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(Article begins on next page)



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Effect of dietary supplementation of vitamin E in pigs to prevent the formation of carcinogenic substances in meat products

Giorgia Meineri ^a, Claudio Medana ^b, Valeria Giancotti ^b, Sonja Visentin ^b, Pier Giorgio Peiretti ^{c*}

^a Department of Animal Production, Epidemiology and Ecology, University of Torino, Italy

^b Department of Molecular Biotechnology and Health Sciences, University of Torino, Italy

^c Institute of Science of Food Production, National Research Council, Grugliasco, Torino, Italy

*Corresponding author. Tel.: +39.11.6709230; fax +39.11.6709297; e-mail address:

piergioorgio.peiretti@ispa.cnr.it (P.G. Peiretti).

Abstract

The aims of this study were threefold: to evaluate the effect of vitamin E supplementation in the diet of pigs (SG group; 0.5 g Vitamin E/kg diet) in order to prevent the formation of carcinogens in salamis after seasoning; to compare these results with those obtained from a control group (CG group) and from a trial in which vitamin E (0.3 g/kg meat) is added directly during the preparation of the meat products (MG group); and to evaluate the nitrosamine content in meat products before and after enzymatic *in vitro* digestion to highlight the action of vitamin E under acidic conditions similar to gastric ambient. It was found that the oxidation products are formed in salamis after seasoning (0.50, 0.27, and 0.15 nM malondialdehyde/g in CG, MG, and SG group, respectively), while the formation of N-nitrosodimethylamine requires enzymatic digestion in an acidic environment. In addition, vitamin E shows greater efficacy when administered to animals through a diet rather than adding it directly to the meat. This work has also demonstrated the absence of pentosidine in meat products, despite the presence of simple sugars and amino protein groups.

Keywords: Pork; Salami; Nitrosamines; Pentosidine; Digestion; Food safety; Food processing; Food analysis; Food composition

1 Introduction

The human diet has changed considerably in recent decades and in particular there has been an increased consumption of food with a high percentage of fats, additives and refined sugars. It is generally accepted that the incidence of human cancer in different organs varies according to the geographical area, and that environmental factors, including the human diet, may be an important cause of cancer (Hussain et al., 2003; Danaei et al., 2005). The induction of a cellular mutation that leads to cancer is the result of an irreversible interaction between the genetic material and some molecules that are harmful to the DNA. The terminal products of lipid oxidation (malondialdehyde), advanced glycation end products (AGEs) and nitrosamines are among the substances that show a high carcinogenic potential (VanderVeen et al., 2003; Cho et al., 2007; Kang et al., 2010; Bryan et al., 2012; Pun and Murphy, 2012).

The process of lipid peroxidation is caused by a direct reaction between the body fats and the free radicals that are derived from molecular oxygen (Hsieh and Kinsella, 1989). One of the most important causes of the deterioration of meat is lipid oxidation, and the thiobarbituric acid reactive substances (TBARS) test seems to be an appropriate method for the routine assessment of secondary oxidation in meat samples (Fernández et al., 1997; Papastergiadis et al., 2012). The rate and extent of lipid oxidation in muscle tissue is governed by a number of factors, the most important being the level of polyunsaturated fatty acids that are found in the intramuscular fat tissue, in the cell membranes and in the intracellular lipid fraction (Buckley et al., 1995). Changes in pork and poultry meat quality are revealed through adverse changes in flavour, colour and nutritive value, as well as through the production of toxic and carcinogenic compounds (Jensen et al., 1998). Terminal oxidation products (malondialdehyde, 4-hydroxy-trans-2-nonenal and other advanced lipoxidation end products) are formed as a result of the lipid oxidation process in animal tissues; these compounds have cytotoxic, mutagenic and carcinogenic effects (Sayre et al., 2006). Lipid peroxidation is an *in vivo* process caused by “free radicals” containing molecular oxygen with a deficiency of electrons (“peroxyl”); oxidized lipids propagate the damage through a chain

reaction, since the lipids tend to replenish the loss of electrons and take electrons from neighbouring molecules and involve nuclear proteins and the DNA, causing cancer (Czapski et al., 2012).

Many other substances, such as nitrosamines and AGEs, are of great interest in human nutrition because they are potentially carcinogenic substances. Nitrosamines are molecules that tend to accumulate in tissues, and they have a strong mutagenic activity against cellular DNA. Extensive experiments and some epidemiological data suggest that humans are susceptible to carcinogenesis through nitrosamines, and that the presence of these compounds in some processed meat food may be regarded as an aetiological risk factor for certain human cancers including cancer of the oesophagus, stomach and nasopharynx (Tricker and Preussmann, 1991). Nitrosamines are formed in the body from meat products that have been supplemented with nitrates and nitrites as preservatives. Nitrite is added to meat because of its antibacterial effect (particularly against *Clostridium perfringens*); it helps to maintain the red colour of meat. Nitrite can also be derived from nitrate, which is converted to nitrite in the body; this transformation is accomplished by reducing bacteria in the oral cavity. The conversion of nitrate into nitrite may take place not only in the body but also during the preparation of food due to bacteria or other reducing agents (Biaudet et al., 1996).

