponding to the oregano and thyme experiments, respectively. The increase in lipid content is likely a result of the frying step (Huidobro *et al*., 1995; Castrillón *et al*., 1997). No differences ($p>0.05$) in the lipid content of the squid rings could be found because of the addition of oregano or thyme or the storage time.

The occurrence of lipid oxidation in the squid rings was assessed via the formation of peroxide, TBARS and interaction compounds. The PV assessment indicated an inhibitory effect of OEO (Table 3), which was especially remarkable for the two batches with the higher OEO concentrations. This preservation effect could also be observed for the secondary (TBARS) and tertiary (fluorescence ratio) lipid oxidation product formation, although in these cases, no differences could be established among

the different OEO concentrations tested (Table 3). In all cases, the values determined for the PV and the TBA-i (below 6.0 and 0.80, respectively) can be considered relatively low.

Other authors have reported that the application of OEO has positive effects when combined with other preservation techniques. Thus, an inhibitory effect on lipid oxidation development (TBARS assessment) was observed when OEO was applied to salted sea bream (*S. aurata*) stored at 4±0.5ºC (Goulas & Kontominas, 2007) and in salted rainbow trout (*O. mykiss*) fillets stored at 4ºC (Pyrgotou *et al*., 2010). The combination of OEO and an oxygen absorber also reduced the rate of lipid oxidation (peroxide value and TBARS assessment) in rainbow trout (*O. mykiss*) fillets stored at 4ºC (Mexis *et al*., 2009).

An inhibitory effect of thyme oil on the lipid oxidation of squid rings was observed (Table 4). Thus, the highest thyme concentration tested (T-3 condition) decreased the formation of peroxides (PV), TBARS and fluorescent compounds as compared with the control batch. Likewise, the T-1 and T-2 batches also exhibited some inhibitory effect; this effect was significant ($p<0.05$) only for TBARS formation on day 12 and on both days 8 and 12 for the T-1 and T-2 batches, respectively.

In agreement with our results, other authors have reported a partial inhibition of lipid oxidation due to the addition of TEO. For example, the use of TEO extended the lipid stability of wild and farmed gilthead sea bream (*S. aurata*) fillets by approximately 5 days during chilled storage (Attouchi & Sadok, 2010). Likewise, the combination of TEO and MAP was also found profitable when compared with MAP alone; thus, lipid oxidation was inhibited in sword fish (*X. gladius*) fillets at 4ºC during 18 days of refrigerated storage (Kykkidou *et al*., 2009) and in hot smoked rainbow trout (*O. mykiss*) stored at 2ºC (Erkan, 2010). Finally, thyme oil was also tested as a component

of a biodegradable film including hake (*Merluccius capensis*) proteins at different concentrations; the addition of TEO reduced the film thickness and water vapour permeability and exerted a significant antioxidant activity (Pires *et al*., 2011).

Conclusions

The present study provides a first approach to the employment of two important essential oils (OEO and TEO) with the aim of extending the period of good quality of an RTE product, pre-fried squid ring, commercialised under MAP and refrigeration conditions. It can be concluded that there is a marked ($p<0.05$) inhibitory effect on microbial activity (aerobes, anaerobes, Enterobacteriaceae and psychrotrophs) due to the addition of OEO to the coating at all OEO concentrations tested, and this effect was more relevant for the highest oregano concentration (0.050%). The addition of OEO also provoked the inhibition of lipid oxidation, as determined by the formation of peroxides, TBARS and interaction compounds. In contrast, the TEO experiment demonstrated that the addition of TEO to the coating did not result in an inhibitory effect ($p>0.05$) of the microbial activity in RTE squid rings, although some inhibitory effect on lipid oxidation development could be noted, especially when using the highest (0.050%) TEO concentration.

