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1. **Predescu CN**, Papuc C, Stefan G, Taşbac B, Temocico G, Sărăcilă M, Untea AE. Combined Effects of Parsnip Fermented Juice and Hawthorn Extract Regarding Pork Mince Stability: Physico-Chemical and Microbiological Aspects. Agriculture. 2023; 13(2):432. ISI (FI – 3,6) https://doi.org/10.3390/agriculture13020432.

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Combined Effects of Parsnip Fermented Juice and Hawthorn Extract Regarding Pork Mince Stability: Physico-Chemical and Microbiological Aspects

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Abstract: Parsnip fermented juice (PFJ) and hawthorn extract (HE) were identified as natural nitrite and antioxidant sources for pork mince. This study aimed to determine the effects of varying levels of HE added to a constant concentration of PFJ on lipids stability, heme pigment conversion degree, residual nitrite content, and spoilage bacteria growth, during refrigeration, compared with the combined effect of synthetic nitrite and sodium ascorbate (SA). Pork mince was formulated in six different ways with sterile distilled water (NC), 100 ppm synthetic nitrite and 50 ppm SA (PC), PFJ in the concentration of 100 ppm NO_2^- (T1), constant level of PFJ (100 ppm NO_2^-), and increased level of HE, 50, 25 and 10 ppm GAE (T2, T3 and T4). During the experiment, pH increased for all the treatments, but the addition of PFJ alone or in combination with HE, it was maintained below the NC pH value. The lowest TBARS values and the highest PUFA concentrations were found in the T3, T4, and PC treatments. Of all the samples, the lowest residual nitrite values were found for T2. The highest NO-heme values were found for T2 and PC. After 9 days of storage, TVC results were higher than 5.69 logs CFU/g for all treatments. Overall, the obtained results showed that the combination of HE and PFJ could be a promising natural preservative for minced meat that could replace synthetic preservatives.

Keywords: parsnip fermented juice; hawthorn extract; natural nitrite; natural antioxidant; lipids stability; spoilage bacteria; heme pigment conversion degree; bioactive compounds

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1. Introduction

Oxidative processes and meat spoilage bacteria are the most important factors in decreasing the shelf life of meat and meat products. Meat oxidation starts during slaughtering and continues in post-slaughtering conditions, handling, processing, and storage [1,2]. Meat is susceptible to spoilage bacteria due to its favourable growth conditions, high water content, nitrogen-containing molecules, carbohydrates, lipids, lactic acid, vitamins, and minerals. These degradative processes result in the development of off-odours, off-flavour, off-taste, loss of colour, loss in nutritional value, slim formation, and toxic compounds generation, making the meat undesirable for human consumption [3–5]. In the meat industry, synthetic antioxidants and nitrates and nitrites inhibit oxidative processes, bacterial growth, and input attractive colour. Lipid oxidation may be slowed down by synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), propyl gallate (PG), and sodium ascorbate and ascorbate.

To inhibit the grow spoilage bacteria and improve meat colour, synthetic additives, such as potassium/sodium nitrite and potassium/sodium nitrate are used. According to commission regulation (EU) No 1129/2011, the amount of nitrite permitted for use as a food additive in cured meat is currently 150 mg kg^{-1}. Many studies have suggested the toxicity and carcinogenicity of synthetic antioxidants with phenolic structures [6–8] and synthetic nitrites and nitrates [9,10] and for this reason, the researchers interested in replacing these synthetic substances with natural ones has increased in recent decades [11–16]. Many research studies have demonstrated the antioxidant and antimicrobial activity of plant extracts rich in phenolic compounds [14]. Vegetables are important sources of natural nitrates, but nitrate concentrations vary widely among plants, plant parts, and growing conditions. During the fermentation process, nitrates from plant juices can be converted into nitrites and, after concentration, concentrated fermented vegetable plant juices or fermented plant powders rich in natural nitrites result. The high nitrate content in the fermented vegetable juice/powder led researchers to study it as a nitrite natural source for the meat industry [17]. In recent years, consumer preferences have shifted towards requiring health-friendly, high-quality, nutrient-rich natural products. Parsnip fermented juice appears to be highly compatible with processed meat products because it has a high content of nitrates, very little vegetable pigment, and a mild, pleasant flavour profile. On the other hand, extracts of hawthorn berries are sources of natural antioxidants with previously proven preservative effects for meat products [5]. In fact, the present research highlights the preservative activity of the active compounds in hawthorn and nitrite-rich fermented juice. This study emphasizes the effects of varying levels of hawthorn berries ethanolic extract (HE) added to a constant concentration of parsnip fermented juice (PFJ) on lipids stability, heme pigment conversion degree, residual nitrite content, and spoilage bacteria growth in pork mince during refrigeration, compared to the combined effect of synthetic nitrite and sodium ascorbate. This study aimed to evaluate the combined effects of parsnip fermented juice and hawthorn phenolics on lipids and colour stability, and on spoilage bacteria growth in pork mince.

2. Materials and Methods

2.1. Parsnip Fermented Juice (PFJ) Obtaining

Parsnip roots were bought from a local market near Bucharest. The parsnip roots were cut into small pieces (1 × 1 cm) and then placed in a homogenizer with sterile distilled water in a ratio of 1:2 (*w*/*v*), and left to rest for 3 h at 4 °C. After homogenization, filtration and sterilization *Staphylococcus xylosus* (ATCC 29971) were added at 10^8 cfu/mL in a shaker incubator at 37 °C for 36 h [18]. Next, the mixture was filtered through Whatman No. 1 filter paper and evaporated using a rotary evaporator (Heidolph Laborota 4000). The concentrated fermented parsnip juice had a pH of 5.31 and 6237.5 ppm nitrite content. The nitrite concentration was assayed by the AOAC method [19]. The concentrated juice was kept in the refrigerator until used.

2.2. Hawthorn Extract (HE) Obtaining

The dried hawthorn fruits were collected from the forests near Câmpulung Muscel, Argeș county. After grinding, using a kitchen milling machine, the obtained powder was mixed with 60% ethanol (*v*/*v*), in an extraction rapport of 1:10 (*w*/*v*). Then was vigorously mixed and left to stand for 5 h. After that, the extraction was continued in a water bath (GFL 1092) at 60 °C for 3 h. The mixture was filtered through Whatman No. 1 filter paper and concentrated with a rotary evaporator (Heidolph Laborota 4000) to a temperature lower than 80 °C. The total phenolic content was assayed using Folin Ciocalteu reagent [20] and was expressed as mg gallic acid equivalent/mL (mg GAE/mL).

2.3. Experimental Setup and Preparation of Meat Samples

Fresh pork leg was purchased from a local butcher. After washing with distilled sterile water, all subcutaneous and intramuscular fat and visible connective tissue were removed

with a knife. The meat was manually chopped into cubes of approximately 2 cm^3 and then minced in a grinder equipped with a 10 mm plate, followed by grinding through a 3.5 mm plate. The meat was divided into 6 portions of one kilogram. The samples were treated with sodium nitrite (SN) and sodium ascorbate (SA), parsnip fermented juice (PFJ), and hawthorn extract (HE) at different concentrations, as shown in Table 1. Sodium ascorbate and sodium nitrite were analytical reagents and were obtained from Sigma-Aldrich, St Louis, MO, USA.

Table 1. Pork mince treatments with sodium nitrite (SN), sodium ascorbate (SA), parsnip fermented juice (PFJ), and hawthorn extract (HE).

Additives / Treatments	SN (ppm NO_2^-)	SA (ppm)	PFJ (ppm NO_2^-)	HE (ppm GAE)
NC	0	0	0	0
PC	100	50	0	0
T1	0	0	100	0
T2	0	0	100	50
T3	0	0	100	25
T4	0	0	100	10

Abbreviation: SN, sodium nitrite; SA, sodium ascorbate; PFJ, parsnip fermented juice; HE, hawthorn extract; NC, negative control; PC, positive control; GAE, gallic acid equivalent; ppm, parts per million and it expresses milligrams per kg (mg/kg); T1, treatment 1; T2, treatment 2; T3, treatment 3; T4, treatment 4.

For the pork-minced formulation, the ingredients (SN, SA, PFJ, HE) were dissolved in cold (4 °C) sterilized distilled water in a 100 mL final volume. For the negative control, 100 mL of sterilized distilled water was used. All meat samples were homogenized using a food processor (Moulinex DP 700), packed in plastic film, and stored at 4 °C for 9 days.

2.4. Physicochemical Analysis

pH Value

The pH value of all samples was measured using a pH meter (Hanna Instruments, Cluj Napoca, Romania) by direct measurement with a glass electrode calibrated with the phosphate buffers 4.0 and 7.0 at room temperature (21 °C). Ten grams of sample was homogenized with distilled water in a ratio of 1:100 (w/v) for 30 min. After filtration, the pH of the filtrate was measured [21].

2.5. Chemical Analysis

2.5.1. Chromatographic Profile of Fatty Acids

Lipid Extraction and Fatty Acid Methyl Esters

The fatty acids profile of lipids from minced meat was determined as fatty acids methyl esters (FAME). Lipid extractions were made according to [22] method and FAME was prepared by transmethylation using 2 M KOH in methanol and normal heptane according to the method described by [23].

Gas-chromatographic Analysis of Fatty Acid Methyl Esters

Fatty acid methyl esters (FAME) were quantified by gas chromatography (GC) using a Perkin-Elmer-Clarus 500 system with a flame ionisation detector (FID), capillary column injection system and a silica capillary column SGE (BPX70, 60 m; 0.25 mm inner diameter, 0.25 µm film, Agilent). Gas-chromatographic conditions were according to the procedure described by [5]. Each fatty acid was expressed as g/100 g fatty acid methyl esters (FAME).

2.5.2. Thiobarbituric Acid Reactive Substances (TBARS) Value

Lipid oxidation in the minced pork was monitored by measuring thiobarbituric acid reactive substances (TBARS) every 3 days during refrigeration storage. TBARS value was assayed by the method described by [24]. Briefly, 0.5 g minced meat was treated with 2.5 mL thiobarbituric acid solution. After homogenisation, the tube with the mixture was

immersed in a boiling water bath for 10 min. After cooling under running tap water, sonication for 30 min, and centrifugation, the absorbance of the supernatant was read at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane and the concentration ranging from 0 to 10 ppm. TBARS value was expressed as mg of malondialdehyde equivalents/kg of the sample (mg MDA/kg).

2.5.3. Nitrosyl Hemochrome, Total Pigment Content, and the Heme Pigment Conversion Degree

Nitrosyl hemochrome and total pigments were measured after extraction with acetone and acidified acetone [25]. For quantification of the nitrosyl hemochrome, a 10 g sample was mixed with 43 mL of acetone-water solution (acetone: water, 40.5:2.5, *v*/*v*) in reduced light. After 5 min, the mixture was filtered through Whatman No. 1 paper, and the filtrate absorbance was measured at 540 nm using acetone water solution (80% acetone, 20% water) for blank. NO-heme pigment concentration was calculated using formula (1).

$$\text{NO-heme concentration (ppm hematin acid)} = \text{sample A540} \times 290 \quad (1)$$

For total pigment measurement, a 10 g sample (65% water) was mixed with 40.4 mL acetone, 1.6 mL water and 1 mL of concentrated HCl. The mixture was stored in dark at the room temperature, stirring from time to time. After 1 h, the homogenates were filtrated and the absorbance of the filtrate was read at 640 nm against the same solvent mixture used for the homogenate preparation. The optical density read at 640 nm was multiplied by 680, according to formula (2), to express the total pigment concentration.

$$\text{Total pigment concentration (ppm hematin acid)} = \text{A640} \times 680 \quad (2)$$

The heme pigment conversion degree was calculated using formula (3).

$$\%\text{Conversion} = (\text{NO-Heme/Total-Heme}) \times 100 \quad (3)$$

2.5.4. Residual Nitrite Level

Residual nitrite levels in the minced samples were assayed using the method recommended by [26]. For nitrite extraction, 5 g of sample was mixed completely with 40 mL hot water and quantitative transferred to a 500 mL volumetric flask. After the addition of about 300 mL, the volumetric flask was immersed in a shaking water bath for 2 h at 80 °C. Then, the mixture was cooled at room temperature, diluted to volume with water, mixed and filtrated. The colourimetric method using Griess diazotization was performed. Residual nitrite level was determined by comparison with the prepared standard curve and was expressed as ppm NO_2^- (μg NO_2^-/g sample).

2.5.5. Volatile Basic Nitrogen (VBN) Value

To determine the extent of protein deterioration during refrigeration storage, volatile basic nitrogen value was performed by the method of [27]. Briefly, 5 g of sample were mixed with 30 mL of 5% (*w*/*v*) trichloroacetic acid (TCA). The homogenate was made up to 50 mL of final volume with 5% (*w*/*v*) TCA and filtered using the Whatman filter paper No. 1. One mL of filtrate and 1 mL of borate buffer were placed in the outer and inner Conway dish, respectively. After 100 min incubation at 37 °C, the inner solution was titrated with 0.01 N HCl and the titration volume was recorded. The results were expressed as mg%.