The currently available data suggest that Asian foods are more frequently found to contain volatile nitrosamine and at higher concentrations than those found in food elsewhere. Food preservation methods have been implicated as the cause of the high incidence of oesophageal and nasopharyngeal cancer in certain provinces in China (Mergens, 1982). Food such as meat is often stored in ambient temperature conditions for two to three days prior to processing in non-industrialized developing countries; under such conditions nitrite levels increase rapidly due to the bacterial reduction of nitrate, and this is often accompanied by noticeable flavour changes. The drop in gastric cancer incidence in the United States over the past few decades has in fact been attributed to the widespread use of refrigeration (Fuchs and Mayer, 1995). In a lifetime bioassay of sodium nitrite administered in drinking water at an average daily doses of approximately 130 mg/kg in male

rats, 150 mg/kg in female rats, 220 mg/kg in male mice, and 165 mg/kg to female mice, the only adverse finding was the occurrence of combined benign and malignant forestomach tumours, and only in female mice (National Toxicology Program, 2001).

Recent findings have highlighted the important role played by other substances, such as AGEs, in the pathogenesis of some neoplastic forms, e.g. colorectal cancer (Johnson and Lund, 2007). Certain types of food, such as meat products, seasoned salamis, hams, wurstel and mortadella, are considered risky to human health because they are proteic products with a high fat content (which may undergo lipid peroxidation), and which contain additives such as nitrate and nitrite (nitrosating agents). Furthermore, simple sugar is added to some of these meat products, which could combine with amino groups of proteins and result in the formation of AGEs.

The importance of some antioxidants (tocopherols, carotenoids, ascorbic acid, etc.) in the prevention of cancer caused by dietary carcinogens is based on epidemiological evidence (Olveria, 1997). Scientific data suggest that the reduction in stomach and colorectal cancer is due to the increase in consumption of fruit and vegetables, which are rich in antioxidant substances (La Vecchia et al., 2001; Agnoli et al., 2012). In a recent review, it has been pointed out that peroxidation and nitrosation may be reduced by adding antioxidant or antinitrosant additives to meat (Corpet, 2011). Vitamin E is a liposoluble antioxidant that plays an important role in the diet preventing undesirable and potentially dangerous reactions, such as lipid oxidation and the formation of nitrosamine and AGEs (Jensen et al., 1998). The mechanism by which the antioxidant vitamin E may prevent formation of nitrosamines could involve a radical scavenging reaction as reported (Anouar et al., 2002).

In the literature there are no data that indicate whether the administration of vitamin E in the diet is more useful than its addition to the meat product. The aim of the present study was to evaluate the effect of vitamin E supplementation in the diet of pigs in order to prevent the formation of carcinogens in meat products (seasoned salamis) and to compare these results with those obtained

from a trial in which vitamin E was not administered orally to animals, but added directly to the preparation of meat products. A second aim was to evaluate the nitrosamine contents in meat products before and after enzymatic *in vitro* digestion to evaluate the generation of nitrosamines and to highlight the action of vitamin E under similar acidic conditions to the human stomach (Boisen and Fernández, 1997). This trial was also conducted to clarify whether the reducing activity of vitamin E could be of assistance in the conversion of nitrate to nitrite and consequently in facilitating nitrosamine production.

2 Materials and methods

2.1 Chemicals

N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR), N-nitroso-nornicotine, sodium nitrite, sodium nitrate, α -tocopherol (vitamin E), epitafluorobutanoic acid and trichloroacetic acid that were used were obtained from Sigma-Aldrich (Milan, Italy). HPLC grade water was acquired from MilliQ System Academic (Millipore, Milan, Italy). HPLC grade methanol and acetonitrile were from Carlo Erba (Milan, Italy) and zinc sulphate, sodium bicarbonate and potassium carbonate (99%) were from Merck (Milan, Italy). Pentosidine was synthesized in our laboratory, as reported in the literature (Visentin et al., 2010).

2.2 Animal diet and sampling

Fourteen pigs were selected at approximately 104 kg live weight and randomly assigned to the three following groups:

- CG group (control group); 6 animals fed standard diet, from their meat were produced salami without the addition of vitamin E;
- SG group (supplemented group), 4 animals fed standard diet supplemented with vitamin E (0.5 g/kg diet); from their meat were produced salamis without the addition of vitamin E;

- MG group (meat group), 4 animals fed standard diet; from their meat were produced salamis with the addition of vitamin E (0.3 g/kg meat).

The pigs were housed at the Interdepartmental Centre of Animal Recovery at the Faculty of Veterinary Medicine, the University of Turin, Grugliasco, Italy, and fed *ad libitum* throughout the course of the experiment. After 8 weeks, the animals (mean live weight 166 kg) were slaughtered at Salumificio Nadia (Arè di Caluso, Italy). The shoulder, ham trimmings and belly were removed from the carcasses 24 h *post mortem* after chilling at 5°C and then made into salami. “Filzetta” type salamis (~75% meat and 25% fat), weighing about 600 g, were prepared by adding salt (25 g/kg batter), spices (1.6 g/kg batter), sugar (4.2 g/kg batter), wine (7.5 g/kg batter), vitamin C (60 mg/kg batter) and potassium nitrate (150 mg/kg batter) to the minced meat and fat. At the end of the seasoning period of 28 days, eight salamis from each group of pigs, considered representative of all the production, were sampled, freeze-dried and stored frozen at –20°C until analysis.

