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Dietary Supplementation of Oregano Essential Oil and α-tocopheryl Acetate on Microbial Growth and Lipid Oxidation of Turkey Breast Fillets During Storage

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Abstract: Twenty four 12-week-old turkeys were divided into four equal groups. One of the groups was given a basal diet containing 30 mg α-tocopheryl acetate/kg feed (CONT), whereas the other groups the basal diet further supplemented with 100 mg α-tocopheryl acetate/kg (TOC), or 100 mg oregano essential oil/kg (OR), or 100 mg oregano essential oil plus 100 mg α-tocopheryl acetate/kg (ORTOC), for 4 weeks prior to slaughter. Lipid oxidation, total viable counts (TVC) and *Pseudomonas spp*. counts were all assessed in breast fillets stored refrigerated at 4°C for 12 days. Results showed that the OR group was more effective ($P<0.05$) in delaying lipid oxidation compared to the CONT group, but inferior ($P<0.05$) to TOC group which in turn was inferior ($P<0.05$) to the ORTOC group. TVC and *Pseudomonas spp*. counts of the TOC group were not different ($P<0.05$) than those of the CONT group, but higher ($P<0.05$) than those of the OR and ORTOC groups, which in their turn did not differ ($P>0.05$) among each other.

Key words: Oregano, α-tocopherol, turkey meat, microbial growth, lipid oxidation, *Pseudomonas spp*.

Introduction

Fresh meat is easily contaminated with a variety of microorganisms and, if not properly handled and preserved, it supports growth of spoilage and pathogenic species, leading to loss of quality and potential public heath problems (Sofos *et al*., 2000). Microbial growth and lipid oxidation are primary factors of meat spoilage during refrigerated storage. To extend storage period, antimicrobial and antioxidant additives especially of synthetic origin, are added in muscle foods. However, consumers and health authorities increasingly dictate that the use of chemical food additives should be phased out and, where possible, only natural products should be used. Hence, new nutritional strategies for the growing animals are introduced and alternative substances from natural sources, such as vitamin E and herbal extracts, are investigated for obtaining muscle food of higher quality.

The use of nutritional strategies to improve the quality of muscle food is a relatively new approach that has emerged at the interface of animal science and food science. It often represents the only technology available to alter the quality of intact muscle food, where utilization of exogenous compounds is difficult if not impossible. Nutritional approaches are often more effective than direct addition of the additive to the muscle food since the compound is preferably deposited where it is most needed (Govaris *et al*., 2004). When the additive is the lipid soluble antioxidant vitamin E, its dietary supplementation allows uniform incorporation of α-tocopherol into the subcellular membranes (Sheehy *et al*., 1993; Packer and Kagan, 1993), where it can effectively inhibit the oxidative reactions at their localized sites improving meat quality due to delayed lipid oxidation and muscle discoloration. The efficacy of the dietary supplemented vitamin E, used as α-tocopheryl acetate, against lipid oxidation of muscle tissues has been well demonstrated in a variety of animals including turkeys; its efficacy, however, against microbial growth of the produced turkey muscle tissue has not been yet evaluated (Higgins *et al*., 1998; Sofos *et al*., 2000; Botsoglou *et al*., 2003a, b; Papageorgiou *et al*., 2003). Herbal extracts of the Labiatae family including rosemary, sage and oregano (Lopez-Bote *et al*., 1998; Botsoglou *et al*., 2002), have presented further the potential to serve as efficient food antioxidants from natural sources when added in animal diets. The essential oil of oregano, in particular, which is obtained by a steam-distillation process of leaves and flowers of *Origanum vulgare subsp. hirtum* plants, is a very promising dietary supplement since it exhibits substantial antimicrobial and antioxidant activity in vitro (Kokkini, 1994; Ultee *et al*., 1999; Marino *et al*., 2001). Several recent studies have shown that incorporation of this essential oil in turkey diets improved the oxidative stability of the produced raw and cooked muscle tissues during refrigerated and long-tern frozen storage (Botsoglou *et al*., 2003 a, b). However, no evidence is yet

Table 1: Composition of basal diet

Components	[g/kg feed]
Corn, grains	519.5
Herring meal	25.0
Soybean meal	310.0
Soybean oil	25.0
Corn gluten feed	60.0
Yeast	25.0
DL-Methionine	1.0
Biolysine	1.0
Choline chloride	0.2
Limestone pulverized	18.0
Dicalcium phosphate	10.0
Sodium chloride, iodized	3.2
Natuphos (phytase)	0.1
Vitamin premix[a]	1.0
Trace-mineral premix[b]	1.0