2.5.6. Microbiological Analysis

Total viable count (TVC) was determined in plate count agar by the pour-plate method [28]. A total of 25 g of sample was aseptically weighed and homogenized with sterile 0.1% peptone water in the ratio 1:9 (*w*/*v*) for 1 min using a stomacher (400 circulators Seward Ltd.UK) at a speed of 6000 rpm. The homogenized sample was serially diluted (1:10) in sterile 0.1% peptone water. One mL sample of serial dilutions was plated into plate

count agar and then incubated at 35–37 °C for 48 h. Microbiological data were expressed as the logarithm of the number of colony-forming units (logCFU/g). All counts were performed in triplicate.

2.6. Statistical Analysis

The effect of treatments on the fatty acids profile of minced pork was performed using one-way ANOVA (XLStat, Addinsoft, New York, NY, USA). The effect of treatments and storage time on pH value, TBARS, NO-heme, total heme, heme pigment conversion degree, residual nitrite level, volatile basic nitrogen, and the total viable count was performed using two-way ANOVA (XLStat, Addinsoft, New York, NY, USA). The Tuckey test was used to predict differences among the criteria; the effects were considered significant if $p < 0.05$. The statistical model included the fixed effects of treatments (NC, PC, T1, T2, T3, T4) and storage time (0, 3, 6, 9 days) and their interactions. For correlations between parameters, Pearson's correlation was used.

3. Results and Discussions

3.1. Physico-Chemical Analysis

pH Value

Several studies demonstrated that increased refrigeration storage increased raw meat's pH [29–31]. It was suggested that increases in pH during refrigeration are due to the volatile basic compounds, such as ammonia, methylamine, dimethylamine and trimethylamine, and microbial catabolites, which result in meat spoilage [4,32,33]. The pH variations for meat samples according to the storage time are shown in Table 2.

Table 2. Changes in pH values of pork mince depending on treatment and storage time.

Days	Treatments						Treatments		Main Effects Time		*p* Values	
	NC	PC	T1	T2	T3	T4	NC	5.788 [a]	0	5.460 [c]	Treatments	<0.0001
0	5.52 [a]	5.40 [b]	5.43 [ab]	5.45 [ab]	5.47 [ab]	5.49 [ab]	PC	5.495 [d]	3	5.492 [c]	Time	<0.0001
3	5.59 [a]	5.43 [b]	5.45 [b]	5.47 [b]	5.49 [b]	5.52 [ab]	T1	5.628 [b]	6	5.625 [b]	Treatments × time	<0.0001
6	5.81 [a]	5.47 [c]	5.7 [b]	5.51 [c]	5.55 [c]	5.71 [b]	T2	5.533 [d]	9	5.867 [a]		
9	6.23 [a]	5.68 [d]	5.93 [b]	5.7 [d]	5.81 [c]	5.85 [bc]	T3	5.580 [c]				
							T4	5.643 [b]				

[a–d] Means in a row without a common letter significantly differ. NC—negative control; PC—positive control; T1 –treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4– treatment with PFJ +HE, 10 ppm GAE.

On day 0, a slight difference was found between the positive control pH values and others, due to the residual acidity of hawthorn phenolics and PFJ. After 3 days of refrigeration, the pH increased for all samples, but the highest increase was found in the NC. The increase in the pH during storage was lower in the first 3 days and higher in the last 6 days. On the 6th day, T2 and T3 pH values were similar to PC. After 9 days of storage, the pH value of T1 was significantly lower than the pH value found in NC ($p < 0.05$). T2 pH value was significantly lower than the pH values found in T3 and T4 ($p < 0.05$). HE addition in pork mince inhibited pH values increasing in a relation depending on HE levels; thus, after 9 days of refrigeration storage, the pH values decreased in order T4, T3, and T2. By comparison of pH values for samples treated with PFJ + HE, it can be seen that samples treated with 25 and 10 ppm GAE (T3 and T4) resulted in a greater increase in pH ($p < 0.05$); formulation with 50 ppm GAE (T2) resulted in an increase in pH values similar to PC. The slight increase in the pH in the meat samples treated with the PFJ and PFJ + HE treatments (T1, T2, T3 and T4) is attributed to the inhibitory effect of antimicrobial compounds found both in HE and PFJ on the growth and proliferation of spoilage microorganisms that metabolize basic nitrogen compounds, such as amino acids, L-carnitine, lecithin, and choline [30,31].

3.2. Chemical Analysis

pH Value

Chromatographic Profile of Fatty Acids

Lipid deterioration is the major cause of the loss of bioavailability and sensory quality of the meat and is due to fatty acids oxidation and, to a lesser extent of fatty acids catabolism by spoilage bacteria. Lipid oxidation in muscle systems is initiated at the membrane level in the intracellular phospholipid fractions, with high polyunsaturated fatty acids (PUFA) content [34]. In minced meat, PUFA oxidation is the result of the interaction with endogenous prooxidants, such as metalloproteins [35], and with exogenous prooxidants, such as iron from grinding machines and singlet oxygen. The effects of PFJ and PFJ + HE, in concentrations 50, 25, and 10 mg GAE/kg, on the fatty acids profile, after 9 days of refrigeration are shown in Table 3.

Table 3. Fatty acid profile of pork mince with different treatments after 9 days of refrigeration.

Fatty Acid [1] (g/100 g)	NC	PC	T1	T2	T3	T4	SEM	*p* Value
C8:0	0.18 ^{ab}	0.17 ^{abc}	0.19 ^{a}	0.16 ^{bc}	0.14 ^{c}	0.16 ^{abc}	0.006	0.0001
C10:0	0.18 ^{a}	0.17 ^{b}	0.18 ^{ab}	0.17 ^{b}	0.05 ^{c}	0.17 ^{b}	0.007	<0.0001
C12:0	0.10 ^{cd}	0.11 ^{d}	0.14 ^{a}	0.11 ^{d}	0.13 ^{bc}	0.14 ^{ab}	0.005	<0.0001
C14:0	1.72 ^{c}	0.75 ^{b}	1.68 ^{a}	1.66 ^{a}	1.68 ^{a}	1.67 ^{a}	0.014	<0.0001
C15:0	0.17 ^{a}	0.17 ^{a}	0.11 ^{b}	0.17 ^{a}	0.11 ^{b}	0.07 ^{c}	0.010	<0.0001
C15:1	0.07 ^{bc}	0.08 ^{ab}	0.07 ^{bc}	0.10 ^{a}	0.05 ^{c}	0.06 ^{bc}	0.008	0.0001
C16:0	23.94 ^{b}	23.35 ^{f}	24.35 ^{a}	23.44 ^{e}	23.66 ^{c}	23.58 ^{d}	0.021	<0.0001
C16:1	3.67 ^{a}	3.39 ^{c}	3.58 ^{b}	3.59 ^{b}	2.73 ^{e}	3.08 ^{d}	0.016	<0.0001
C17:0	0.18 ^{c}	0.19 ^{bc}	0.22 ^{b}	0.19 ^{bc}	0.26 ^{a}	0.20 ^{bc}	0.010	<0.0001
C17:1	0.20 ^{a}	0.19 ^{a}	0.19 ^{a}	0.18 ^{ab}	0.04 ^{c}	0.15 ^{b}	0.011	<0.0001
C18:0	10.61 ^{a}	10.13 ^{c}	10.40 ^{b}	10.20 ^{c}	10.42 ^{b}	10.32 ^{bc}	0.062	<0.0001
C18:1	44.67 ^{b}	44.6 ^{c}	44.79 ^{a}	44.75 ^{a}	44.58 ^{c}	44.45 ^{d}	0.021	<0.0001
C18:2n−6	12.21 ^{d}	13.80 ^{a}	12.24 ^{d}	12.99 ^{c}	13.52 ^{b}	13.52 ^{b}	0.044	<0.0001
C18:3n−6 (γ)	0.14 ^{b}	0.22 ^{a}	0.16 ^{b}	0.17 ^{b}	0.20 ^{a}	0.20 ^{a}	0.009	<0.0001
C18:3n−3(α)	0.54 ^{c}	0.71 ^{a}	0.55 ^{c}	0.55 ^{c}	0.65 ^{ab}	0.60 ^{bc}	0.021	<0.0001
C20:2n−6	0.64 ^{a}	0.56 ^{b}	0.43 ^{d}	0.49 ^{cd}	0.54 ^{bc}	0.46 ^{d}	0.082	<0.0001
C20:3n−6	0.45 ^{bc}	0.57 ^{a}	0.30 ^{d}	0.50 ^{b}	0.46 ^{bc}	0.43 ^{c}	0.017	<0.0001
C20:4n−6	0.43 ^{c}	0.72 ^{a}	0.39 ^{d}	0.52 ^{b}	0.7 ^{a}	0.69 ^{a}	0.014	<0.0001
Others fatty acids	0.08 ^{a}	0.07 ^{a}	0.03 ^{b}	0.06 ^{ab}	0.03 ^{b}	0.05 ^{ab}	0.008	0.001
∑FA	100	100	100	100	100	100	-	-
∑SFA	40.98 ^{d}	35.04 ^{e}	35.71 ^{a}	36.10 ^{c}	36.46 ^{b}	36.31 ^{bc}	0.037	<0.0001
∑MUFA	44.61 ^{a}	48.26 ^{b}	48.63 ^{a}	48.62 ^{a}	47.40 ^{d}	47.74 ^{c}	0.038	<0.0001
∑PUFA	14.41 ^{e}	16.67 ^{a}	14.30 ^{f}	15.22 ^{d}	16.12 ^{b}	15.90 ^{c}	0.060	<0.0001

[1] FAME—fatty acids methyl esters. FA—fatty acids; SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids. $^{a–f}$ Means in a row without a common superscript letter significantly differ ($p < 0.05$). NC—negative control; PC—positive control; T1—treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4—treatment with PFJ +HE, 10 ppm GAE.

In all samples, the monounsaturated fatty acids (MUFA) showed the highest degree (44.61–48.63%), with the oleic acid (C18:1) being the most abundant fatty acid (44.58–44.79%), whereas polyunsaturated fatty acids (PUFA) showed the lowest percent (14.30–16.67%), with linoleic acid (C18:2n−6) the most abundant PUFA (12.21–13.80%). Saturated fatty acids (SFA) were found in the intermediate degree (35.04–40.98%), with palmitic (C16:0) and stearic (C18:0) acids the most abundant (23.35–24.35% and 10.13–10.61%, respectively). The results are in agreement with the results obtained by [36] that found a similar fatty acid profile in pork meat. Results show differences in fatty acid profile for all treatments, although some of them were statistically significant. For PFJ + HE treatments, (T2, T3 and T4), the total PUFA degree was significatively higher than the degree found in NC ($p < 0.05$), on the last day of determination. The highest total PUFA degree was found in PC

samples, treated with sodium nitrite and sodium ascorbate (PC). The total PUFA degree in sample T3, treated with PFJ + HE, 25 ppm GAE, follows an increasing path as PC ($p < 0.05$). Except for the formulation with PFJ + HE 50 ppm GAE/kg (T2), the other treatments with PFJ + HE (T3 and T4), significantly ($p < 0.05$) increased the stability of unsaturated fatty acids to oxidation, compared with samples formulated with PFJ alone (T1). The lowest degree for total PUFA was found for T1. Comparing the PUFA results obtained for T2, T3, and T4, a prooxidant effect on minced pork lipids by HE added at 50 ppm GAE/kg can be suggested. According to the results from the present study, the concentrations of 25 and 10 mg GAE/kg, seem to protect the essential fatty acids, linoleic (C18:2n−6), linolenic (C18:3n−3), and arachidonic (C20:4n−6) acids against oxidative damage, comparatively with T1. Some authors have shown that colonial-type salami treated with rosemary extract alone or in association with celery powder had a higher PUFA content compared with control samples. [37]. Some authors used Mediterranean berries' ethanolic extracts as antioxidants in pork burgers subject to cooking and chilled storage and found that the protective effect of ethanolic extracts on PUFA decreased in the following order: *Rosa canina*, *Rubus ulmifolius*, *Arbusto unedo*, and *Crataegus monogyna*. The highest level of linoleic acid they found in patties treated with the ethanolic extract obtained from *Arbusto unedo* berries [38].

3.3. Thiobarbituric Acid Reactive Substances (TBARS) Value

Generally, lipid oxidation affects bioavailability and sensory quality in minced meat. A good indicator of lipid oxidation level is the TBARS value. It was suggested that the TBARS value at 0.5 mg/kg was a threshold value for rancidity perception by consumers [39]. The results regarding the changes in TBARS values for the six evaluated treatments are presented in Table 4.

Table 4. Changes in thiobarbituric acid reactive substances values of minced pork depending on treatment and storage time.