2.3 Determination of lipid oxidation

The TBARS assay was modified from that of Witte et al. (1970) and was performed for each meat sample in triplicate; 10 g of freeze-dried meat were homogenized for 30 s at high speed with 20 mL of 10% trichloroacetic acid (TCA) using a Polytron tissue homogenizer (Type PT 10-35; Kinematica GmbH, Luzern, Switzerland). After centrifugation of the homogenate (600 rpm for 5 min at 4°C), the supernatant was filtered through Whatman #1 filter paper. One mL of filtrate was combined with 1 mL of a 0.02 M aqueous 2-thiobarbituric acid solution (TBA), heated in a boiling water bath for 20 min together with a blank containing 1 mL of a TCA/water mix (1/1) and 1 mL of TBA reagent and subsequently cooled under running tap water. The samples were analysed in triplicate and the results were expressed as mM malondialdehyde/kg DM, using a standard curve that covered the concentration range of 1 to 10 mM 1,1,3,3-tetramethoxypropane (Sigma-Aldrich, Steinheim, Germany). The absorbance was measured at 532 nm using a Helios spectrophotometer (Unicam Limited, Cambridge, UK) against a blank that contained all the reagents but no meat.

2.4 Sample preparation for HPLC-MS/MS analysis

Two extraction procedures were carried out on each lyophilized salami sample. In order to measure N-nitrosamine concentration, 500 mg of dried meat were extracted using 10 mL of ZnSO₄-saturated water, centrifuged (11,200g) and spiked with N-nitroso-nornicotine as the internal standard (final concentration 200 µg/L). Protein hydrolysis was carried out for pentosidine quantification by treating 50 mg of dried meat with 0.25 mL of 6 M HCl for 18 h at 110°C and diluting the obtained sample with 0.25 mL of 5 mM eptafluorobutanoic acid before injection into the HPLC-MS.

2.5 Determination of N-nitrosamines

The chromatographic separations of N-nitrosamines were run on a Varian 920-LC HPLC coupled with a triple quadrupole mass spectrometer 320-MS (Varian, Leinì, Italy) through an atmospheric pressure interface and an ESI ion source. We used a procedure described in literature (Krauss and Hollender, 2008) with slight modifications: we obtained better results with the triple quadrupole mass analyzer and so we decided to run the analyses on this kind of instrumentation. Samples were analyzed using a RP C18 column (Phenomenex Luna 150 mm × 2.1 mm, 3 µm particle size) at a 200 µL/min flow rate. A gradient mobile phase composition was adopted: 85/15 to 0/100 in 18 min 0.1 mM ammonium acetate/methanol. The analyses were run using MS/MS acquisition. The tuning parameters adopted for the ESI source were: source voltage 4.5 kV, capillary voltage 82 V, shield voltage 450 V, drying gas temperature 300°C. The followed transitions were 75 to 43 *m/z* (collision energy 13 V) for NDMA, 101 to 55 *m/z* (collision energy 10 V) for NPYR and 178 to 148 *m/z* (collision energy 8 V) for N-nitroso-nornicotine. The retention times of the analytes were 3.5 min (NDMA), 6.3 min (NPYR) and 16.3 min (N-nitroso-nornicotine). Injection volume was 20 µL.

In order to validate the methodology, we determined linearity, limit of quantification (LOQ), precision and accuracy using the extraction procedure described for the salami samples. The method show an acceptable linearity in the range 0.02 (LOQ)–50 mg/kg.

2.6 Determination of pentosidine

In order to detect pentosidine, the hydrolyzed samples were analysed the method described by a previous work of our research group (Peiretti et al., 2012) using another RP C18 column (Phenomenex Synergi 150 mm × 2.1 mm, 3 µm particle size) on a Dionex Ultimate 3000 HPLC coupled with a LTQ-Orbitrap mass spectrometer at 200 µL/min flow rate. A gradient mobile phase composition was adopted: 95/5–40/60 in 25 min 5 mM eptafluorobutanoic acid/acetonitrile. The retention time of the analyte was 7.1 min. Phenylephrine was used as internal standard (retention time 4.1 min). Injection volume was 20 µL.

2.7 In vitro digestion

The *in vitro* technique was slightly modified from that of Boisen and Fernández (1997) and was based on the two-step multi-enzymatic incubation assay without the use of bile salts. The *in vitro* incubation conditions simulated the protein digestion processes in the stomach and small intestine with the action of pepsin (porcine, 2000 FIP-U/g, Merck, Milan, Italy, No. 7190), followed by pancreatin (porcine, grade IV, Sigma, Milan, Italy, No. P-1 750). The method allowed one to obtain the protein digestion of meat product, after a filtration process (0.45 µm), and to highlight the action of vitamin E under similar acidic conditions to the human stomach.

2.8 Determination of nitrite and nitrate ions

A Metrohm 861 IC (Metrohm, Origgio, Italy) ion chromatograph, equipped with a conductimetric detector, was employed. The anions (nitrite and nitrate) were analyzed using a Metrosep A 150 mm × 4.0 mm anionic column (Metrohm, Origgio, Italy) with an isocratic eluent composed of 1.0 mM NaHCO₃ and 3.2 mM K₂CO₃ in HPLC-grade water (pH 10.8) at flow rate of 1.0 mL/min. The injection volume was 20 µL.