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TABLE 1: Microbiological count (log CFU g^{-1} muscle) assessment* in refrigerated squid rings treated with oregano essential oil (OEO)**

Microbiological parameter	Storage time (days)	OEO treatment			
		C-O	O-1	O-2	O-3
Aerobes	Raw squid rings	a 3.39 (0.12)	c 3.39 (0.12)	b 3.39 (0.12)	b 3.39 (0.12)
	3	a 2.85 y (1.21)	a 1.99 z (0.00)	a 1.99 z (0.00)	a 1.99 z (0.00)
	7	ab 4.16 y (1.66)	a 1.99 z (0.00)	a 1.99 z (0.00)	a 1.99 z (0.00)
	10	b 5.94 y (0.41)	abc 2.63 z (0.91)	a 1.80 z (0.28)	b 2.77 z (0.66)
	14	ab 5.35 y (1.70)	b 2.57 z (0.38)	b 2.95 zy (0.83)	ab 2.85 zy (1.21)
Anaerobes	Raw squid rings	b 3.76 (0.63)	b 3.76 (0.63)	c 3.76 (0.63)	b 3.76 (0.63)
	3	ab 2.15 (1.20)	a 1.74 (0.37)	a 1.39 (0.12)	a 1.74 (0.37)
	7	a 1.99 (0.00)	a 1.99 (0.00)	b 1.99 (0.00)	a 1.99 (0.00)
	10	c 5.45 x (0.52)	b 3.92 y (0.45)	b 1.80 z (0.28)	a 1.65 z (0.49)
	14	c 5.42 y (1.03)	a 1.99 z (0.00)	abc 1.99 z (1.81)	a 1.99 z (0.00)
Enterobacteriaceae	Raw squid rings	b 1.89 (0.27)	b 1.89 (0.27)	b 1.89 (0.27)	b 1.89 (0.27)
	3	a 0.99 (0.00)	a 0.99 (0.00)	a 0.99 (0.00)	a 0.99 (0.00)
	7	b 2.56 y (0.78)	a 0.99 z (0.00)	a 0.99 z (0.00)	a 0.99 z (0.00)
	10	a 0.99 (0.00)	a 0.99 (0.00)	a 0.99 (0.00)	a 0.99 (0.00)
	14	b 2.36 y (0.52)	b 1.65 y (0.49)	a 0.99 z (0.00)	a 0.99 z (0.00)
Psychrotrophes	Raw squid rings	a 1.30 (0.43)	b 1.30 (0.43)	b 1.30 (0.43)	a 1.30 (0.43)
	3	ab 1.95 (1.34)	a 0.99 (0.00)	a 0.99 (0.00)	a 1.49 (0.43)
	7	b 1.99 (0.00)	c 1.99 (0.00)	c 1.99 (0.00)	b 1.99 (0.00)
	10	c 5.56 y (0.79)	d 3.62 z (0.74)	abc 2.15 z (1.63)	ab 2.33 z (1.89)
	14	abc 4.10 y (2.99)	c 1.99 z (0.00)	abc 1.99 y (1.08)	b 1.99 z (0.00)

* Mean values of three independent replicates (n = 3); standard deviations are indicated in brackets. For each storage time, mean values in the same row followed by different letters (z, y, x) indicate significant ($p<0.05$) differences as a result of the OEO treatment. For each treatment, mean values in the same column followed by different letters (a-d) denote significant differences ($p<0.05$) as a result of the refrigeration time. No letters are indicated when significant differences are not found ($p>0.05$).

** OEO concentrations (TEO/squid ring, w/w) employed in the coating medium: 0.000% (C-O; control), 0.010% (O-1), 0.025% (O-2) and 0.050% (O-3).

TABLE 2: Microbiological count (log CFU g^{-1} muscle) assessment* in refrigerated squid rings treated with thyme essential oil (TEO)**