Days	Treatments						Treatments		Main Effects Time		*p* Values	
	NC	PC	T1	T2	T3	T4	NC	0.845 [a]	0	0.123 [b]	Treatments	<0.0001
0	0.121 [a]	0.122 [a]	0.121 [a]	0.130 [a]	0.122 [a]	0.121 [a]	PC	0.198 [e]	3	0.328 [a]	Time	<0.0001
3	0.650 [a]	0.17 [d]	0.45 [b]	0.27 [c]	0.16 [d]	0.27 [c]	T1	0.523 [b]	6	0.632 [d]	Treatments × time	<0.0001
6	1.340 [a]	0.22 [e]	0.87 [b]	0.65 [c]	0.27 [e]	0.44 [d]	T2	0.373 [c]	9	0.557 [c]		
9	1.27 [a]	0.28 [e]	0.65 [b]	0.44 [c]	0.35 [d]	0.35 [d]	T3	0.226 [e]				
							T4	0.295 [d]				

[a–e] Means in a row without a common letter significantly differ. NC—negative control; PC—positive control; T1 –treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4– treatment with PFJ +HE, 10 ppm GAE.

The analysis of variance for the TBARS data indicates that the TBARS values for pork mince were influenced by the refrigeration period ($p < 0.001$) and treatments. Overall, TBARS values increased during refrigeration storage, but the lowest TBARS values were found in PC pork mince ($p \leq 0.05$). The sample treated with PFJ alone (T1) had lower TBARS values than NC, but higher than PC ($p \leq 0.05$). The addition of HE in the minced pork affects the TBARS values in a concentration-dependent manner, after 3 days of refrigeration. The best antioxidant activity was found in the sample treated with PFJ + HE in a concentration equal to 25 ppm GAE (T3), followed by the samples treated with PFJ + 10 ppm GAE HE (T4), on the 6th day of the determination. On the last day of the investigation (9th day), the samples treated with PFJ + 50 ppm HE (T2) showed the highest TBARS values, comparatively with T3 and T4. The result obtained showed that the capacity of PFJ + HE to inhibit lipid oxidation in minced pork depends on the amount of HE used. HE in concentrations of 50 ppm GAE/kg meat has prooxidant activity, but HE in a concentration of 25 and 10 mg GAE/kg influenced the antioxidant activity as the association of sodium nitrite with sodium ascorbate (PC).

Another study showed the TBARS values after the preparation of beef patties using resveratrol as an antioxidant in concentrations as 110 µmol/kg meat and 550 µmol/kg meat, during 9 days of refrigeration. The highest TBARS values were found in samples treated with 550 µmol/kg meat, comparatively with TBARS values found in samples treated with 110 µmol/kg meat, but both were lower than those found in control samples [40]. It was reported different TBARS values for cooked pork patties, prepared with ethanolic berry extracts, and stored by refrigeration than those found in control samples (1.071 mg MDA/kg) [38]. So, higher TBARS values were reported for *Crataegus monogyna* (0.181 mg MDA/kg) and *Rosa canina* (0.143 mg MDA/kg) treatment extracts, but lower TBARS values for *Rubus ulmifolius* (0.082 mg MDA/kg) and *Arbusto unedo* (0.113 mg MDA/kg) treatment extracts.

3.4. Nitrosyl Hemochrome, Total Pigment Content, and the Heme Pigment Conversion Degree

Nitrosyl hemochrome (NO-heme), the pink pigment of cured meat, is formed mainly from NO(II)Mb resulting in the reaction of myoglobin (Mb) with NO generated from nitrite conversion in the presence of ascorbate [41]. Some studies suggested that the addition of ascorbate in the muscle reduces met(III)Mb to deoxy(II)Mb and the simultaneously generated NO from nitrite binds to deoxy(II)Mb or can reduce nitrosylate met(III)Mb under anaerobic conditions, resulting in the formation of NO(II)Mb [41].

The studies regarding the effect of pH on the stability of nitrosyl hemochromogen suggest that NO-Heme is extremely unstable at weakly acid pH, and this explains why pH affects the apparent colour of cured meat products with storage time [42]. Table 5 showed the results obtained for nitrosyl hemochrome (NO-Heme) values of minced pork formulated with parsnip fermented juice (PFJ) alone, parsnip fermented juice + hawthorn extract (PFJ + HE) compared to untreated minced pork (NC), and synthetic added compounds (PC).

Table 5. Changes in NO-heme value, total heme value, and heme pigment conversion degree of minced pork depending on treatment and storage time.

Treatments	NO-Heme (ppm Hematin Acid)	Total Heme (ppm Hematin Acid)	Heme Pigment Conversion Degree (%)
0 days			
NC	0.00 ^{c}	121.23	0.00 ^{c}
PC	26.23 ^{a}	120.99	21.68 ^{a}
T1	20.50 ^{b}	121.45	16.87 ^{b}
T2	26.20 ^{a}	123.09	21.30 ^{a}
T3	26.00 ^{a}	121.40	21.41 ^{a}
T4	25.89 ^{a}	120.97	21.42 ^{a}
3 days			
NC	0.00 ^{d}	120.46	0.00 ^{c}
PC	44.60 ^{a}	120.90	36.68 ^{a}
T1	26.70 ^{c}	123.77	21.76 ^{b}
T2	30.50 ^{b}	123.09	23.99 ^{b}
T3	30.70 ^{b}	121.41	24.47 ^{b}
T4	27.98 ^{bc}	121.56	23.02 ^{b}
6 days			
NC	0.00 ^{e}	119.00 ^{b}	0.00 ^{e}
PC	55.60 ^{a}	120.35 ^{ab}	46.11 ^{a}
T1	32.5 ^{d}	123.56 ^{a}	26.31 ^{d}
T2	45.81 ^{b}	122.67 ^{ab}	37.34 ^{b}
T3	43.21 ^{bc}	120.50 ^{ab}	34.70 ^{bc}
T4	40.76 ^{c}	121.67 ^{ab}	33.50 ^{c}

Table 5. *Cont.*

Treatments	NO-Heme (ppm Hematin Acid)	Total Heme (ppm Hematin Acid)	Heme Pigment Conversion Degree (%)
9 days			
NC	0.00 ^{d}	118.00	0.00 ^{d}
PC	60.50 ^{ab}	119.30	50.71 ^{ab}
T1	38.45 ^{c}	120.15	32.00 ^{c}
T2	63.50 ^{a}	120.75	52.58 ^{a}
T3	43.21 ^{ab}	119.50	50.79 ^{ab}
T4	40.76 ^{b}	119.59	47.99 ^{b}
Main effects Treatments			
NC	0.000 ^{e}	119.672 ^{c}	0.000 ^{e}
PC	46.733 ^{a}	120.438 ^{bc}	38.794 ^{a}
T1	29.540 ^{d}	121.482 ^{ab}	24.235 ^{d}
T2	41.503 ^{b}	122.400 ^{a}	33.803 ^{b}
T3	40.153 ^{b}	120.703 ^{abc}	32.843 ^{bc}
T4	38.008 ^{c}	120.950 ^{abc}	31.482 ^{c}
Time			
0	20.803 ^{d}	121.522 ^{a}	17.113 ^{d}
3	26.747 ^{c}	121.364 ^{a}	21.653 ^{c}
6	36.315 ^{b}	121.327 ^{a}	29.660 ^{b}
9	46.758 ^{a}	119.550 ^{b}	39.012 ^{a}
p value			
Diet	<0.0001	0.001	<0.0001
Time	<0.0001	0.0001	<0.0001
Diet × time	<0.0001	0.769	<0.0001

$^{a–e}$ Means in a row without a common letter significantly differ. NC—negative control; PC—positive control; T1–treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4–treatment with PFJ +HE, 10 ppm GAE

The highest value for NO-Heme during 9 days of refrigeration was found in PC. In the sample formulated only with PFJ (T1), NO-Heme content was significantly lower, compared with PC and samples formulated with PFJ + HE (T2, T3 and T4) ($p < 0.05$). The formulation of the minced meat with PFJ and HE increased NO-Heme content, in a relation dependent on the HE level in samples (Table 5).

After 9 days of refrigeration storage, in the samples treated with PFJ + HE, the content of NO-Heme increased in the order: T2, T3, and T4. Obtained results showed that HE accelerates the heme pigments to react with NO in a concentration-dependent manner. The nitrosylation reaction stimulated by HE and SA may be due to the lowering of the pH values on day 0 and of the reaction between phenolics and nitrite [43]. It was reported that a pH decreasing only 0.2 unit, doubles the rate of colour formation through nitrite-myoglobin reaction [44]. It was suggested that the final colour properties of nitrite also increase with decreasing pH and this constitutes the basis for product formulation with acidulants to accelerate curing [44,45].

Table 5 presents the results regarding total heme pigment values obtained for all treatments during 9 days of refrigeration. In all samples, a slight decrease in total heme pigment was found after 9 days of storage. Significant differences ($p < 0.05$) in total heme pigment were found in the treated minced pork after 9 days of refrigeration storage compare with previous determinations (0, 3 and 6 days).

Heme pigment conversion degrees for all minced pork treatments are presented in Table 5. After 9 days of refrigeration, the results showed that the minced pork with PFJ (T1) produced an increase in the heme pigment conversion % equal to 32.00%. When HE was

added to PFJ, even the lowest HE concentration, 10 ppm GAE (T4) produced an increase in heme pigment conversion degree (47.99%), compared to the formulated sample with PFJ alone. So, compared to PC, the samples formulated with PFJ + HE provided increases in the heme pigment conversion degree in T2 and T3 or slower in T4 (Table 5). Moreover, PFJ + HE and SN + SA have the same effect on the heme pigment conversion degree, meaning an increased percentage. Results found for pH values, NO—heme and heme pigment conversion degree show important aspects related to sample formulation. The results can be attributed to the reducing and acidic properties of SA or HE that favour met(III)Mb reduction, NO generation, and the reaction of heme pigments with NO also.

3.5. Residual Nitrite Level

Nitrite added in the meat reacts especially with heme proteins (myoglobin, haemoglobin), and less with nonheme proteins, lipids, and carbohydrates. Unreacted nitrite, named residual nitrite, can be depleted by the formation of nitrogen gasses [46]. Nitrite depletion in cured meat is influenced by pH, temperature, time, meat type, salt, and nitrite concentration. The residual nitrite level in meat treated with PFJ and PFJ + HE is shown in Table 6. For all samples, residual nitrite level decreased during refrigeration storage, but the most rapidly decreasing was found after 3 days of storage. Throughout the refrigeration, the lowest residual nitrite level was found in the negative control (NC), without adding any nitrite, and the highest level was found in pork mince treated with PFJ alone (T1). The addition of HE to the PFJ favours the decrease in the residual nitrite level. Therefore, the residual nitrite level in minced pork treated with PFJ + HE decreased with increasing levels of HE. In the pork mince treated with the PFJ + 50 ppm HE (T2), the residual nitrite level was significantly ($p < 0.05$) lower than those found in the positive control. Regarding the residual nitrite level in the minced pork treated with the PFJ + 50 ppm HE (T3), the rate of nitrite depletion with storage time was similar to the one found for the positive control. The residual nitrite depletion was a bit slower in the meat treated with PFJ + HE, at level 10 ppm GAE (T4), compared with the PC. The addition of HE showed a statistically significant effect in the nitrite depletion with storage time, and so, the decrease in the residual nitrite level ($p < 0.001$).

Table 6. Changes in residual nitrite level and volatile basic nitrogen of minced pork depending on treatment and storage time.

Treatments	Residual Nitrite Level (ppm)	Volatile Basic Nitrogen (VBN) mg%
0 days		
NC	0.00 ^{b}	12.89
PC	99.67 ^{a}	12.82
T1	99.33 ^{a}	12.89
T2	98.67 ^{a}	12.84
T3	100.00 ^{a}	12.81
T4	98.33 ^{a}	12.88
3 days		
NC	0.00 ^{e}	23.55 ^{a}
PC	20.30 ^{c}	16.84 ^{c}
T1	80.50 ^{a}	19.89 ^{b}
T2	13.50 ^{d}	17.87 ^{bc}
T3	25.60 ^{c}	18.02 ^{bc}
T4	33.50 ^{b}	20.10 ^{b}

Table 6. *Cont.*

Treatments	Residual Nitrite Level (ppm)	Volatile Basic Nitrogen (VBN) mg%
	6 days	
NC	0.00 ^{d}	30.45 ^{a}
PC	16.00 ^{c}	18.86 ^{c}
T1	75.00 ^{a}	23.78 ^{b}
T2	10.50 ^{c}	10.87 ^{bc}
T3	15.50 ^{c}	21.17 ^{bc}
T4	23.00 ^{b}	21.70 ^{bc}
	9 days	
NC	0.00 ^{d}	46.7 ^{a}
PC	10.00 ^{bc}	20.11 ^{d}
T1	69.00 ^{a}	33.70 ^{b}
T2	5.80 ^{c}	22.39 ^{cd}
T3	13.50 ^{b}	24.50 ^{c}
T4	14.00 ^{b}	31.80 ^{b}
	Main effects Treatment	
NC	0.000 ^{e}	28.398 ^{a}
PC	36.492 ^{c}	17.158 ^{d}
T1	80.958 ^{a}	22.565 ^{b}
T2	32.117 ^{d}	18.493 ^{c}
T3	38.650 ^{c}	19.125 ^{c}
T4	42.208 ^{b}	21.620 ^{b}
	Time	
0	82.667 ^{a}	12.855 ^{d}
3	28.900 ^{b}	19.378 ^{c}
6	23.333 ^{c}	22.805 ^{b}
9	18.717 ^{d}	29.867 ^{a}
	p value	
Diet	<0.0001	<0.0001
Time	<0.0001	<0.0001
Diet × time	<0.0001	<0.0001

$^{a–e}$ Means in a row without a common letter significantly differ. NC—negative control; PC—positive control; T1—treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4—treatment with PFJ +HE, 10 ppm GAE.