2.9 Statistical analysis

The statistical analyses were performed using the SPSS software package (version 11.5.1 for Windows, SPSS Inc., Chicago, IL, USA). The data were reported as the mean ± standard deviation

and subjected to analysis of variance. Differences among the means were determined by means of the Duncan test with significance defined at $P < 0.05$.

3 Results and discussion

The composition of the experimental diets and vitamin-mineral premix are shown in Table 1. Vitamin and mineral premix was added in the diets fed to all groups at the same percentage, in order to cover the recommended levels for pig requirements and not influence the results of the trials. Only the SG group was supplemented with a level of vitamin E in excess of recommended levels in the diet.

The results obtained in our work are summarized in Tables 2 and 3. They show that vitamin E administered orally to animals reduces the TBARS values in meat products more significantly than adding vitamin E directly during the preparation of the products. The MG and SG group salamis have significantly lower TBARS values than the CG group salamis. Vitamin E or α -tocopherol is defined as a radical-chain breaker, which operates in a lipid environment due to its hydrophobic nature. The effects of α -tocopherol as an antioxidant are thus restricted to its direct effects in membranes and lipoprotein domains (Fuchs-Tarlovsky, 2013).

The biochemical changes that accompany the post-slaughter metabolism and post mortem aging in the conversion of muscle to meat give rise to conditions whereby the process of lipid oxidation is no longer tightly controlled and as a consequence there may be some peroxidative damage. In the post-slaughter phase, it is highly unlikely that the set of antioxidant defensive systems (superoxide dismutase, glutathione peroxidase, ceruloplasmin, transferrin) available to the cell in the live animal are still functioning, due to the quantitative changes that occur in several metabolites and physical properties.

The rate and extent of oxidation of foods from muscles are also influenced by pre-slaughter events, such as stress, and post-slaughter events such as early post-mortem, pH, carcass temperature,

shortage of cold, and techniques such as electrical stimulation (Buckley et al., 1995). In addition, any disruption of the integrity of muscle membranes by mechanical boning, mincing, restructuring or cooking alters the cellular compartmentalization. This facilitates the interaction of pro-oxidants with unsaturated fatty acid and leads to the production of free radicals and the propagation of the oxidative reaction (Asghar et al., 1988).

Many researchers believe that the presence of transition metals, notably iron, is pivotal in facilitating the generation of chemical species that are capable of extracting a proton from an unsaturated fatty acid (Gutteridge et al., 1979). As a result of this chemical process, some oxidation end products that have cytotoxic, mutagenic and carcinogenic effects (malondialdehyde and 4-hydroxy aldenale) are formed (Buckley et al., 1995).

Tocopherols react with free radicals, notably peroxide radicals, and with singlet molecular oxygen ($\dot{\text{O}}_2$), which is the base of its function as an antioxidant (Azzi, 2007). In membranes such high reactivity is important because tocopherols react with lipid peroxy radicals to yield a relatively stable lipid hydroperoxide, and the tocopheroxyl radical interrupts the radical chain reaction, thereby affording protection against lipid peroxidation (Burton et al., 1982, 1983). Vitamin E cannot be synthesized in animals, and therefore its presence in animal tissue reflects dietary availability. Due to the fat soluble characteristics of vitamin E, its absorption is dependent on the ability of the animals to digest and absorb fat (Wiss et al., 1962). Studies on pigs (Asghar et al., 1991; Monahan et al., 1993), chickens (Asghar et al., 1990; Lauridsen et al., 1997) and steers (Arnold et al., 1993) showed that dietary vitamin E supplementation significantly increased the α -tocopherol content of muscle membranes.

Different tissues responded to dietary intake according to their metabolic activities. Several reports showed that α -tocopherol accumulation is muscle-dependent (Arnold et al., 1993; Jensen et al., 1988). Jensen et al. (1998) showed that the striate muscle, which has the highest oxidative capacity, also possess the greatest storage capacity for α -tocopherol.

In our work, the supplementation of vitamin E in the animal diet had a better effect than the incorporation of vitamin E during the preparation of the meat products. As shown in Table 2, the TBARS of the CG samples are four times those of the SG samples obtained from animals fed the vitamin E enriched diet. This is in agreement with other scientific studies which stated that the post-mortem addition of α -tocopherol to meat was not as effective as dietary supplementation, since the vitamin was not incorporated directly into the membrane in which lipid oxidation was initiated (Asghar et al., 1990; Schaefer et al., 1995).

In fact, α -tocopherol is associated with the biomembrane; the protection of these membranes can therefore be achieved through the neutralization of the oxidation induced by free radicals (Morrissey et al., 1994). α -tocopherol acts as an antioxidant by donating a hydrogen atom to a free radical, and thus a stable α -tocopherol radical is formed (Yin et al., 1993).