Microbiological parameter	Storage time (days)	TEO treatment			
		C-T	T-1	T-2	T-3
Aerobes	Raw squid rings	b 3.72 (0.02)	b 3.72 (0.02)	c 3.72 (0.02)	b 3.72 (0.02)
	5	a 2.00 (0.00)	a 1.99 (0.00)	a 1.99 (0.00)	a 2.15 (0.21)
	8	ab 2.84 (1.21)	a 1.99 (0.00)	ab 2.65 (0.93)	a 1.99 (0.00)
	12	b 3.09 (0.87)	ab 2.74 (1.06)	b 3.34 (0.18)	b 3.27 (0.70)
Anaerobes	Raw squid rings	c 3.16 (0.45)	b 3.16 (0.45)	b 3.16 (0.45)	b 3.16 (0.45)
	5	bc 2.87 zy (0.80)	ab 2.42 zy (0.60)	b 3.00 y (0.00)	a 2.15 z (0.21)
	8	a 1.99 (0.00)	a 1.99 (0.00)	a 1.99 (0.00)	a 1.99 (0.00)
	12	ab 2.15 (0.21)	a 1.99 (0.00)	a 2.00 (0.01)	a 2.00 (0.01)
Enterobacteriaceae	Raw squid rings	c 3.39 (0.54)	b 3.39 (0.54)	b 3.39 (0.54)	b 3.39 (0.54)
	5	b 1.50 (0.71)	a 0.99 (0.00)	a 0.99 (0.00)	a 0.99 (0.00)
	8	a 0.99 (0.00)	a 0.99 (0.00)	a 0.99 (0.00)	a 0.99 (0.00)
	12	a 0.99 (0.00)	a 0.99 (0.00)	a 0.99 (0.00)	a 0.99 (0.00)
Psychrotrophes	Raw squid rings	b 3.14 (0.56)	b 3.14 (0.56)	b 3.14 (0.56)	b 3.14 (0.56)
	5	a 2.00 (0.01)	a 2.15 (0.22)	a 1.99 (0.00)	a 1.99 (0.00)
	8	a 1.99 (0.00)	a 1.99 (0.00)	a 1.99 (0.00)	a 1.99 (0.00)
	12	a 2.00 (0.01)	a 1.99 (0.00)	a 2.00 (0.01)	a 2.00 (0.01)

* Mean values of three independent replicates (n = 3); standard deviations are indicated in brackets. For each storage time, mean values in the same row followed by different letters (z, y) indicate significant ($p<0.05$) differences as a result of the TEO treatment. For each treatment, mean values in the same column followed by different letters (a, b, c) denote significant differences ($p<0.05$) as a result of the refrigeration time. No letters are indicated when significant differences are not found ($p>0.05$).

** TEO concentrations (TEO/squid ring, w/w) employed in the coating medium: 0.000% (C-T; control), 0.010% (T-1), 0.025% (T-2) and 0.050% (T-3).

TABLE 3: Assessment of chemical quality parameters* in refrigerated squid rings treated with oregano essential oil (OEO)**

Chemical parameter	Storage time (days)	OEO treatment			
		C-O	O-1	O-2	O-3
pH	Raw squid rings	a 6.42 (0.00)	a 6.42 (0.00)	a 6.42 (0.00)	a 6.42 (0.00)
	3	b 7.09 (0.30)	b 7.09 (0.30)	b 6.90 (0.21)	b 6.89 (0.04)
	7	b 7.06 y (0.08)	b 7.06 y (0.08)	b 6.98 zy (0.03)	b 6.87 z (0.10)
	10	b 7.15 (0.19)	b 7.15 (0.19)	b 7.07 (0.07)	b 6.99 (0.04)
	14	b 7.09 (0.06)	b 7.09 (0.06)	b 7.02 (0.19)	b 6.90 (0.13)
Peroxide value (meq oxygen kg^{-1} lipids)	Raw squid rings	ab 2.36 (0.62)	c 2.36 (0.62)	b 2.36 (0.62)	a 2.36 (0.62)
	3	a 2.18 y (0.33)	a 0.82 z (0.24)	a 1.43 z (0.11)	a 1.43 z (0.41)
	7	bc 3.79 zy (1.18)	d 3.52 y (0.37)	b 2.16 z (0.50)	a 2.79 zy (1.08)
	10	c 3.90 y (0.21)	b 1.86 z (0.06)	b 2.16 z (0.55)	a 2.03 z (0.39)
	14	d 5.70 y (0.05)	e 5.70 y (0.33)	c 4.80 z (0.25)	b 5.21 z (0.11)
Thiobarbituric acid index (mg malondialdehyde kg^{-1} muscle)	Raw squid rings	a 0.53 (0.15)	ab 0.53 (0.15)	a 0.53 (0.15)	a 0.53 (0.15)
	3	ab 0.74 (0.06)	bc 0.74 (0.06)	b 0.79 (0.05)	c 0.79 (0.03)
	7	b 0.78 y (0.04)	b 0.69 z (0.02)	ab 0.73 zy (0.12)	bc 0.75 zy (0.04)
	10	b 0.75 (0.03)	c 0.79 (0.04)	ab 0.74 (0.10)	bc 0.74 (0.04)
	14	b 0.79 y (0.01)	a 0.54 z (0.06)	a 0.65 z (0.05)	ab 0.66 z (0.07)
Fluorescence ratio	Raw squid rings	a 2.10 (0.10)	a 2.10 (0.10)	a 2.10 (0.10)	a 2.10 (0.10)
	3	a 2.15 (0.13)	a 1.98 (0.07)	a 1.98 (0.04)	ab 2.27 (0.29)
	7	b 2.54 (0.11)	b 2.43 (0.05)	b 2.36 (0.08)	b 2.47 (0.27)
	10	c 2.86 y (0.07)	ab 2.24 z (0.21)	ab 2.24 z (0.23)	b 2.44 z (0.11)
	14	d 3.38 y (0.12)	b 2.69 z (0.35)	b 2.43 z (0.12)	b 2.42 z (0.15)