These results were in agreement with those found by other researchers [47,48], who reported continuous depletion of the residual nitrite level in cured meat during the storage time. A more pronounced decrease in residual nitrite level in minced pork formulated with SA and HE, compared to the sample formulated with PFJ alone may be the result of the pH decreasing and the interaction of nitrite with the SA and the HE. Some researchers reported a decrease in free nitrite in aqueous solutions of nitrite and sodium ascorbate [49]. Other results suggested that NO resulting in the reaction of free nitrite with SA is responsible for nitration reactions and residual nitrite depletion during meat curing [50]. A good correlation ($p < 0.001$) was found between pH value and residual nitrite depletion rate and between HE concentrations and residual nitrite depletion rate. Similar results were achieved in different research regarding cured meat products [51–54].

3.6. Volatile Basic Nitrogen (VBN) Value

Volatile basic nitrogen is a product of bacterial spoilage and endogenous enzyme action [55]. VBN is mainly composed of ammonia and primary, secondary, and tertiary

amines [56] resulting from amino acid degradation [57,58]. The changes in the VBN value of all samples with storage time are shown in Table 6.

The results show the progressively increasing VBN for all samples. The highest VBN values were found in NC and the lowest in the PC, throughout the refrigeration period. In pork mince formulated with PFJ alone, VBN values were lower than those found in NC but higher than those found in PC. Meat formulation with PFJ + HE decreased VBN values in total phenolics in HE concentration-dependent manner, the lowest values were found for PFJ + HE, in the concentration of 50 ppm GAE (T2) and the highest for PFJ +HE, 10 ppm GAE (T4). In the T2 samples, the geometry of the VBN values concentration dependence was similar to one obtained for PC, but the values were higher by an average of 11.14%. A previous study used fermented spinach for pork loin curing and reported similar results [15]. VBN values in samples treated with fermented spinach were higher than those found in the negative control and lower than those found in the positive control treated with synthetic nitrite.

3.7. Microbiological Analysis

The effect of PFJ and PFJ + HE on total viable count (TVC) is shown in Table 7. The initial number of bacteria in samples was between 2.60 log CFU/g and 2.77 log CFU/g which indicated the good quality of pork used in this study. For all samples, TVC increased with storage time and the value of NC and T1 increased faster than PC, T2, T3, and T4. From day 0 to day 3, there were strong significant differences between NC and T1 ($p < 0.05$). After 6 days of storage, significant ($p < 0.05$) differences between NC and T1, which indicated that PFJ inhibited the growth of TVC alone, were found. After 6 days of storage significant differences ($p < 0.05$) between T1 and samples T2 were found and these results indicated that HE added in PJF increased the antimicrobial activity of PFJ. A significant correlation was established between PC and samples T2, T3, and T4 ($p < 0.001$).

Table 7. Changes in total viable count of minced pork depending on treatment and storage time.

Days	Treatments						Treatments		Main Effects Time		*p* Values	
	NC	PC	T1	T2	T3	T4	NC	7.325 [a]	0	2.687 [d]	Treatments	<0.0001
0	2.77	2.67	2.67	2.75	2.6	2.66	PC	3.863 [d]	3	4.143 [c]	Time	<0.0001
3	5.2 [a]	2.87 [c]	4.66 [a]	3.34 [bc]	4.22 [ab]	4.57 [a]	T1	5.945 [b]	6	5.890 [b]	Treatments × time	<0.0001
6	8.22 [a]	4.11 [e]	6.89 [b]	4.56 [de]	5.55 [cd]	6.01 [bc]	T2	4.358 [d]	9	8.400 [a]		
9	13.11 [a]	5.8 [d]	9.56 [b]	6.78 [cd]	7.2 [c]	7.95 [c]	T3	4.893 [c]				
							T4	5.298 [c]				

[a–e] Means in a row without a common letter significantly differ. NC—negative control; PC—positive control; T1—treatment with PFJ; T2—treatment with PFJ + HE, 50 ppm GAE; T3—treatment with PFJ + HE, 25 ppm GAE; T4—treatment with PFJ +HE, 10 ppm GAE.

After 9 days of storage in all samples, TVC was higher than 5.69 logs CFU/g and so, not acceptable for safe consumption, but TVC values were lower for T1, T2, T3, and T4 than NC. Similar results were reported by the literature, showing that fermented spinach extract, celery juice powder, and cherry juice powder can inhibit the growth of microorganisms in different meat models [15,59]. Other researchers tested the antimicrobial activity of the ethanolic extracts obtained from rosemary and cloves and found important antimicrobial activity for extracts used alone or in combination [31].

3.8. Correlations between Analysed Parameters

The Pearson correlation coefficient (r) is the simplest way to measure a linear correlation. It takes values between −1 and 1 and indicates the relationship between two variables in strength and direction. When one variable changes, the other variable also changes. In this study, we analysed the effects of various concentrations of HE in combination with a constant PFJ concentration on the pH, fatty acids profile, TBARS value, NO-heme, total heme pigment, heme pigment conversion degree, residual nitrite, VBN, and TVC. pH value is a very important parameter for pork mince and was influenced by VBN value (r = 0.2488,

$p < 0.0001$) and TVC, in a positive and linear manner. NO-heme is a parameter related to meat colour stability. According to the results, a weak positive relationship was found between NO-heme and TBARS (r = 0.9349, $p < 0.0001$), but negative correlations were found between TBARS value and MUFA (r = −0.7275, $p < 0.0001$), and between TBARS value and PUFA (r = −0.8643, $p < 0.0001$). Colour stability is influenced by TBARS and could be related to fatty acids profile. A negative correlation was found between NO-heme and residual nitrite (r = −0.7786, $p < 0.0001$). The pH values determined in the present study established positive correlations with heme conversion degree, (r = 0.5848, $p < 0.0001$), and NO-heme (r = 0.5787, $p < 0.0001$) (data in Table S1).

4. Conclusions

The use of PFJ in combination with HE exerted an inhibitory effect on the growth of spoilage bacteria that metabolize nitrogen compounds and significantly increased the stability of unsaturated fatty acids to oxidation in minced pork. The addition of HE in the minced pork decreased the TBARS values compared to T1. At any time during the experiment, NO-heme increased in a concentration-dependent manner with HE. So, HE may influence the NO-heme concentration. The concentration of HE of 25 ppm together with PFJ was the most effective to delay the FA oxidation in pork mince, whereas the higher concentration of HE (50 ppm GAE) had decreased the total PUFA and increased TBARS, suggesting a prooxidant effect on the minced pork lipids. Colour and lipids stability may depend on the HE and PFJ combination. However, T1, T2, T3, and T4 showed lower TVC values than NC. Between them, T2 was the most effective against microbial growth. In conclusion, these combinations of natural sources of nitrites and antioxidants could be considered promising meat alternatives to synthetic nitrites and antioxidants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13020432/s1, Table S1: Pearson's correlation coefficients of analysed parameters.

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Microalgae (*Chlorella vulgaris* and *Spirulina platensis*) as a Protein Alternative and Their Effects on Productive Performances, Blood Parameters, Protein Digestibility, and Nutritional Value of Laying Hens' Egg

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Abstract: Protein is an essential nutrient for laying hens, playing a crucial role in egg production and supporting their overall health. An 8-week feeding trial was conducted on 120 Lohmann Brown laying hens (aged 38 weeks). The layers were assigned randomly to three groups and housed in cages (twenty replicates × two birds/cage). All groups were fed a corn–soybean meal basal diet (2750 kcal/kg metabolizable energy (ME) with 17.8% crude protein (CP)). In contrast to conventional diet (CON), the experimental groups were supplemented primarily at the expense of soybean meal with 2.0% *Chlorella vulgaris* (CV2%) and 2.0% *Spirulina platensis* (SP2%). Their high concentrations of chlorophyll *a* (5.56; 9.06 mg/g), chlorophyll *b* (0.88; 1.34 mg/g), and antioxidant activity expressed as 2,2-diphenyl-1-picrylhydrazyl (73.29; 81.27 DPPH% inhibition) improved egg yolk quality. At the end of the trial, eighteen eggs/group (six yolk samples/group, three eggs/sample) were collected to determine the egg quality and nutritional parameters (fatty acids profile, cholesterol, β-carotene, yolk color, and antioxidant capacity). To determine the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH), and total antioxidant capacity (TAC), blood samples were collected at the end of the period. Microalgae inclusion increased ($p < 0.05$) the fatty acid content, ß-carotene concentration ($p < 0.001$), antioxidant capacity ($p < 0.0001$), and yolk color intensity ($p < 0.001$) significantly, especially the yolk redness a* color parameter, but without any significant results concerning cholesterol concentration. Boiling the eggs for 10 min significantly ($p < 0.001$) increased the b* color parameter on microalgae treatments. The supplementation of laying hens' diet with microalgae positively influenced egg quality and nutritional properties.

Keywords: antioxidant capacity; blood antioxidant enzymes; cholesterol; fatty acids; laying hens; microalgae; yolk color

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1. Introduction

The protein source in a hen's diet is of utmost importance as it directly influences the bird's growth and health, egg quality, and overall production performances [1].

Soybean meal is considered a high-quality protein source, widely used in poultry diet formulation [2], which contains a balanced amino acid profile, crucial for egg production and overall growth. These amino acids contribute to the synthesis of proteins, enzymes, and hormones, supporting various physiological functions in hens [3]. Soybean protein can be compared to proteins found in meat, milk, and eggs. Among plant-based protein

sources, soybean protein is widely regarded as having the highest biological value [4]. Alshelmani et al. [5] consider that the increasing competitiveness of feedstuffs for poultry nutrition presents a challenge to food security; therefore, ongoing efforts are made to explore alternative protein sources that can partially replace soybean meal in poultry diets.

Microalgae are being increasingly explored as a valuable and sustainable alternative in animal and poultry nutrition due to high protein content [6], and are primary sources of omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [7]. Moreover, they are considered environmentally friendly due to their minimal impact on land and water resources [8]. Furthermore, microalgae contain bioactive compounds that exhibit antioxidant [9], antimicrobial, and immunomodulatory properties [10], thereby contributing to disease prevention and supporting the immune system.

Chlorella (*Chlorella vulgaris*) is a naturally single-celled green microalga considered as an alternative for partially replacing soybean meal included in poultry diets [11]. Previous studies found that *Chlorella* has a positive impact on egg production and quality, enhancing the intensity of the yolk color due to carotenoid transfer (canthaxanthin and β-carotene) [12]. Furthermore, it promotes the growth of lactic-acid-producing bacteria in the intestines and lowers the total cholesterol and triglyceride level concentration in serum and liver [13].

Spirulina (*Sp. platensis*), a blue-green spiral filamentous alga [14], is a natural product with high nutritional value and increased antioxidant potential. Its utilization improves production efficiency, egg production [15], and yolk redness, while also exhibiting favorable amino acid profiles and high digestibility [16]. Spirulina is recognized as a sustainable protein source with a low-impact environmental footprint that can vary significantly depending on factors such as the production system and regional climate [17].

Despite previous research on this topic, the results of previous experiments involving the inclusion of microalgae in poultry diets have generated inconsistent findings with regard to both poultry productivity and egg quality. As a result, our study aims to investigate the partial substitution of soybean meal in the diet of laying hens, and to examine the potential impacts and effects of chlorella and spirulina, both at equivalent inclusion levels, on these specific parameters.

2. Materials and Methods

2.1. Ethical Statement

The study was carried out at the Laboratory of Animal Physiology, National Research-Development Institute for Animal Biology and Nutrition (IBNA), Balotesti, Romania. The feeding, handling, and slaughtering procedures of the study were performed in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes, and the experimental procedures, according to an experimental protocol (No. 6252/27.10.2021), were approved by the Research Ethics Committee for Animal Production studies of IBNA.

2.2. Microalgae Purchase and Chemical Analyses

Microalgae chlorella and spirulina powder were purchased from the agri-food market. Triplicate analyses were conducted on samples of chlorella and spirulina powder to determine the following: dry matter (DM), ash, organic matter (OM), crude protein (CP), ether extract (EE), crude fiber (CF), and non-fermentable extractive substance (NFE); in vitro nutrient digestibility of protein, dry matter, and organic matter (DCP, DDM, DOM); and antioxidant activity and fatty acid profile.