Vitamin E is usually incorporated in the diet as α -tocopheryl acetate, and it does not act as an antioxidant until the ester is hydrolysed in the gastrointestinal tract (Buckley et al., 1995). The nutritional recommendation for vitamin E supplementation for an optimum animal performance under normal management was 15–40 mg α -tocopheryl acetate/kg feed in fattening pigs, and 15–20 mg α -tocopheryl acetate/kg feed in broilers (Albers et al., 1984). However, numerous studies showed that the use of supra-nutritional levels of α -tocopherol was an efficient way of improving the quality and storage stability of pork and poultry. The effects of feeding pigs with α -tocopheryl acetate for either basic (30 mg/kg of feed) or extra food rations (200 mg/kg of feed) from weaning until slaughter were evaluated by Monahan et al. (1990). The plasma and tissue levels of α -tocopherol were 2.5 – 3.0 times higher in the pigs fed the supplemented diet than in those on the basic diet. A similar trend was reported by Asghar et al. (1991) for pigs receiving 10, 100 or 200 I.U. vitamin E/kg of feed.

With regard to the presence of nitrosamines in the examined meat products, the results of our study indicate that NDMA is not formed from the nitrates in the salamis after seasoning (Table 2). Traces

of NPYR are detected in the MG and SG groups, but at low values. The great standard deviation value is due to the fact that many of the samples within each of the 8 salami groups did not show any nitrosamine. Therefore, the reported standard deviation values are relative to the entire sample treatment/analysis process. We confirm that the R.S.D. of the HPLC-MS/MS determination was < 5%.

Moreover, in order to verify whether vitamin E could contribute to the conversion of nitrate to a nitrite ion, we put nitrate solutions in similar acidic conditions to those found in the stomach (HCl, pH 1.5, 37°C for 1 h and 24 h) in the presence of vitamin E, and determined the nitrate and nitrite ion concentration by means of ion chromatography. The concentration of nitrate was deduced from a potential intake of 50 g salami in 1 kg meal, on the basis of sodium nitrate and vitamin E meat adding up of 20 g/100 kg (10 mg/kg of sodium nitrate). Vitamin E was added in an equimolar concentration with nitrate and the obtained suspension is kept under stirring. No significant formation of nitrite ions was detected after 1 h or after 24 h.

In another experiment, we performed the nitrosamine content determination in the studied samples simulating gastric digestion. Table 3 shows that after enzymatic *in vitro* digestion in an acid environment, NDMA is formed in the CG group, even though with a large standard deviation, and that vitamin E could contribute to prevent the formation of NDMA in the other two experimental groups (salamis from pigs with nutritional supplementation of vitamin E and salamis in which vitamin E is added during their preparation). The formation of NPYR seems to be unaffected by vitamin E treatments, and the great dispersion of data illustrates that the reaction is promoted by some uncontrolled factor(s), maybe one or many. The formation of nitrosamines obtained in our work with the *in vitro* enzymatic digestion reflects what has been shown in the studies of *in vivo* digestion published by Ohshima and Bartsch (1981). These authors showed that the generation of a nitrosating agent is pH dependent and occurs in different steps: the first step involves the addition of a hydrogen ion to the nitrite ion to form unstable nitrous acid (HNO₂); this reaction is promoted in an acidic condition similar to that which occurs within the gastric cavity. The further addition of H⁺

and the subsequent loss of water molecules leads to the formation of a nitrosonium ion (NO^+), which is capable of reacting with the nitrogen of amines or the amides of food proteins. It was found that carcinogenic nitrosamines were obtained from the reaction of nitrite with secondary amines, which can also be present within a protein structure of food (Biaudet et al., 1996). This study shows that proline is subsequently nitrosated to nitrosoproline under the acidic conditions of the stomach and the simultaneous ingestion of 500 mg α -tocopherol inhibited *in vivo* nitrosation in humans by 50%. Several authors offered an explanation for the principle by which vitamin E was employed as a nitrosamine blocking agent (Kamm et al., 1977). In this case, vitamin E also acted as an antioxidant agent that could reduce the nitrosating agent NO^+ to a non nitrosating compound (Mergens, 1982). This inhibition occurred because vitamin E could compete effectively with susceptible amines or amides for the nitrosating agent. The above-mentioned vitamin E action in the prevention of nitrosamine formation was similar to that of vitamin C, but while vitamin E is fat soluble, vitamin C is water soluble (Mergens, 1982). In addition, fat soluble vitamin E added to the diet could be effectively absorbed and deposited in the tissues of animals.

As far as the glycation process is concerned, it is known that it begins in the body tissues with a non-enzymatic reaction between simple sugars (glucose, fructose, ribose) and protein amino-groups. When glycation is accompanied by oxidation, harmful AGEs are formed; one of these molecules is named pentosidine. AGEs were identified in the body tissues as well as in food of prosperous industrialized countries (Johnson and Lund, 2007).

As far as the assessment of AGEs is concerned, our work indicates the absence of pentosidine in the examined meat products, despite the presence of simple sugars and amino groups of proteins. This may be due to the fact that the reactions leading to the formation of AGEs, particularly pentosidine, are catalyzed at high temperatures, while the meat products in our study were not heat-treated. It has therefore not been possible to demonstrate the effect of vitamin E on reducing advanced glycation end products, an effect that was shown and explained in the work by Baragetti et al. (2006). In this

case, the protective role of vitamin E was due to the antioxidant action; it prevented glycosylation accompanied by oxidation and the formation of AGEs and pentosidine.