[a]Supplying per kg of feed: 14,000 IU all-trans retinol acetate, 5,000 IU cholecalciferol, 30 mg α-tocopheryl acetate, 7 mg menadione sodium bisulphite, 5 mg thiamine hydrochloride, 10 mg riboflavin, 10 mg pyridoxine hydrochloride, 0.02 mg cyanocobalamine, 85 mg niacin, 25 mg pantothenic acid, 2 mg folic acid, 0.25 mg biotin, and 10 mg ascorbic acid.
[b] Supplying per kg of feed: 100 mg Zn, 120 mg Mn, 20 mg Fe, 20 mg Cu, 0.2 mg Co, 1 mg I, and 0.3 mg Se.

available on the potential antimicrobial properties of the essential oil of oregano when added in animal diets.

The use of nutritional strategies to improve the oxidative stability of animal products can extend the shelf life and increase the acceptability of muscle food during retail display. However, delayed lipid oxidation caused by use of supplemental dietary antioxidants to the growing animal may mask microbial growth without warning consumers of product spoilage expressed as undesirable off-flavors or odors. A major concern with extending the shelf life of muscle food is associated to the extent and type of microbial growth that may occur during the additional time of product acceptability. The present study was designed to evaluate the effects of dietary oregano essential oil and α-tocopheryl acetate supplementation on microbial growth and lipid oxidation of turkey breast fillets during refrigerated storage.

Materials and Methods

Animals and diets: Twenty-four 10-week-old female turkeys of black strain of local type divided into four equal groups were used in this study. The birds were allowed to acclimate for a period of two weeks. During the acclimatization period, they were fed *ad libitum* on a commercial turkey diet supplemented with a basal amount of 30 mg α-tocopheryl acetate/kg. The ingredients and the composition of the commercial basal diet are presented in Table 1. Ambient temperature was controlled, continuous lighting was provided, and conventional breeding and management procedures were employed throughout the experiment. After the end of the acclimatization period, feeding of the commercial diet was discontinued to all but one of the groups. The birds within this control group (CONT) were given the commercial diet for a further four weeks. The experimental diets given to the remaining three groups were based on the same commercial diet but contained an additional 100 mg α-tocopheryl acetate/kg (TOC), or oregano essential oil at 100 mg/kg feed (OR), or 100 mg oregano essential oil plus 100 α-tocopheryl acetatekg (ORTOC). Oregano oil was from Ecopharm Hellas S.A. (Kilkis, Greece) in form of a feed supplement called Orego-Stim (Meriden Animal Health Ltd. (Luton, UK) that contains 5% essential oil of *Origanum vulgare subsp. hirtum* plants and 95% natural feed grade inert carrier. α-Tocopheryl acetate was obtained from Roche Products Ltd. (Hertfordshire, UK). Feeding of the experimental diets to turkeys lasted four weeks.

Sampling procedure: At 16 weeks of age, turkeys were slaughtered, and carcasses were immediately trimmed for breast fillets by removing skin, bones and connective tissue. Following trimming, breast fillet samples from each bird were individually divided into two sections each of which was placed in a sterile polypropylene bag and stored in a non-illuminated refrigerated cabinet at 4°C for up to 12 days. One section was used for microbiological analysis whereas the other for oxidative stability studies.

Microbiological analysis: Sampling for microbiological analysis was carried out at 0 day and at 2-d intervals for up to 12 days of refrigerated storage. Duplicate samples were taken by cutting aseptically two sections (2cm x 2cm x 0.5 cm deep) of surface tissues of turkey breast fillets. Each sample was placed in a sterile Stomacher bag, diluted 1:10 with 0.1% sterile peptone water (Oxoid, Basingstoke,UK) and homogenized for 2 min (Stomacher, Seward, Medical Ltd, London, England). After maceration, serial decimal dilutions were made in 0.1% peptone water and 0.1 ml was plated on appropriate media. Microbiological analysis included total viable counts (TVC) and *Pseudomonas spp*. counts. TVC were determined on plate count agar (Oxoid, Basingstoke, UK) at 25° C for 72 h. *Pseudomonas spp*. were determined on *Pseudomonas* agar base, CM 559 (Oxoid, Basingstoke, UK) supplemented with cetrimide, fucidin and cephaloridine (Pseudomonas CFC supplement, SR 103; Oxoid, Basingstoke, UK) at 25°C for 48 h.