* Mean values of three independent replicates (n = 3); standard deviations are indicated in brackets. For each storage time, mean values in the same row followed by different letters (z, y) indicate significant ($p<0.05$) differences as a result of the OEO treatment. For each treatment, mean values in the same column followed by different letters (a-e) denote significant differences ($p<0.05$) as a result of the refrigeration time. No letters are indicated when significant differences are not found ($p>0.05$).

** OEO concentrations employed as expressed in Table 1.

TABLE 4: Assessment of chemical quality parameters* in refrigerated squid rings treated with oregano essential oil (TEO)**

Chemical parameter	Storage time (days)	TEO treatment			
		C-O	O-1	O-2	O-3
pH	Raw squid rings	a 6.32 (0.01)	a 6.32 (0.01)	a 6.32 (0.01)	a 6.32 (0.01)
	5	b 7.47 (0.14)	b 7.57 (0.29)	b 7.38 (0.04)	b 7.45 (0.05)
	8	b 7.47 (0.14)	b 7.37 (0.05)	b 7.45 (0.24)	b 7.47 (0.14)
	12	b 7.48 (0.21)	b 7.46 (0.33)	b 7.59 (0.26)	b 7.48 (0.17)
Peroxide value (meq oxygen kg^{-1} lipids)	Raw squid rings	2.42 (0.45)	2.42 (0.45)	2.42 (0.45)	b 2.42 (0.45)
	5	2.38 (1.32)	1.89 (1.00)	1.67 (0.85)	a 1.59 (0.24)
	8	1.95 y (0.11)	2.07 y (0.14)	1.89 y (0.10)	a 1.57 z (0.16)
	12	2.66 y (0.70)	1.97 zy (0.74)	1.89 zy (0.27)	a 1.57 z (0.21)
Thiobarbituric acid index (mg malondialdehyde kg^{-1} muscle)	Raw squid rings	a 0.28 (0.04)	a 0.28 (0.04)	a 0.28 (0.04)	0.28 (0.04)
	5	b 0.51 (0.14)	b 0.41 (0.04)	b 0.48 (0.03)	0.45 (0.14)
	8	b 0.53 y (0.10)	b 0.39 zy (0.07)	a 0.33 z (0.03)	0.31 z (0.02)
	12	c 1.64 x (0.33)	c 0.96 y (0.25)	ab 0.41 z (0.16)	0.52 z (0.14)
Fluorescence ratio	Raw squid rings	a 2.26 (0.08)	a 2.26 (0.08)	a 2.26 (0.08)	a 2.26 (0.08)
	5	a 2.32 (0.11)	a 2.41 (0.17)	b 2.42 (0.03)	a 2.33 (0.05)
	8	b 2.61 (0.12)	b 2.84 (0.13)	c 2.75 (0.25)	b 2.70 (0.12)
	12	b 2.82 y (0.15)	b 2.76 zy (0.11)	c 2.68 zy (0.07)	b 2.60 z (0.05)

* Mean values of three independent replicates (n = 3); standard deviations are indicated in brackets. For each storage time, mean values in the same row followed by different letters (z, y, x) indicate significant ($p<0.05$) differences as a result of the TEO treatment. For each treatment, mean values in the same column followed by different letters (a, b, c) denote significant differences ($p<0.05$) as a result of the refrigeration time. No letters are indicated when significant differences are not found ($p>0.05$).

** TEO concentrations as expressed in Table 2.