4 Conclusions

Our work shows that vitamin E supplementation of animal diets protects the meat products from the formation of carcinogenic substances (malondialdehyde, NDMA) even more than when the vitamin E is added directly to the minced meat. These results agree with Corpet's expectation (Corpet, 2011) concerning the amelioration of red meat with an improvement of the animal diet.

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References

- Agnoli, C., Gioni, S., Sieri, S., Palli, D., Masala, G., Sacerdote, C., Vineis, P., Tumino, R., Giurdanella, M.C., Pala, V., Berrino, F., Mattiello, A., Panico, S., Krogh, V., 2013. Italian mediterranean index and risk of colorectal cancer in the Italian section of the EPIC cohort. *International Journal of Cancer*, 132, 1404-1411.
- Albers, N., Behm, G., Klaus, W., Kùthner, K., Lidner, H., 1984. Vitamins in animal nutrition. In: *Arbeitsgemeinschaft für Wirkstoffe in der Tierernàhrung*, Bonn, Germany, p. 34.

- Anouar, E., Košinová, P., Kozłowski, D., Mokrini, R., Duroux, J.L., Trouillas, P., 2002. New aspects of the antioxidant properties of phenolic acids: a combined theoretical and experimental approach. *Physical Chemistry Chemical Physics* 11, 7659–7668.
- Arnold, R.N., Arp, S.C., Scheller, K.K., Williams, S.N., Schaefer, D.M., 1993. Tissue equilibration and subcellular distribution of vitamin E relative to myoglobin and lipid oxidation in displayed beef. *Journal of Animal Science* 71, 105–118.
- Asghar, A., Gray, J.I., Buckley, D.J., Pearson, A.M., Booren, A.M., 1988. Perspectives on warmed-over flavor. *Food Technology* 42, 102–108.
- Asghar, A., Lin, C.F., Gray, J.I., Buckley, D.J., Booren, A.M., Flegal, C.J., 1990. Effects of dietary oils and α -tocopherol supplementation on membranous lipid oxidation in broiler meat. *Journal of Food Science* 55, 46–50.
- Asghar, A., Gray, J.I., Booren, A.M., Gomaa, E.A., Abouzied, M.M., Miller, E.R., Buckley, D.J., 1991. Effects of supranutritional dietary vitamin E Levels on subcellular deposition of α -tocopherol in the muscle and on pork quality. *Journal of the Science of Food and Agriculture* 57, 31–41.
- Azzi, A., 2007. Molecular mechanism of α -tocopherol action. *Free Radical Biology and Medicine* 43, 16–21.
- Baragetti, I., Furiani, S., Vettoretti, S., Raselli, S., Maggi, F.M., Galli, F., Catapano, A.L., Bucciante, G., 2006. Role of vitamin E-coated membrane in reducing advanced glycation end products in hemodialysis patients: a pilot study. *Blood Purification* 24, 369–376.
- Biaudet, H., Pignatelli, B., Debry, G., 1996. N-nitroso compounds. In: Nolle L.M.L. (Ed.), *Handbook of Food Analysis*. NY, Marcel Dekker.

- Boisen, S., Fernández, J.A., 1997. Prediction of the total tract digestibility of energy in feedstuffs and pig diets by in vitro analyses. *Animal Feed Science Technology* 68, 277–286.
- Bryan, N.S., Alexander, D.D., Coughlin, J.R., Milkowski, A.L., Boffetta, P., 2012. Ingested nitrate and nitrite and stomach cancer risk: An updated review. *Food and Chemical Toxicology* 50, 3646–3665.
- Buckley, D.J., Morrissey, P.A., Gray, J.I., 1995. Influence of dietary vitamin E on the oxidative stability and quality of pig meat. *Journal of Animal Science* 73, 3122–3130.
- Burton, G.W., Joyce, A., Ingold, K.U., 1982. First proof that vitamin E is the major lipidsoluble, chain breaking antioxidant in human blood plasma. *Lancet* 2, 327–328.
- Burton, G.W., Joyce, A., Ingold, K.U., 1983. Is vitamin E the only lipid-soluble, chainbreaking antioxidant in human blood plasma and erythrocyte membranes? *Archives of Biochemistry and Biophysics* 221, 281–290.
- Czapski, G.A., Czubowicz, K., Strosznajder, R.P., 2012. Evaluation of the antioxidative properties of lipoxygenase inhibitors. *Pharmacological Reports* 64, 1179-1188.
- Cho, S.-J., Roman, G., Yeboah, F., Konishi, Y., 2007. The road to advanced glycation end products: A mechanistic perspective. *Current Medicinal Chemistry* 14, 1653-1671.
- Corpet, D.E., 2011. Red meat and colon cancer: should we become vegetarians, or can we make meat safer? *Meat Science* 89, 310–316.
- Danaei, G., Vander Hoorn, S., Lopez, A.D., Murray, C.J.L., Ezzati, M., 2005. Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. *The Lancet* 366, 1784–1793.