Lipid oxidation studies: To access the effect of the dietary treatments on the oxidative stability of turkey breast fillets during refrigerated storage, samples were sliced, and oxidative changes were monitored on the basis of the malondialdehyde (MDA) formed at 0 day and at 2-d intervals for up to 12 days of storage. MDA, the

Table 2: Effect of dietary oregano oil and α-tocopheryl acetate supplementation on α-tocopherol concentrations in turkey breast fillet

Treatments	α-Tocopheryl acetate added in feed (μg/g)	Mean α-tocopherol found in breast fillet (μg/g)
CONT**	30	1.17 ± 0.26[a]
OR	30	1.28 ± 0.17a
ORTOC	130	1.96 ± 0.24[b]
TOC	130	1.90 ± 0.41[b]

*Mean value± standard deviation from 6 samples analyzed
**CONT = turkeys fed control diet; OR = turkeys dietary supplemented with 100 mg oregano oil/kg; ORTOC = turkeys dietary supplemented with 100 mg oregano oil plus 100 mg α-tocopheryl acetate/kg; TOC = turkeys dietary supplemented with 100 mg α-tocopheryl acetate/kg

compound used as an index of lipid peroxidation, was determined by a selective third-order derivative spectrophotometric method (Botsoglou *et al.*, 1994). In brief, samples were homogenized in presence of 8 ml of 5% aqueous trichloroacetic acid (Merck, Darmstadt, Germany) and 5 ml of 0.8% butylated hydroxytoluene (Sigma Chemical Co, St. Louis, MO) in hexane, and the mixture was centrifuged. The top layer was discarded, and a 2.5-ml aliquot from the bottom layer was mixed with 1.5 ml of 0.8% aqueous 2-thiobarbituric acid (Sigma Chemical Co, St. Louis, MO) to be further incubated at 70°C for 30 min. Following incubation, the mixture was cooled under tap water and submitted to conventional spectrophotometry (Shimadzu, Model UV-160A, Tokyo, Japan) in the range of 400-650 nm. Third-order derivative spectra were produced by digital differentiation of the normal spectra using a derivative wavelength difference setting of 21 nm. The concentration of MDA in analyzed samples was calculated on the basis of the height of the third-order derivative peak at 521.5 nm by referring to slope and intercept data of the computed least-squares fit of standard calibration curve prepared using 1,1,3,3-tetraethoxypropane (Sigma Chemical Co, St. Louis, MO).

Assay for α-tocopherol in breast samples: For the extraction of α-tocopherol, muscle and feed samples (0.5 g) were homogenized with 5 ml of saturated methanolic solution of KOH in presence of 100μl pyrocatechol (Merck, Darmstadt, Germany) solution (200 mg/ml) and, and then immersed in a water bath at 80 °C for 15 min (Botsoglou *et al.*, 1998). Following saponification, 5 ml hexane and 1 ml water were added, and the mixture was vortex-mixed and centrifuged at 2000 g. An aliquot of the upper phase was evaporated to dryness to be further reconstituted in methanol and injected into the liquid chromatograph (Shimadzu, Model 6AV, Tokyo, Japan). Liquid chromatography was carried using a Nucleosil C_{18}, 5 mm, 250 x 4.6 mm, column (Reading, UK), and a mobile phase of methanol/water (97:3, v/v) that was delivered to the system at a flow rate of 2 ml/min (Sheehy *et al.*, 1994). A fluorimetric detector set at excitation wavelength of 290 nm and emission wavelength of 330 nm was used for monitoring column effluents. Detector signals were quantified on the basis of peak heights and a calibration curve using α-tocopherol (Sigma Chemical Co, St. Louis, MO) as reference standard.

Statistical analysis: Each individual turkey within each group served as experimental unit in the statistical analysis of all data. Data were subjected to analysis of variance (ANOVA) using the SPSS 12.00 statistical package (SPSS Ltd., Woking, Surrey, UK). The homogeneity of the variances was tested by Bartlett's test. When significant treatment effects were disclosed at the probability level of $P<0.05$, the Tukey's test was applied in order to determine statistical differences between means (Anderson and McLean, 1974).