Metabolizable energy (ME) of the microalgae was calculated using formula (1), according to [18] and cited by [19]:

$$\text{ME (kcal/kg)} = (35.3 \times \text{CP \%}) + (79.5 \times \text{EE \%}) + (40.6 \times \text{NFE \%}) + 199.0 \quad (1)$$

2.3. Animals, Housing, and Experimental Diets

An eight-week feeding trial was conducted on 120 Lohmann Brown layers (38 weeks), individually weighed and assigned in 3 treatments (CON, CV2%, and SP2%). The layers

were randomly placed in twenty replicates with 2 birds per treatment, housed in metabolic cages (50 cm width × 40 cm height × 50 cm length) under controlled environmental conditions monitored by a ViperTouch computer (16 h light/24 h; T = 23.08 ± 0.98 °C; H = 66.35 ± 5.68%). Each replicate was considered an experimental unit and performance parameters were evaluated per pen. The feed was administrated daily at 08:30 a.m. and water was available at all times. Throughout the experimental period, no vaccination treatment was applied to the birds.

The isocaloric and isonitrogenous three experimental treatments (in mash form) were formulated by a nutritional optimization program (HYBRIMIN® Futter5) to meet the nutrient requirements for laying hens as given by [20]. All groups were fed a corn–soybean meal basal diet (17% crude protein and 2750 kcal ME/ kg feed) as follows: CON—a commercial diet without microalgae (chlorella or spirulina); CV2%—a control diet containing 2.0% chlorella powder; and SP2%—a control diet containing 2.0% spirulina powder, as shown in Table 1. A quantity of 500 g feed samples from each group were taken and analyzed by chemical composition as described previously for the microalgae samples. Following the manufacturing of the diets, the feed was packaged, appropriately labeled, and stored under optimal conditions, specifically in a cool environment, in preparation for the experimental procedures.

Table 1. Ingredients and chemical composition of the diets (% as fed).

Ingredients, %	Experimental Diets		
	Control (CON)	Chlorella Powder (CV2%)	Spirulina Powder (SP2%)
Corn	40.00	40.00	40.00
Wheat bran	22.49	23.72	23.60
Chlorella vulgaris	-	2.00	-
Spirulina platensis	-	-	2.00
Soybean meal	24.36	21.58	21.63
Vegetable oil	1.48	0.97	1.04
L-lysine HCl	-	0.01	0.01
DL–methionine	0.16	0.17	0.17
Calcium carbonate	8.83	8.84	8.84
Monocalcium phosphate	1.32	1.33	1.33
Salt	0.33	0.33	0.33
Choline premix	0.04	0.04	0.04
Vitamin–mineral premix *	1.00	1.00	1.00
Total	100.00	100.00	100.00
	Calculated analysis (%) **		
Metabolizable energy (Kcal/kg)	2.750.00	2.750.00	2.750.00
Crude protein	17.00	17.09	17.00
Lysine	0.87	0.80	0.80
Methionine+Cystine	0.73	0.71	0.71
Threonine	0.64	0.59	0.59
Calcium	3.90	3.90	3.90
Phosphorus	0.63	0.62	0.62

Where: CON, conventional diet; CV2%, conventional diet supplemented with 2% chlorella powder; SP2%, conventional diet supplemented with 2% spirulina powder; * 1 kg diet contains: = 11,000 IU/kg vit. A; 2000 IU/kg vit. D3; 27 IU/kg vit. E; 3 mg/kg vit. K; 2 mg/kg Vit. B1; 4 mg/kg vit. B2; 14.85 mg/kg pantothenic acid; 27 mg/kg nicotinic acid; 3 mg/kg vit. B6; 0.04 mg/kg Vit. B7; 1 mg/kg vit. B9; 0.018 mg/kg vit. B12; 20 mg/kg vit. C; 80 mg/kg manganese; 80 mg/kg iron; 5 mg/kg copper; 60 mg/kg zinc; 0.37 mg/kg cobalt; 1.52 mg/kg iodine; 0.18 mg/kg selenium. ** Calculated according to NRC [20].

2.4. Laying Hens Performance

During the 8-week feeding trial, the performance parameters of the laying hens (daily feed intake (DFI; g/day/layer), feed conversion ratio (FCR; g feed/g egg), hen day egg production (HDEP; %), egg weight (EW; g), and egg size classification (%)) were monitored. At the initial and the final period, body weight (g/hen) was measured, and eggs were collected and weighed every day. Hen day egg production was calculated using the

following formula [(100 × number of eggs laid)/(number of hens × days)] and classified according to the European Council Directive (2006). Data on feed intake and egg mass were used to calculate the feed conversion ratio (feed intake/egg mass; g/g). All performance parameters were determined for each replicate of treatment groups.

2.5. Nutrient Digestibility Trial

During the last week of the feeding trial (the 8th wk), 6 cages per group (2 birds per cage) were randomly selected from the digestibility trial to measure the apparent nutrient digestibility. For 5 days, both feed leftovers and excreta were collected and weighed daily to determine nutrient intake. During the balance period, fecal samples were stored in a refrigerator at a constant temperature of 4 °C. Finally, each sample was homogenized, and approximately 200 g samples were extracted and dried for 48 h at a constant temperature of 65 °C in an oven (ECOCELL Blueline Comfort, Nuremberg, Germany). After drying, the samples were ground (using a Grindomix GM 200 knife mill, Retsch, Germany) and analyzed for chemical composition. The values obtained from the laboratory chemical analysis were used to calculate the apparent digestibility of nutrients (DDM, DOM, DCP, DEE, and DNFE) as described earlier by [21] using the following formula:

$$\text{Apparent nutrient digestibility } (\%) = \frac{(\text{nutrient intake} - \text{nutrient excreta})}{\text{nutrient intake}} \times 100 \quad (2)$$

2.6. Blood Collection and Analysis

On the final day of the experiment, approximately 3 mL of venous blood samples per birds were aseptically collected from 18 laying hens from the sub-axial region into 9 mL anticoagulant-free Vacutainers containing 14.3 U/mL of lithium heparin (Vacutest®, Arzergrande, Italy). Further, these samples were used to determine the activity of blood antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH), and total antioxidant capacity (TAC). Blood samples were separated by centrifugation at 3000× g in a refrigerated centrifuge (Eppendorf Centrifuge 5430R; Eppendorf, Hamburg, Germany) for 25 min at 4 °C. Afterwards, the supernatant obtained from serum samples were carefully transferred to plastic vials and stored at −20 °C until the analysis.

2.7. Egg Quality Measurement

A total of 306 eggs were collected during the experiment. The collected eggs (18 eggs/group: 3 eggs/cage, 6 cages/lot; each cage representing a sample) were analyzed at the end of the experiment (2 months) to evaluate the impact of microalgae-based diets, specifically those containing chlorella and spirulina, on the fatty acid composition. The antioxidant profile of the yolk, as well as the internal and external quality parameters of the eggs, were determined at the end of the experiment (8 weeks) using a Digital Egg Tester DET-6500 (NABEL Co., Ltd., Kyoto, Japan). First, the eggs were weighed whole and then cracked, and the yolks were separated from the albumen and shell; every yolk was rolled onto a paper towel to remove any adherent albumen or chalazae membrane as described by [22]. Each egg component was weighed with a Kern scale (precision 0.001). The yolk color intensity was measured using the portable colorimeter 3 nh YS3020 (Shenzhen ThreeNH Technology Co., Ltd., Beijing, China), and the temperature and pH of yolk and albumen were measured using a portable pH meter (Five Go F2-Food, Greifensee, Switzerland) and Haugh unit. After measuring the internal and external quality parameters of the eggs, the yolk samples were dried for 48 h at a constant temperature of 65 °C in an oven (ECOCELL Blueline Comfort, Nuremberg, Germany) for further chemical analysis, such as the concentration of β-carotene (μg/g), total polyphenols (mg/g GAE), antioxidant activity (expressed as DPPH % inhibition and μM Trolox), fatty acid profile (g acid/100 g total FAME), and cholesterol concentration (g/egg).

To assess the yolk color stability after boiling, at the end of the experiment, 90 eggs were collected (10 eggs/group, 30 eggs/period) and boiled for 10, 15, and 20 min, respectively.

2.8. Chemical Analysis of Samples

2.8.1. Determination of In Vitro Digestibility of Nutrients

The in vitro digestibility of nutrients was determined following the method proposed by [23] and adapted for poultry as described by [21] using a Daisy Incubator (ANKOM Technology, Macedon, NY, USA) in a 2-step procedure: two successive incubations with pepsin and pancreatin. The samples were introduced into F57 bags (Ankom) and incubated in Daisy Incubator jars with 0.1 M phosphate pH 2.0 buffer with 0.3 g of pepsin (porcine, 2000 FIP U/g) per liter for 6 h at 39 °C. After draining the buffer and washing bags with slightly warm tap water, the next 0.04 M phosphate buffer pH 6.8 with 1 g of pancreatin (porcine, grade IV, reference Sigma P-1750) per liter was added to the jars. Incubation lasted for 18 h at 39 °C and finally the bags were dried in a forced draught oven at 65 °C for 48 h. The final weight after digestion of each bag was recorded for in vitro digestibility of dry matter calculation. Some of the bags was retained for nitrogen analysis and consequently for calculation of the in vitro digestibility of nitrogen. The remaining bags were subsequently subjected to incineration in a muffle furnace at a temperature of 550 °C for a duration of 5 hours. The resulting ash was utilized for the purpose of residue digestion and in vitro calculation of organic matter digestibility. The results were expressed as mean ± standard deviation of five replicate analyses.

2.8.2. Pigment Extraction from *Spirulina platensis* and *Chlorella*

To extract pigments from feed and dried *Spirulina platensis* and *Chlorella*, we used a combined method of sonication–solvent extraction followed by stirring on a magnetic stirrer [24]. Acetone solvent ratio was 1:100, w:v; sonication was performed for 30 min, and magnetic stirring was applied for 60 min. The extract was obtained by centrifugation (SIGMA 2-16KL refrigerated centrifuge) at 2599× *g* for 10 min. The resulting precipitate was extracted until no color was observed. Pigment extracts were then analyzed using a spectrum UV-Vis with wavelengths between 400 and 700 nm and absorbance at 470, 645, and 663 nm (JASCO V-670 spectrophotometer), in triplicate. The pigment levels, including chlorophyll *a* (C*a*), chlorophyll *b* (C*b*), and total carotenoids (Cc), were estimated with Equations (3)–(5). The results were reported, taking into account the dilution factor (DF) as mg/g DW (dry weight) for *Spirulina platensis* and *Chlorella vulgaris* powder and µg/g feed.

$$\mathrm{C}a = 11.24 \times A_{663} - 2.04 \times A_{645} \times \mathrm{DF} \quad (3)$$

$$\mathrm{C}b = 20.13 \times A_{645} - 4.19 \times A_{663} \times \mathrm{DF} \quad (4)$$

$$\mathrm{Cc} = \frac{(1000 \times A_{470} - 1.90 \times \mathrm{C}a - 63.14 \times \mathrm{C}b)}{214} \times \mathrm{DF} \quad (5)$$

2.8.3. Measurement of Some Antioxidant Enzyme Activity and GSH in Blood Serum

The activity of superoxide dismutase (SOD) was determined following the method described by [25]. Blood serum was added to the assay mixture containing 66 mM phosphate buffer with a pH of 7.8, 0.1 mM EDTA, 5.7 M nitro blue tetrazolium (NBT), 9.9 mM L-methionine, and 2.5% (*w*/*v*) Triton X100 and riboflavin (0.01 mL of 4.4%, *w*/*v*) was finally added to initiate the reaction. NBT reduction was measured at 560 nm in a Jasco V-670. The activity of SOD was calculated in units of enzyme/mL.

The activity of catalase (CAT) was determined by the classical method developed by [26]. CAT decomposes H_2O_2 (the substrate) and can be directly measured by decreased absorbance at 240 nm. Freshly prepared reagents prior to assays were phosphate buffer (66 mM, pH 7.0) and 30 mM H_2O_2 in a phosphate buffer. The final volume was 1 mL and the reaction was started by the addition of H_2O_2. To correct for any non-enzymatic reaction, a blank assay containing buffer instead of substrate was used. CAT activity is defined in specific units/mL.

The level of reduced glutathione (GSH) was measured according to the method described by [27] and was determined based on the reaction of GSH with 5,5′-dithiobis (2-nitrobenzoic acid). The resulting chromophore, TNB (5-thio-2-nitrobenzoic acid), has a maximum absorbance of 412 nm. The TNB formation rate is proportional to the sample GSH level. Blood serum was treated with 0.6% sulfosalicylic acid and centrifugated. The supernatant was added to the assay mixture containing 100 mM phosphate buffer with a pH of 7.5. A 3 mM stock solution of the DTNB reagent was prepared in phosphate buffer with a pH 7.5, and diluted to a final concentration of 10 µM. The reaction between GSH and DTNB was monitored at a wavelength of 412 nm using a Jasco UV/Vis V-670 spectrophotometer. The concentration of GSH in blood serum was calculated with the linear equation generated from a GSH standard curve.