- Fernández, J., Pérez-Álvarez, J.A., Fernández-López, J.A., 1997. Thiobarbituric acid test for monitoring lipid oxidation in meat. *Food Chemistry* 59, 345–353.
- Fuchs, C.S., Mayer, R.J., 1995. Gastric Carcinoma. *New England Journal of Medicine* 333, 32–41.
- Fuchs-Tarlovsky, V., 2013. Role of antioxidants in cancer therapy. *Nutrition* 29, 15-21.
- Gutteridge, J.M., Richmond, R., Halliwell, B., 1979. Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. *Biochemical Journal* 184, 469–472.
- Hsieh, R.J., Kinsella, J.E., 1989. Oxidation of polyunsaturated fatty acids: mechanisms, products and inhibition with emphasis on fish. *Advances in Food and Nutrition Research*, 33, 233–341.
- Hussain, S.P., Hofseth, L.J., Harris, C.C., 2003. Radical causes of cancer. *Nature Reviews Cancer* 3, 276–285.
- Jensen, M., Essen-Gustavsson, B., Hakkarainen, J., 1988. The effect of a diet with high or low content of vitamin E on different skeletal muscles and myocardium in pigs. *Journal of Veterinary Medicine Series A* 35, 487–497.
- Jensen, C., Lauridsen, C., Bertelsen, G., 1998. Dietary vitamin E: quality and storage stability of pork and poultry. *Trends in Food Science and Technology* 9, 62–72.
- Johnson, I.T., Lund, E.K., 2007. Review article: nutrition, obesity and colorectal cancer. *Alimentary Pharmacology and Therapeutics* 26, 161–181.
- Kamm, J.J., Dashman, T., Newmark, H., Mergens, W.J., 1977. Inhibition of amine-nitrite hepatotoxicity by α -tocopherol. *Toxicology and Applied Pharmacology* 41, 575–583.
- Kang, R., Tang, D., Schapiro, N.E., Livesey, K.M., Farkas, A., Loughran, P., Bierhaus, A., Lotze, M.T., Ze, H.J., 2010. The receptor for advanced glycation end products (RAGE) sustains

autophagy and limits apoptosis, promoting pancreatic tumor cell survival. *Cell Death and Differentiation* 17, 666–676.

Krauss, M., Hollender, J., 2008. Analysis of nitrosamines in wastewater: exploring the trace level quantification capabilities of a hybrid linear ion trap/orbitrap mass spectrometer. *Analytical Chemistry* 80, 834–842.

Lauridsen, C., Buckley, D.J., Morrissey, P.A., 1997. Influence of dietary fat and vitamin E supplementation on alfa-tocopherol levels and fatty acid profiles in chicken muscle membranal fractions and on susceptibility to lipid peroxidation. *Meat Science* 46, 9–22.

La Vecchia, C., Altieri, A., Tavani, A., 2001. Vegetables, fruit, antioxidants and cancer: a review of Italian studies. *European Journal of Nutrition* 40, 261–267.

Mergens, W.J., 1982. Efficacy of vitamin E to prevent nitrosamine formation. *Annals of the New York Academy of Sciences* 393, 61–69.

Monahan, F.J., Buckley, D.J., Gray, J.I., Morrissey, P.A., Asghar, A., Hanrahan, T.J., Lynch, P.B., 1990. Effect of dietary α -tocopherol supplementation on α -tocopherol levels in porcine tissues and on susceptibility to lipid peroxidation. *Food Science and Nutrition* 42F, 203–212.

Monahan, F.J., Gray, J.I., Asghar, A., Haug, A., Shi, B., Buckley, D.J., Morrissey, P.A., 1993. Effect of dietary lipid and vitamin E supplementation on free radical production and lipid oxidation in porcine muscle microsomal fractions. *Food Chemistry* 46, 1–6.

Morrissey, P.A., Buckley, D.J., Sheehy, P.J.A., Monahan, F.J., 1994. Vitamin E and meat quality. *Proceedings of the Nutrition Society* 53, 289–295.

National Toxicology Program, 2001. Toxicology and Carcinogenesis Studies of Sodium Nitrite (CAS NO. 7632–00-0) in F344/N Rats and B6C3F1 Mice (Drinking Water Studies), National Toxicology Program Tech. Rep. Ser., United States, pp. 7–273.

- Ohshima, H., Bartsch, H., 1981. Quantitative estimation of endogenous nitrosation in humans by monitoring *N*-nitrosoproline excreted in the urine. *Cancer Research* 41, 3658–3662.
- Olveria, S.A., 1997. The role of epidemiology in cancer prevention. *Proceedings of the Society for Experimental Biology and Medicine*, 216, 142–150.
- Papastergiadis, A., Mubiru, E., Van Langenhove, H., De Meulenaer, B., 2012. Malondialdehyde measurement in oxidized foods: evaluation of the spectrophotometric thiobarbituric acid reactive substances (TBARS) test in various foods. *Journal of Agriculture and Food Chemistry* 60, 9589–9594.
- Peiretti, P.G., Medana, C., Visentin, S., Dal Bello, F., Meineri, G., 2012. Effect of cooking method on carnosine and homologues, pentosidine and thiobarbituric acid reactive substances contents in beef and turkey meats. *Food Chemistry* 132, 80–85.
- Pun, P.B., Murphy, M.P., 2012. Pathological significance of mitochondrial glycation. *International Journal of Cell Biology* 2012, 1–13.
- Sayre, L.M., Lin, D., Yuan, Q., Zhu, X., Tang, X., 2006. Protein adducts generated from products of lipid oxidation: focus on HNE and ONE. *Drug Metabolism Reviews* 38, 651–675.
- Schaefer, D.M., Liu, Q., Faustman, C., Yin, M.C., 1995. Supranutritional administration of vitamins E and C improves oxidative stability of beef. *Journal of Nutrition* 125, 1792–1798.
- Tricker, A.R., Preussmann, R., 1991. Carcinogenic *N*-nitrosamines in the diet: occurrence, formation, mechanisms and carcinogenic potential. *Mutation Research* 259, 277–289.
- VanderVeen, L.A., Hashim, M.F., Shyr, Y., Marnett, L.J., 2003. Induction of frameshift and base pair substitution mutations by the major DNA adduct of the endogenous carcinogen malondialdehyde. *Proceedings of the National Academy of Sciences of the United States of America* 100, 14247–14252.