Results and Discussion

The susceptibility of raw turkey breast fillets to lipid oxidation as a function of storage time and dietary treatments is illustrated in Figure 1. Refrigerated storage increased the levels of malondialdehyde (MDA), the compound used as an index of lipid oxidation, the increase being higher in the control group. However, the extent of lipid oxidation was also influenced by the dietary treatments. The OR group presented MDA values that were significantly ($P<0.05$) lower than the CONT group but higher ($P<0.05$) than the TOC and ORTOC groups after 3, 6 and 9 days of refrigerated storage. Among the latter two groups dietary supplemented with α-tocopheryl acetate, the ORTOC group exhibited MDA values that were significantly ($P<0.05$) lower than the TOC group.

The difference in the MDA values between the ORTOC and TOC groups should be due to antioxidant constituents of oregano essential oil that entered the circulatory system, distributed and retained in tissues. Studies on the composition of the oregano essential oil have shown more than 35 substances most of which are phenolic antioxidants (Vekiari *et al.*, 1993). Major components of the total oil are carvacrol and thymol with known *in vitro* antioxidant activity, whereas the activity of other main constituents such as *Y*-terpinene and p-cymene is uncertain, as it is the effect of each of the other minor constituents, not mentioning the effect of all of these substances working together (Adam *et al.*, 1998). Analytical method capable for identification and quantification of any of the oregano oil constituents at trace levels in tissues has not been yet developed. Therefore, the bioavailability of any of these compounds cannot be yet directly demonstrated.

Considering these analytical limitations and the fact that the greater the amount of α-tocopherol in tissues, the

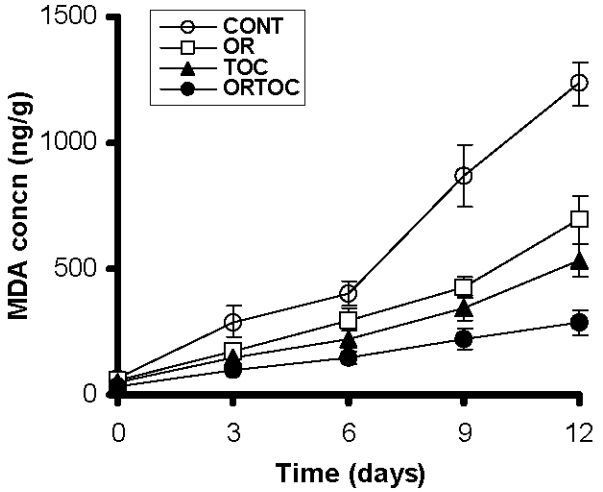

Fig. 1: Effect of refrigerated storage on lipid oxidation of breast fillets as a function of dietary supplementation with 100 mg oregano oil/kg (OR), or 100 mg oregano oil plus 100 mg α-tocopheryl acetate/kg (ORTOC), or 100 mg α-tocopheryl acetate/kg (TOC). Data points represent mean malondialdehyde concentrations from six samples analyzed and standard deviations, some of which, however, lie within the data points.

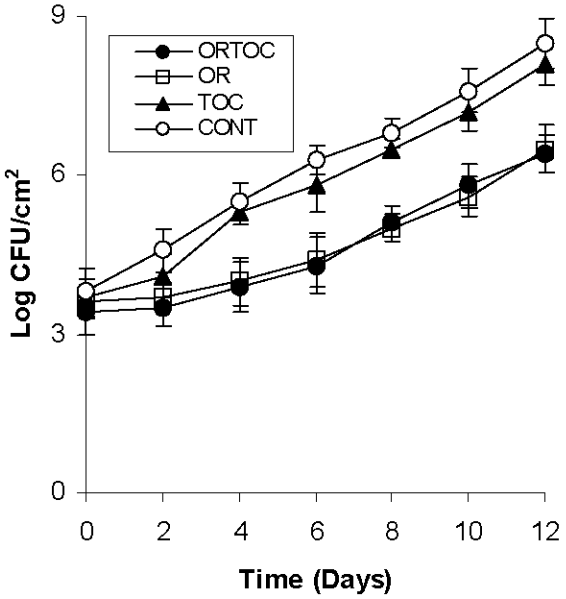

Fig. 2: Changes in total viable counts during refrigerated storage at 4°C of breast fillets from turkeys dietary supplemented with 100 mg oregano oil/kg (OR), or 100 mg oregano oil plus 100 mg α-tocopheryl acetate/kg (ORTOC), or 100 mg α-tocopheryl acetate/kg (TOC).

better protection tissues would have against oxidative attack, samples from all groups were analyzed for their α-tocopherol content. Table 2 shows that the concentration of α-tocopherol in breast fillet increased as the incorporation level of α-tocopheryl acetate in feed increased from 30 mg/kg to 100 mg/kg. The CONT and OR groups presented α-tocopherol values that did not differ significantly among each other and were significantly lower than those of the ORTOC and TOC groups that also did not differ significantly among each other. The concentrations of α-tocopherol appear low, however, α-tocopherol is not efficiently absorbed and deposited in the cell membranes of turkeys (Wen *et al.*, 1996). These values compare well with literature values (Higgins *et al.*, 1998; Botsoglou *et al.*, 2003a; Papageorgiou *et al.*, 2003).