Total antioxidant capacity (TAC) was analyzed by scavenging of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical activity [28]. Blood serum proteins were removed with one volume of acetonitrile, incubated for 5 min and centrifugated for 10 min at 9000× g. Supernatant (25 µL) was added to the assay mixture containing 970 µL of methanol and 5 µL of 10 mM of DPPH radical methanolic solution. After 30 min, the absorbance was read at 517 nm by a Jasco UV/Vis V-670 spectrophotometer. In parallel, a negative control with 25 µL acetonitrile, instead of deproteinated blood serum was prepared. All determinations were performed in triplicate and the serum scavenging effect (Sc%) was calculated according to Equation (6).

$$\text{Scavenging } \% = \frac{1 - A_{517\ sample}}{A_{\ 517\ negativ\ control}} \times 100 \quad (6)$$

2.8.4. Egg Yolk β-Carotene and Antioxidant Activity Determination

The β-carotene concentration of egg yolk was determined using spectroscopy method [29]. A quantity of 0.5 g of well-mixed egg yolk from each fresh or lyophilized form was taken in a 50 mL conical flask. First, 25 mL of acetone was added and the vortex was used to make a smooth paste. The solution was mixed well for 10 min and filtered (Whatman No. 1, Merck KGaA, Darmstadt, Germany). The remaining solid was re-extracted with another 20 mL of acetone using the vortex. The two filtrates were combined and the acetone extract was diluted to 50 mL. The egg yolk pigments expressed as µg β-carotene/g were measured at 450 nm wavelength (E1% 2500) using a JASCO V-670 spectrophotometer.

The total phenolic content of egg yolk samples was determined by the Folin–Ciocalteu colorimetric method [30]. The absorbance was recorded at 732 nm using a spectrophotometer (Jasco V-530, Japan Servo Co., Ltd., Tokyo, Japan). Gallic acid was used as standard solution. The total phenolic content is expressed as mg gallic acid equivalents (GAE)/ g of the sample on the basis of a standard curve of gallic acid.

The antioxidant capacity of egg yolk samples was measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity method described by [31]. The absorbance of the solution was measured at 517 nm with the help of a spectrophotometer (Jasco V-530, Japan Servo Co., Ltd., Japan). Trolox solution was used as standard. The results were expressed as mM Trolox equivalents (TE).

2.8.5. Egg Yolk Cholesterol Content and Fatty Acids Profile

The cholesterol content of dried yolk was determined using the gas chromatography (GC) method (AOAC, 1996) as described by [32]. The sample was saponified in a methanol–potassium hydroxide solution, extracted with petrol ether, concentrated using a rotavapor, and subjected to chloroform addition before being analyzed using a GC (Perkin Elmer Clarus-500, with a flame ionization detector). Separation was achieved using an HP-5 capillary column (30 m length, 0.32 mm internal diameter, 0.1 um film thickness), and the results were expressed as grams of cholesterol per whole egg. Fatty acid profile of dried yolk was determined as described by [21], using GC (Perkin ElmerClarus 500, Mass Spectrometer System) of fatty acid methyl esters (FAME) equipped with a flame ionization detector (FID) and a BPX70 capillary column (60 m × 0.25 mm ID, 0.25 µm film thickness).

The column temperature was set at 5 °C/min^{-1} ramped from 180 °C to 220 °C. The carrier gas was hydrogen (linear velocity 35 cm/s at 180 °C), and the split ratio was 1:100. The injector and detector temperatures were 250 °C and 260 °C, respectively. The results were expressed as g fatty acid per 100 g total fatty acids. The average amount of each fatty acid was used to calculate the sum of the total saturated (SFAs), total monounsaturated (MUFAs), and total polyunsaturated (PUFAs) fatty acids.

2.8.6. Color Measurement of Fresh and Boiled Eggs

Yolk color intensity was measured using a portable colorimeter as previously described by [32]. The yolk was separated from the albumen and subsequently positioned on a Petri dish (∅ = 50 mm) prior to measurement. The color parameters of L* (lightness), a* (red-green intensity), and b* (yellow-blue intensity) of the CIE-Lab system (Commission Internationale de l'Eclaraige) were determined by reflectance CIE—L* a* b* color coordinates. The instrument was calibrated with a white calibration before the measurements. All measurements were performed in triplicate.

2.9. Statistical Analysis

The results obtained from feed nutritional composition, apparent nutrient digestibility, laying hens' performances, antioxidant enzyme activity, egg quality parameters, fatty acids, and yolk cholesterol content were analyzed using a randomized complete block design and the general linear model (GLM) procedures of SAS (Statistical Analysis System, Minitab version 17, SAS Institute Inc., Cary, NC, USA) considering a cage as an experimental unit, according to the following linear model:

$$Y_{ij} = \mu + A_j + e_{ij}, \quad (7)$$

where Y_{ij} means value of trait (the dependent variable); μ, overall mean; A_j, the treatment effect; and e_{ij}, random observation error.

The effects of boiling time on fresh vs. boiled yolk color were analyzed to determine whether the factors studied (treatment and boiling time) affected the fatty acid concentration and yolk color of eggs for different time periods. The data obtained were analyzed by two-way ANOVA using the Tukey test, following the statistical model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha_i\beta_j + e_{ijk} \quad (8)$$

where Y_{ijk} = variable measured for the k^{th} observation of the i^{th} treatment and j^{th} feeding or boiling time; μ is the sample mean; α_i is the effect of the i^{th} treatment; βj is the effect of the j^{th} feeding or boiling time; $\alpha i\beta j$ is the interaction of the i^{th} treatment and j^{th} feeding or boiling time, and ε_{ijk} is the effect of error. The differences were highly significant when $p < 0.001$, significant if $p < 0.05$, and a tendency of influence was considered when $p < 0.10$.

The graphs for antioxidant enzyme activities were created using GraphPad Prism 9.1.2 software (GraphPad Software, La Jolla, CA, USA). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Nutritional Value of Chlorella and Spirulina Powder

The nutritional values of the microalgae are presented within Table 2. Both microalgae present a high nutrient content, with a high concentration of easily digestible proteins, metabolizable energy, and a low level of cellulose. Compared to chlorella, spirulina is characterized by a higher antioxidant capacity, with a significantly higher content of chlorophylls *a* and *b*. The concentration of carotenoids is high in both chlorella and spirulina. Chlorophylls are green pigments found in plants and algae. Chlorella has a chlorophyll *a* content of 5.56 mg/g and chlorophyll *b* content of 0.88 mg/g. Spirulina platensis has a higher chlorophyll content compared to chlorella, with chlorophyll *a* at 9.06 mg/g and chlorophyll *b* at 1.34 mg/g. It is known that carotenes are orange or red pigments that

serve as powerful antioxidants. Chlorella contains 1.52 mg/g of carotenes and spirulina has a slightly higher carotene content at 1.68 mg/g. Polyphenols are compounds found in plants and algae known for their antioxidant properties and potential health benefits. The total polyphenol content of chlorella was assessed to be 1.16 mg/g of total polyphenols, while spirulina has a slightly higher content of 1.35 mg/g of total polyphenols. DPPH is a common method used to evaluate the antioxidant activity of substances. Trolox is a synthetic antioxidant used as a standard for comparison. Chlorella exhibited an antioxidant capacity of 73.29% inhibition of DPPH (2,2-diphenyl-1-picrylhydrazyl) and an antioxidant capacity of 15.49 μM Trolox. On the other hand, spirulina demonstrated a higher antioxidant capacity compared to chlorella, with 81.27% inhibition of DPPH and an antioxidant capacity of 16.78 μM Trolox. Both chlorella and spirulina exhibited antioxidant activity, with spirulina generally showing higher values in terms of chlorophyll content, carotenes, total polyphenols, and antioxidant capacity. These findings suggest that spirulina demonstrated stronger antioxidant properties compared to chlorella.

Concerning the fatty acid concentrations, spirulina had a higher content of total SFAs at 51.03 g FAME/100 g Total FAME compared to chlorella, which had a total SFA content of 33.77 g FAME/100 g Total FAME. The monounsaturated fatty acids (MUFAs) of chlorella contained 9.05 g FAME/100 g Total FAME, while spirulina contained a higher content of MUFA at 27.12 g FAME/100 g Total FAME. MUFAs are considered to be healthier fats compared to SFAs. Polyunsaturated fatty acids (PUFAs) include both omega-3 and omega-6 fatty acids. Chlorella has a higher total PUFA content of 56.56 g FAME/100 g Total FAME compared to spirulina (21.85 g FAME/100 g Total FAME). The data presented suggest that chlorella has a higher content of total PUFAs, particularly PUFA n-3, compared to spirulina. On the other hand, spirulina has higher contents of SFAs, MUFAs, and PUFA n-6. The nutritional value and potential health benefits of these microalgae may be influenced by their fatty acid composition. Overall, the fatty acid profile indicates that chlorella contains a significantly higher amount of polyunsaturated fats (PUFAs), with over 67% being represented by omega-3 polyunsaturated fats, particularly α-linolenic acid (ALA).

3.2. Nutritional Value of the Experimental Diets

The nutritional content of the diets is presented in Table 3. The antioxidant activity characterized by chlorophyll a is significantly higher ($p < 0.0001$) in the SP2% group compared to the CV2% and CON groups, but the CV2% group is also significantly higher ($p < 0.0001$) compared to the CON group. Chlorophyll *b* was found in significantly higher ($p < 0.0001$) concentrations in both the CV2% and SP2% groups compared to the CON group. The antioxidant capacity was significantly higher ($p < 0.0001$) in the SP2% group compared to the CV2% and CON groups, but the CV2% group also registered a significantly higher ($p < 0.0001$) antioxidant capacity compared to the CON group. The ΣSFA was significantly higher ($p < 0.001$) in the CON group compared to the SP2% and CV2% groups. Additionally, ΣMUFA concentration was significantly higher ($p < 0.03$) in the CON group compared to the CV2% group. The ∑ PUFA concentration was observed to be significantly higher ($p < 0.003$) in the SP2% and CV2% groups compared to the CON group. The highest value concentration of Σ n-3 PUFA ($p < 0.007$) was noticed in the CV group compared to the SP2% and CON groups. Concerning the ∑ n-6 PUFA, a statistically higher concentration was determined for the CV2% and SP2% groups compared to the CON group. The ∑ n-6/∑ n-3 ratio was highly significant for the SP2% and CV2% groups compared to the CON group.

Table 2. Proximate composition, antioxidant activity, and fatty acid profile of chlorella and spirulina.

Parameters	Chlorella Powder	Spirulina Powder
	$\overline{X} \pm s_{\overline{X}}$	
Proximate composition *		
Calculated metabolizable energy (ME), MJ/kg	9.77 ± 0.41	9.68 ± 0.25
Crude protein (CP), %	51.06 ± 0.35	67.02 ± 0.04
Dry matter (DM), %	94.68 ± 0.80	92.70 ± 0.62
Organic matter (OM), %	88.33 ± 0.75	87.75 ± 0.50
Ether extract (EE), %	3.56 ± 0.50	0.48 ± 0.03
Crude fiber (CF), %	0.49 ± 0.09	0.19 ± 0.11
Non-fermentable extractive substance (NFE), %	33.22 ± 0.39	20.06 ± 0.07
Ash, %	6.35 ± 0.25	4.95 ± 0.18
In vitro nutrient digestibility **		
Digestible crude protein (DCP), %	96.59	96.71
Digestible dry matter (DDM), %	99.56	99.05
Digestible organic matter (DOM), %	99.59	99.12
Antioxidant activity *		
Chlorophyll *a*, mg/g	5.56 ± 1.08	9.06 ± 0.79
Chlorophyll *b*, mg/g	0.88 ± 0.20	1.34 ± 0.34
Carotenes, mg/g	1.52 ± 0.19	1.68 ± 0.31
Total polyphenols, mg/g GAE	1.16 ± 0.16	1.35 ± 0.05
Antioxidant capacity (DPPH % inhibition)	73.29 ± 2.93	81.27 ± 1.60
Antioxidant capacity (μM Trolox)	15.49 ± 3.87	16.78 ± 2.47
Fatty acids (g FAME/100 g Total FAME) *		
Caproic (C 6:0)	0.65 ± 0.05	0.16 ± 0.02
Caprilic (C 8:0)	0.25 ± 0.02	7.73 ± 0.65
Capric (C 10:0)	1.20 ± 0.10	-
Lauric (C 12:0)	0.07 ± 0.001	0.68 ± 0.06
Miristic (C 14:0)	0.96 ± 0.08	0.88 ± 0.07
Pentadecanoic (C 15:0)	0.11 ± 0.001	-
Palmitic (C 16:0)	27.25 ± 2.32	34.71 ± 3.12
Stearic (C 18:0)	2.51 ± 0.21	6.87 ± 0.55
Heneicosanoic (C 21:0)	0.17 ± 0.01	-
Behenic (C 22:0)	0.60 ± 0.05	-
∑ SFA	33.77 ± 3.00	51.03 ± 4.34
Pentadecenoic (C 15:1)	0.05 ± 0.004	-
Palmitoleic (C 16:1)	0.19 ± 0.02	4.87 ± 0.46
Heptadecenoic (C 17:1)	0.08 ± 0.007	-
Oleic cis (C 18:1)	8.73 ± 0.72	22.25 ± 2.14
∑ MUFA	9.05 ± 0.19	27.12 ± 1.3
Linoleic cis (C 18:2n6)	14.13 ± 1.20	16.84 ± 1.43
Linolenic γ (C 18:3n6)	0.16 ± 0.01	-
Linolenic α (C 18:3n3)	37.37 ± 3.18	4.41 ± 0.39
Octadecatetraenoic (C18:4n3)	0.73 ± 0.06	-
Eicosadienoic (C20:2n6)	0.33 ± 0.03	-
Eicosatrienoic (C20:3n6)	3.61 ± 0.31	-
Arachidonic (C20:4n6)	0.21 ± 0.01	0.60 ± 0.05
∑ PUFA	56.56 ± 4.80	21.85 ± 1.94
∑ PUFA n-3	38.10 ± 3.65	4.41 ± 0.41
∑ PUFA n-6	18.45 ± 1.77	17.44 ± 1.48
∑ PUFA n-6/∑ PUFA n-3	0.48 ± 0.05	3.95 ± 0.35
Other fatty acids	0.62 ± 0.05	-

Where: ∑SFA, sum of saturated fatty acid; ∑MUFA, sum of monounsaturated fatty acid; ∑P sUFA, sum of polyunsaturated fatty acid; * Mean ± standard deviation of three replicate analyses; ** Mean ± standard deviation of five replicate analyses.