- Visentin, S., Medana, C., Barge, A., Giancotti, V., Cravotto, G., 2010. Microwave-assisted Maillard reactions for the preparation of advanced glycation end products (AGEs). *Organic and Biomolecular Chemistry* 8, 2473–2477.
- Wiss, O., Bunnell, R.B., Gloor, U., 1962. Absorption and distribution of vitamin E in the tissue. *Vitamins and Hormones* 20, 441–455.
- Witte, V.C., Krause, G.F., Bailey, M.E., 1970. A new extraction method for determining 2-thiobarbituric acid values for pork and beef during storage. *Journal of Food Science* 35, 585–592.
- Yin, M.C., Faustman, C., Riesen, J.W., Williams, S.N., 1993. α -Tocopherol and ascorbate delay oxymyoglobin and phospholipid oxidation in vitro. *Journal of Food Science* 58, 1273–1276.

Table 1

Composition of the experimental diets fed to the pigs to produce meat without vit. E (CG), meat supplemented with 0.3 g vit. E/kg meat (MG) and the diet containing 0.5 g α -tocopheryl acetate/kg feed (SG).

| Raw materials | CG and MG groups | SG group |
|----------------------------|------------------|----------|
| Corn (%) | 54 | 54 |
| Barley (%) | 20 | 20 |
| Soybean (%) | 8.8 | 8.8 |
| Bran (%) | 14 | 14 |
| Lignosulphite (%) | 1 | 1 |
| Vitamin-mineral premix (%) | 2.2 | 2.2 |
| Vitamin E (mg/kg) | - | 500 |

Composition of the vitamin-mineral premix (per kg of diet): vit. A 312,000 IU; vit. D₃ 48,800 IU; α -tocopheryl acetate 68 mg; vit. B₁ 39 mg; vit. B₂ 125 mg; vit. B₆ 39 mg; vit. B₁₂ 0.75 mg; vit. PP 623 mg; biotin, 0.75 mg; choline chloride, 12,500 mg; folic acid, 40 mg; D-panthothenic acid 500 mg; sodium menadione bisulphate, 25 mg; lysine, 18,300 mg; Zn, 275 mg; Fe, 275 mg; Cu, 25 mg; Mn, 17 mg; J, 800 μ g; Se, 300 μ g.

Table 2

Pentosidine (mg/kg), N-nitrosodimethylamine (NDMA, mg/kg), N-nitrosopyrrolidine (NPYR, mg/kg), and TBARS (nM malondialdehyde/g) contents in salamis produced using: meat without vit. E (CG), meat supplemented with 0.3 g vit. E/kg meat (MG) and meat obtained from the pigs fed with a diet containing 0.5 g vit. E/kg feed (SG).

| Group | N of salamis | TBARS | NDMA | NPYR | Pentosidine |
|-------|--------------|----------------------------|------|---------------|-------------|
| CG | 8 | 0.497 ± 0.080 ^a | ND | ND | ND |
| MG | 8 | 0.266 ± 0.037 ^b | ND | 0.056 ± 0.059 | ND |
| SG | 8 | 0.153 ± 0.034 ^c | ND | 0.117 ± 0.041 | ND |

Result expressed as the mean ± standard deviation of three determinations for each salami.

^{a,b,c} Means in the same column with different superscripts differ significantly ($P < 0.05$) according to Duncan's multiple-range test.

Data without superscript were not significantly different.

ND, not detectable.

Table 3

N-nitrosodimethylamine (NDMA, mg/kg) and N-nitrosopyrrolidine (NPYR, mg/kg) after *in vitro* enzymatic digestion of salamis produced using: meat without vit. E (CG), meat supplemented with 0.3 g vit. E/kg meat (MG) and meat obtained from the pigs fed a diet containing 0.5 g vit. E/kg feed (SG).

| Group | N of salamis | NDMA | NPYR |
|-------|--------------|-------------|---------------|
| CG | 8 | 5.81 ± 12.5 | 0.023 ± 0.034 |
| MG | 8 | ND | 2.40 ± 6.26 |
| SG | 8 | ND | 0.101 ± 0.190 |

Result expressed as the mean ± standard deviation of three determinations for each salami.
ND, not detectable.