Wada and Fang (1992) reported that a mixture of α-tocopherol with rosemary extract exerted stronger antioxidant effect than either α-tocopherol or rosemary extract alone, in a sardine model system. These workers postulated that the synergistic action of rosemary might be due to regeneration of α-tocopherol through donation of hydrogen to the tocopheroxyl radicals. An analogous hypothesis on the function of oregano essential oil might possibly be considered strong enough to explain the antioxidant activity shown by the OR group. However, this hypothesis cannot explain the synergistic action exhibited by the ORTOC group, which although did not result in significantly ($P<0.05$) higher concentrations of α-tocopherol in fillets than the TOC group, presented significantly ($P<0.05$) higher antioxidant activity. Therefore, oregano essential oil should contribute to the antioxidant activity by additional mechanisms.

A common concern associated with extending the shelf life of muscle foods is associated with the extent and type of microbial growth that may occur during the additional time of product acceptability. Apart from total viable counts (TVC), *Pseudomonas spp.* counts were also determined in the examined samples, as these species are predominant microbial species among psychrotrophic bacteria in natural meat microflora constituting major cause of poultry meat spoilage during refrigerated storage (Salvat *et al.*, 1997; Sofos *et al.*, 2000).

Throughout the refrigerated storage, TVC and *Pseudomonas spp.* counts in breast fillets of all groups were significantly increased ($P<0.05$) as shown in Figures 2 and 3, respectively. TVC in the OR and ORTOC groups increased from an initial number of ca 3.6 log CFU/cm^2 at day 0 to ca 6.4 log CFU/cm^2 at day 12. The TOC and CONT groups presented significantly higher TVC counts that were ca 8.5 log CFU/cm^2 at day 12. On the other hand, *Pseudomonas spp.* counts in the OR and

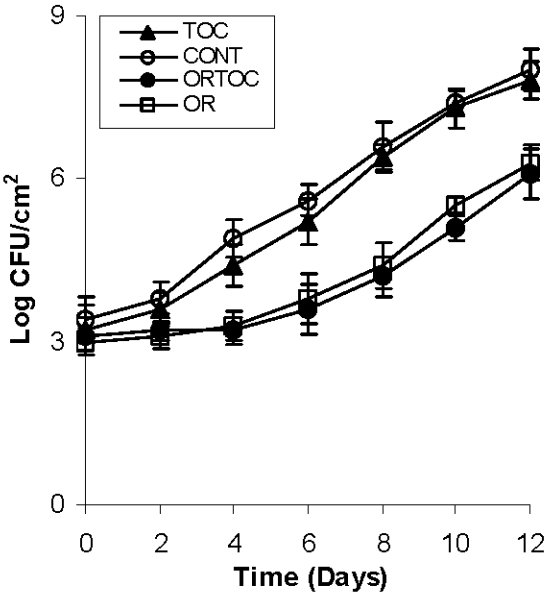

Fig. 3: Changes in total *Pseudomonas spp.* counts during refrigerated storage at 4°C of breast fillets from turkeys dietary supplemented with 100 mg oregano oil/kg (OR), or 100 mg oregano oil plus 100 mg α-tocopheryl acetate/kg (ORTOC), or 100 mg α-tocopheryl acetate/kg (TOC).

ORTOC groups increased from an initial number of ca 3.2 log CFU/cm^2 at day 0 to ca 6.1 log CFU/cm^2 at day 12. The TOC and CONT groups presented significantly higher *Pseudomonas spp.* counts that were ca 8.0 log CFU/cm^2 at day 12.

Initial signs of spoilage with the development of off-flavors or indications of slime formation were observed after the 10th day of storage for the CONT and TOC groups when TVC reached 7.0 log CFU/cm^2. These findings are in agreement with previous reports suggesting that when total bacteria counts reach 7 log CFU/cm^2 meat spoilage is observed (Saucier *et al.*, 2000; Sofos *et al.*, 2000). Mano *et al.* (2000) reported similar findings on microbial growth and onset of spoilage in refrigerated stored turkey meat.