Table 3. Nutritional compounds of the feed (% as fed).

Parameters	Dietary Treatments			SEM	*p*-Value
	CON	CV2%	SP2%		
Antioxidant activity					
Chlorophyll a, µg/g	10.31 ^{c}	344.76 ^{b}	383.22 ^{a}	5.41	<0.0001
Chlorophyll b, µg/g	2.77 ^{b}	40.93 ^{a}	38.92 ^{a}	1.44	<0.0001
Carotenes, µg/g	31.45 ^{b}	84.92 ^{a}	81.70 ^{a}	1.08	<0.0001
Total polyphenols, mg/g GAE	1.49	1.67	1.64	0.177	0.933
Antioxidant capacity (DPPH % inhibition)	32.00 ^{c}	57.57 ^{b}	67.23 ^{a}	1.23	<0.0001
Antioxidant capacity (µM Trolox)	5.19	6.25	5.79	0.403	0.620
Fatty acid composition (% of total fat)					
ΣSFA	29.03 ^{a}	19.72 ^{b}	22.44 ^{b}	0.893	0.001
ΣMUFA	47.42 ^{a}	37.06 ^{b}	40.12 ^{ab}	2.06	0.030
∑ PUFA, from which:	23.16 ^{b}	42.95 ^{a}	37.11 ^{a}	2.36	0.003
Σ n-3 PUFA	1.03 ^{b}	1.43 ^{a}	1.21 ^{ab}	0.06	0.007
∑ n-6 PUFA	22.14 ^{b}	41.52 ^{a}	35.90 ^{a}	2.32	0.003
∑ n-6/∑ n-3	21.58 ^{b}	29.18 ^{a}	29.47 ^{a}	1.22	0.006

Where: CON, conventional diet; CV2%, conventional diet supplemented with 2% chlorella powder; SP2%, conventional diet supplemented with 2% spirulina powder; n = 5 samples per group; SEM, standard error of the mean; ^{abc} Mean values within a row with different letters are significantly different at $p \leq 0.05$. Abbreviations: ΣSFA, sum of saturated fatty acids; ∑MUFA, sum of monounsaturated fatty acid; ΣPUFA, sum of polyunsaturated fatty acids; Σ n-3 PUFA = C18:3n-3 + C18:4n-3; Σ n-6 PUFA = C18:2n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6.

3.3. Production Performances

The production performance values are shown within Table 4. There were no significant differences concerning the initial or final body weight (p = 0.580; p = 0.688) of hens in the CON, CV2%, and SP2% groups. The feed conversion ratio (g feed/g egg) registered the optimum value in the SP2% group compared to CON and CV2% ($p < 0.0001$). There was no significant difference in the daily feed intake among the dietary treatments (p = 0.608). The mean daily feed intake was 112.78 g/day/layer for CON, 112.40 g/day/layer for CV2%, and 113.46 g/day/layer for SP2%. A highly significant difference ($p < 0.0001$) was noticed in the feed conversion ratio between all groups. There was a highly significant difference ($p < 0.0001$) concerning egg weight among the dietary treatments. The mean egg weight was 59.78 g for CON, 62.42 g for CV2%, and 62.44 g for SP2%. There was a significant difference (p = 0.046) in the hen day egg production among the dietary treatments; the highest percentage was registered for the SP2% group (95.56%) compared to CON and CV2%. There were significant differences in the percentages of eggs classified into different sizes (XL, L, M, and S) categories among the dietary treatments ($p < 0.0001$). The data show that the proportions of eggs in each size category varied among the treatment groups, with significant differences observed for all categories ($p < 0.0001$). The CV2% group presented a higher percentage of eggs classified as "M" (medium) compared to the SP2% and CON groups. This suggests that chlorella supplementation might have influenced egg size distribution, potentially leading to a significantly higher proportion of medium-sized eggs ($p < 0.0001$). The SP2% group showed a significantly higher ($p < 0.0001$) percentage of eggs classified as "L" (large) compared to the CV2% and CON groups.

3.4. Serum Antioxidant Status

The effect of experimental diets CV2% and SP2% on the blood antioxidant enzymes SOD, CAT, GSH, and TAC activity is presented within Figure 1. A significant increase ($p < 0.001$; $p < 0.0001$) of SOD concentration in serum of laying hens fed with microalgae was noticed compared to the CON group. Also, a highly significant difference ($p < 0.001$) was registered between experimental groups, where SP2% was characterized by an increased antioxidant activity which assumes a high antioxidant status of this group. The chlorella and spirulina powder diet supplementations improved the enzymatic activity of CAT,

increasing ($p < 0.01$) its serum concentration value significantly compared to CON. There were no differences ($p \geq 0.05$) noticed between the two experimental groups.

Table 4. Performances of laying hens fed with *Ch. vulgaris* and *Sp. platensis*.

Parameters	Dietary Treatments			SEM	*p*-Value
	CON	CV (2%)	SP (2%)		
Initial body weight (g/layer)	1561.30	1599.17	1556.88	23.23	0.580
Final body weight (g/layer)	1670.68	1681.52	1643.96	23.63	0.688
Daily feed intake (g/day/layer)	112.78	112.40	113.46	0.441	0.608
Feed conversion ratio (g feed/g egg)	2.04 ^{b}	2.17 ^{a}	1.92 ^{c}	0.024	<0.0001
Egg weight (g)	59.78 ^{b}	62.42 ^{a}	62.44 ^{a}	0.110	<0.0001
Hen day egg production (%)	94.61 ^{ab}	93.32 ^{b}	95.56 ^{a}	0.412	0.046
Egg classification *, %					
"XL" (>73 g), extra large	0.15 ^{b}	2.99 ^{a}	2.08 ^{a}	0.350	<0.0001
"L" (63–73 g), large	22.87 ^{b}	38.50 ^{a}	39.65 ^{a}	1.01	<0.0001
"M" (53–63 g), medium	71.22 ^{a}	57.321 ^{b}	56.50 ^{b}	1.03	<0.0001
"S" (<53 g), small	5.76 ^{a}	1.19 ^{b}	1.77 ^{b}	0.346	<0.0001

Where: CON, conventional diet; CV2%, conventional diet supplemented with 2% chlorella powder; SP 2%, conventional diet supplemented with 2% spirulina powder; * C.E Regulation no. 852/2004 on the general rules of food hygiene with subsequent amendments and completions and Directive 2000/13 / C.E.; SEM, standard error of the mean. ^{abc} Mean values within a row with different letters are significantly different at $p \leq 0.05$.

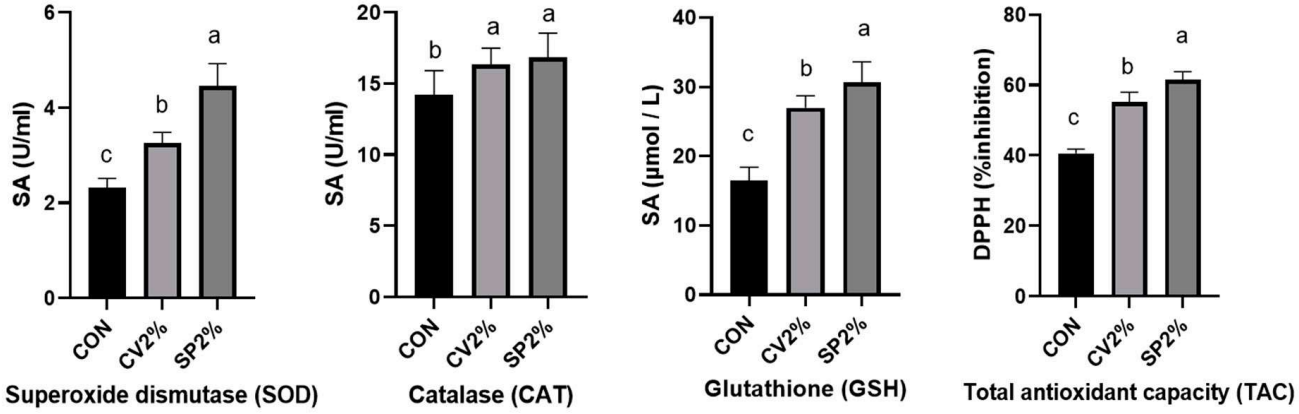

Figure 1. Effect of chlorella (CV2%) and spirulina powder (SP2%) supplementation on the enzymatic specific activity (SA) of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and total antioxidant capacity (TAC), expressed as U /l or % inhibition of sanguine serum; ($n = 6$). ^{abc} Mean values within a column having different letters are significantly different at $p \leq 0.05$.

GSH serum concentration significantly increased ($p < 0.0001$) in the SP2% and CV2% groups compared to CON. Also, significant statistically differences ($p < 0.05$) were observed between the experimental groups. The same trend was noticed for total antioxidant capacity value from serum, but with highly statistical differences ($p < 0.001$) between the two experimental groups.

3.5. Digestibility Trial

The coefficients of apparent nutrient digestibility of laying hens fed with chlorella and spirulina powder diets are presented within Table 5. The supplementation of the conventional diet with chlorella or spirulina powder had no effect on the digestibility of dry matter, organic matter, crude protein, or digestible NFE because there were no significant differences ($p > 0.05$) between the CON diet and algae-supplemented diets. We registered only a tendency for crude fat digestibility to decrease just for chlorella powder.

Table 5. Apparent nutrient digestibility (%) of laying hens fed with *Ch. vulgaris* and *Sp. platensis* powder diets.

Parameters	Dietary Treatments			SEM	*p*-Value
	CON	CV2%	SP2%		
Digestible dry matter (DDM),%	73.17	71.29	72.61	0.835	0.288
Digestible organic matter (DOM), %	73.05	70.52	71.62	0.806	0.112
Digestible crude protein (DCP), %	85.81	85.20	84.85	0.607	0.539
Digestible crude fat (DCF), %	90.58 ^{a}	87.82 ^{b}	89.07 ^{ab}	0.740	0.052
Digestible non-fermentable extractive substance (DNFE), %	68.60	66.27	67.59	0.768	0.128

Where: CON, conventional diet; CV2%, conventional diet supplemented with 2% chlorella powder; SP2%, conventional diet supplemented with 2% spirulina powder; n = 6 samples per group; SEM, standard error of the mean. ^{ab} Mean values within a row having different letters are significantly different at $p \leq 0.05$.

3.6. Nutritional Egg Quality Parameters

Table 6 presents the effect of dietary chlorella and spirulina powder on laying hens' egg nutrients and external/internal quality parameters. The microalgae dietary supplementation influenced the nutritional egg quality positively. The β-carotene concentration registered a highly significant increase ($p < 0.0001$) in experimental diets, the results being positively correlated with the concentration of chlorophylls *a* and *b* and carotene of both dietary microalgae. The same observation could be noted for both antioxidant capacity expressed as DPPH% inhibition ($p < 0.0001$) and μM Trolox ($p < 0.033$).

Table 6. Effect of dietary *Ch. vulgaris* and *Sp. platensis* powder in laying hens' diets on egg yolk nutrients and external and internal egg quality parameters.