Statistical analysis of drawn results showed that the α-tocopheryl acetate treatment presented TVC and *Pseudomonas spp.* counts that did not differ (P>0.05) from the control. In contrast, the treatments with only oregano essential oil and withe oregano essential oil plus α-tocopheryl acetate presented TVC and *Pseudomonas spp.* counts that were significantly lower compared to control, after the 2nd day of refrigerated storage (Fig. 2 and 3). Between these two treatments, there was no significant (P>0.05) difference in the TVC and *Pseudomonas spp.* counts compared to control, a finding indicating that there was no synergistic effect between oregano essential oil and α-tocopheryl acetate. These results suggested that incorporation of oregano essential oil in the diet at the level of 100 mg/kg exerted an inhibitory effect on microbial growth of the breast fillets during refrigerated storage.

The *in-vitro* antimicrobial activity of oregano essential oil and its major biologically active components carvacrol and thymol against pathogenic and food spoilage bacteria has been repeatedly reported (Aureli *et al.*, 1992; Helander *et al.*, 1998; Aligiannis *et al.*, 2001; Marino *et al.*, 2001; Skandamis and Nychas, 2001). Oregano essential oil has also shown considerable inhibitory activity against *Pseudomonas spp.* (Lambert *et al.*, 2001). In addition, direct use of oregano essential oil to raw meat has been found to result in significant delay of microbial growth during refrigerated storage (Skandamis and Nychas, 2001).

Fig. 2 and 3 suggested that incorporation of α-tocopheryl acetate in the diet at the level of 100 mg/kg exerted no inhibitory effect on microbial growth of breast samples during refrigerated storage. This is consistent with previous research on raw pork meat (Cannon *et al.*, 1995) and raw or ground beef (Chan *et al.*, 1995; Cabedo *et al.*, 1998), reporting no influence of vitamin E on microbial growth. Recent studies (Zerby *et al.*, 1998) confirmed that increases in psychotrophic bacterial counts during retail display of beef were not affected by vitamin E supplementation. According to these studies, increased vitamin E levels in meat were not able to mask color deterioration caused by high levels of initial contamination, which could be present due to unsanitary practices at slaughter, dressing, fabrication, and retailing. However, Asghar *et al.* (1991) reported that bacterial growth was greater in chops from pigs fed diets with supplemental vitamin E, which had lower drip losses. This difference in bacterial counts was ascribed to potentially higher water activity or reduced antimicrobial activity due to lack of lipid oxidation products because of the action of vitamin E.

The potentially higher water activity was attributed to the presence of α-tocopherol, which as a lipid-soluble compound, is found in the phospholipid fraction of cell membranes, where it acts as a scavenger of radicals, resulting in their neutralization, stabilizing polyunsaturated fatty acids, and acting as an antioxidant to delay oxidative processes (Faustman *et al.*, 1998). Oxidation of membrane phospholipids leads to a decrease in membrane fluidity and disruption of normal membrane structure and function, while inhibition of oxidation by antioxidants such as lipid-soluble vitamin E would maintain biological fluidity and protect its function (Wen *et al.*, 1997). Therefore, α-tocopherol favours cell membrane integrity that could decrease drip loss of meat, which would enhance growth of bacteria. Release of fluids from muscle might have an effect on microbial growth. It should not always be expected that increased drip losses might result in reduced microbial growth unless the water activity has decreased significantly.

Furthermore, microbial growth may be more pronounced in the exuded fluid where water-soluble nutrients may be concentrated and available for microbial metabolism. These higher microbial loads could be transferred from the drippings onto the meat (Sofos *et al.*, 2000).

As far as the reduced antimicrobial activity due to lack of lipid oxidation products is concerned, it deserves mentioning that lipid hydrolysis results in release of free fatty acids, and their oxidation yields peroxides, aldehydes, ketones, alcohols, and organic acids, which may exhibit antimicrobial activity (Sofos *et al.*, 2000). Considering this, a question that may need to be asked is whether prevention of free radical formation and lipid oxidation by vitamin E could result in reduced antimicrobial activity that might be due to free fatty acids and products of their oxidation. In searching for an answer to this question, it should be noted that antimicrobial activity of free fatty acids and their oxidation products in a complex food system such as meat may be negligible, and that any such contribution may take place following product oxidation and associated spoilage changes that are offensive to consumers; at this stage, the product may also contain high levels of microbial contamination.

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