Parameters	Dietary Treatments			SEM	*p*-Value
	CON	CV2%	SP2%		
Nutrition quality of egg yolk					
β-carotene, (μg/g)	30.77 ^{b}	38.82 ^{a}	38.25 ^{a}	0.432	<0.0001
Total polyphenols (mg/g GAE)	0.534	0.598	0.574	0.027	0.271
Antioxidant capacity (DPPH% inhibition)	16.84 ^{b}	25.14 ^{a}	28.15 ^{a}	0.869	<0.0001
Antioxidant capacity (μM Trolox)	0.74 ^{b}	0.79 ^{a}	0.82 ^{a}	0.019	0.033
External and internal egg quality parameters					
Egg weight (g), of which:	61.28	61.53	61.82	0.389	0.617
albumen white (g)	37.00	37.76	37.38	0.352	0.320
egg yolk (g)	16.37	15.65	16.26	0.242	0.087
eggshell (g)	7.91	8.12	8.17	0.137	0.358
Albumen pH (value)	8.62 ^{ab}	8.44 ^{b}	8.80 ^{a}	0.069	0.002
Yolk pH (value)	6.51	6.38	6.46	0.093	0.625
t° albumen (°C)	19.10 ^{a}	18.57 ^{ab}	17.79 ^{b}	0.258	0.003
t° yolk (°C)	19.99 ^{a}	19.12 ^{b}	19.92 ^{a}	0.134	0.011
White height, mm	10.96	11.74	11.48	0.292	0.168
Haugh units (value)	102.92	106.04	104.82	1.150	0.163
Yolk color, (value)	4.06 ^{c}	8.11 ^{b}	11.06 ^{a}	0.113	<0.0001

Where: CON, conventional diet; CV2%, conventional diet supplemented with 2% chlorella powder; SP2%, conventional diet supplemented with 2% spirulina powder; n = 6 samples per group (3 eggs/ sample); SEM, standard error of the mean. ^{abc} Mean values within a row having different letters are significantly different at $p \leq 0.05$.

Concerning the egg quality parameters, no effects ($p > 0.05$) of chlorella or spirulina powder were observed on egg weight or its components (albumen, yolk, and shell). The albumen pH was influenced by the presence of algae compared to the CON diet: lower value for chlorella diet, and higher value for spirulina diet. The yolk pH and the albumen height were not influenced by the dietary treatment. The Haugh unit, the most widely accepted

indicator of internal egg quality, had an increasing tendency in eggs obtained from algae supplementation diets, especially for the chlorella diet. The egg yolk color in groups fed diets with chlorella or spirulina were significantly higher ($p < 0.0001$) compared to the CON diet.

3.7. Yolk Cholesterol Content and Fatty Acid Profile

Table 7 presents the effects of chlorella and spirulina powder dietary inclusion on egg yolk cholesterol content and fatty acid profile. There were no statistical differences ($p = 0.061$) concerning cholesterol content groups. When examining the fatty acid composition, there were variations observed among the different experimental groups depending on how the results were reported. For the health of consumers, it is important that results are be expressed as mg fatty acids/egg. Concerning the total SFAs ($p = 0.825$) and the total MUFAs ($p = 0.280$), there were no differences registered. Regarding polyunsaturated fatty acids (PUFAs), expressed as mg fatty acids/egg, there were no differences ($p = 0.102$) between groups, even though when expressed as g FAME/ 100 g Total FAME, both CV2% and SP2% groups led to a decrease in the overall amount of PUFAs compared to the control group. The highest ($p < 0.0001$) concentration of omega-3 was observed in the chlorella group (1.72 g FAME/100 g Total FAME; 25.001 mg fatty acids/egg) compared to spirulina (1.21 g FAME/100 g Total FAME; 18.364 mg fatty acids/egg) and control groups (1.23 g FAME/100 g Total FAME; 18.887 mg fatty acids/egg), which influenced the Ω6/Ω3 ratio; highly significantly ($p < 0.0001$) lower for the chlorella group.

3.8. The Effect of Dietary Chlorella and Spirulina Powder on Egg Yolk Color in Fresh and Boiled Eggs

Table 8 presents the effects of chlorella and spirulina powder dietary inclusion on egg yolk color in fresh and boiled eggs. The treatment factor (CON, CV2%, SP2%) exhibited a significant effect on the L*, a*, and b* parameters. The *p*-values registered for all three parameters indicated a highly significant difference ($p < 0.0001$) between the treatments. The boiling time factor (fresh yolk, 10, 20, and 30 min.) also had a highly significant effect ($p < 0.0001$) on the L*, a*, and b* parameters, which indicated that the boiling time had a significant impact on the egg yolk color. The interaction between treatment and boiling time showed highly significant ($p < 0.0001$) effects on the a* and b* parameters. However, the interaction effect was not statistically significant for the L* parameter ($p = 0.293$). The L* parameter represents the lightness of the color, and therefore higher values indicate lighter or brighter colors, while lower values indicate darker colors. Comparing the values across different treatments and boiling times, it can be observed that as the boiling time increased, the L* values showed a tendency to increase as well. This suggests that the egg yolks become lighter in color as they are boiled for a longer duration. The darkest color values were noticed for SP2%, followed by CV2% ($p < 0.0001$). The a* parameter values (red-green color axis, positive values indicate redness, negative values indicate greenness) varied across different treatments and boiling times. The highest value for a* parameters was noticed on SP2%, followed by CV2% ($p < 0.0001$), compared to CON. However, there was no consistent trend concerning boiling time. The interaction effect between treatment and boiling time was highly significant ($p < 0.0001$), indicating that the combination of treatment and boiling time influenced the red-green color component. Positive values indicate more yellowness, while negative values indicate more blueness. Similar to the a* parameter, the b* values also varied across treatments and boiling time. The b* parameter (yellow-blue color) registered the highest value on CV2%, followed by SP2% ($p < 0.0001$), compared to CON. The interaction effect between treatment and boiling time was highly significant ($p < 0.0001$), suggesting that the combination of treatment and boiling time affects the yellow-blue color component.

Table 7. Fatty acid composition in total lipids of egg yolks (average values/group).

	Experimental Groups									
Parameters	**CON**	**CV2%**	**SP2%**	**SEM**	***p*-Value**	**CON**	**CV2%**	**SP2%**	**SEM**	***p*-Value**
Yolk fat (% DM)	29.454	29.570	30.412	0.771	0.640					
Cholesterol (mg col/egg)	224.00	173.00	192.00	0.013	0.061					
Fatty acid content	g FAME/100 g Total FAME					mg fatty acid/egg				
Miristic C14:0	0.308 ^{b}	0.346 ^{a}	0.346 ^{a}	0.008	0.005	4.716	5.063	5.266	0.196	0.167
Pentadecanoic C15:0	0.060	0.140	0.060	0.052	0.399	0.932	2.182	0.918	0.760	0.423
Palmitic C16:0	25.44	26.14	26.05	0.242	0.121	389.9	380.2	395.0	13.500	0.738
Heptadecanoic C17:0	0.128 ^{b}	0.128 ^{b}	0.145 ^{a}	0.003	0.001	1.974 ^{ab}	1.869 ^{b}	2.227 ^{a}	0.093	0.043
Stearic C18:0	10.09	10.11	9.61	0.232	0.256	154.71	146.93	145.79	5.990	0.533
∑SFA	36.03	36.87	36.22	0.409	0.339	552.2	536.3	549.2	19.20	0.825
Miristioleic C14:1	0.079 ^{c}	0.092 ^{b}	0.108 ^{a}	0.003	<0.0001	1.217 ^{b}	1.345 ^{b}	1.645 ^{a}	0.066	0.001
Pentadecenoic C15:1	0.100	0.101	0.417	0.184	0.394	1.53	1.46	6.35	2.810	0.392
Palmitoleic C16:1	4.37	3.98	4.34	0.421	0.765	65.58	57.95	65.86	5.170	0.488
Heptadecenoic C17:1	0.069 ^{b}	0.075 ^{b}	0.089 ^{c}	0.003	0.002	1.056 ^{b}	1.095 ^{b}	1.358 ^{a}	0.070	0.016
Oleic C18:1	36.09 ^{b}	36.80 ^{ab}	37.59 ^{a}	0.406	0.050	552.8	535.8	570.1	20.100	0.499
Erucic C22 (1n9)	0.121 ^{a}	0.120 ^{a}	0.100 ^{b}	0.004	0.002	1.854 ^{a}	1.456 ^{b}	1.819 ^{a}	0.077	0.004
Nervonic C24 (1n9)	0.277	0.279	0.272	0.006	0.640	4.246	4.063	4.128	0.161	0.724
∑MUFA	41.11	41.43	42.94	0.508	0.050	628.3	603.2	651.2	20.4	0.280
Linoleic C18:2	15.66 ^{a}	14.27 ^{b}	13.97 ^{b}	0.108	<0.0001	239.94 ^{a}	207.67 ^{b}	211.94 ^{ab}	8.320	0.031
Linolenic γ C18:3n6	0.126 ^{b}	0.134 ^{ab}	0.138 ^{a}	0.002	0.0007	1.931	1.944	2.089	0.080	0.326
Linolenic α C18:3n3	0.218 ^{b}	0.319 ^{a}	0.233 ^{b}	0.017	0.001	3.343 ^{b}	4.651 ^{a}	3.541 ^{b}	0.283	0.011
Eicosadienoic C20 (2n6)	0.229 ^{b}	0.232 ^{b}	0.269 ^{a}	0.007	0.002	3.515	3.389	4.084	0.190	0.046
Eicosatrienoic C20 (3n6)	0.290 ^{a}	0.257 ^{b}	0.289 ^{a}	0.005	<0.0001	4.451 ^{a}	3.743 ^{b}	4.393 ^{ab}	0.178	0.023
Eicosatrienoic C20 (3n3)	0.252 ^{b}	0.234 ^{b}	0.274 ^{a}	0.006	0.001	3.866 ^{ab}	3.401 ^{b}	4.154 ^{a}	0.172	0.023
Arachidonic C20 (4n6)	3.613 ^{a}	3.548 ^{a}	3.178 ^{b}	0.093	0.010	55.420	51.570	48.230	2.34	0.129
Docosatetraenoic C22 (4n6)	1.501 ^{a}	1.318 ^{b}	1.537 ^{a}	0.043	0.005	23.01 ^{a}	19.16 ^{b}	23.32 ^{a}	1.01	0.019
Docosapentaenoic C22 (5n3)	0.071 ^{b}	0.109 ^{a}	0.071 ^{b}	0.003	<0.0001	1.087 ^{b}	1.593 ^{a}	1.073 ^{b}	0.054	<0.0001
Docosahexaenoic C22 (6n3)	0.691 ^{a}	1.056 ^{b}	0.632 ^{b}	0.021	<0.0001	10.591 ^{b}	15.357 ^{a}	9.595 ^{b}	0.483	<0.0001
∑PUFA	22.65 ^{a}	21.45 ^{b}	20.59 ^{c}	0.183	<0.0001	347.2	312.5	312.4	12.30	0.102
∑Ω3	1.23 ^{b}	1.72 ^{a}	1.21 ^{b}	0.024	<0.0001	18.887 ^{b}	25.001 ^{a}	18.364 ^{b}	0.784	<0.0001
∑Ω6	21.42 ^{a}	19.76 ^{b}	19.39 ^{b}	0.168	<0.0001	328.270	294.059	287.475	11.5	0.053
∑Ω6/Ω3	17.40 ^{a}	11.51 ^{c}	16.06 ^{b}	0.228	<0.0001	266.27 ^{a}	167.44 ^{b}	243.36 ^{a}	8.59	<0.0001
Other fatty acids	0.207	0.214	0.245	0.025	0.517	3.164	3.108	3.749	0.393	0.460

Where: CON, conventional diet; CV2%, conventional diet supplemented with 2% chlorella powder; SP (2%), conventional diet supplemented with 2% spirulina powder; n = 6 samples per group (3 eggs/sample); SEM, standard error of the mean; ^{abc} Mean values within a row with different letters are significantly different at $p < 0.05$.

Table 8. Effects of chlorella and spirulina powder on egg yolk color in fresh and boiled eggs.

Yolk Color Parameter		**L***	**a***	**b***
CON	fresh yolk	45.76 ^{f}	1.321 ^{e}	14.03 ^{f}
	10 min boiling time	79.94 ^{e}	−1.389 ^{g}	19.32 ^{ef}
	20 min boiling time	104.33 ^{c}	−3.836 ^{h}	23.46 ^{de}
	30 min boiling time	125.97 ^{a}	−5.514 ^{i}	25.65 ^{d}
CV2%	fresh yolk	43.82 ^{f}	3.156 ^{d}	17.74 ^{ef}
	10 min boiling time	76.56 ^{e}	0.883 ^{e}	33.14 ^{c}
	20 min boiling time	100.05 ^{cd}	0.073 ^{ef}	51.60 ^{a}
	30 min boiling time	122.15 ^{a}	−1.070 ^{fg}	48.21 ^{a}
SP2%	fresh yolk	40.49 ^{f}	4.924 ^{c}	14.18 ^{f}
	10 min boiling time	74.86 ^{e}	6.414 ^{ab}	32.16 ^{c}
	20 min boiling time	95.11 ^{d}	7.601 ^{a}	39.83 ^{b}
	30 min boiling time	116.46 ^{b}	5.591 ^{bc}	49.31 ^{a}
Main effect				
Treatment	CON	88.99 ^{a}	−2.354 ^{c}	20.61 ^{c}
	CV2%	85.65 ^{b}	0.761 ^{b}	37.68 ^{a}
	SP2%	81.73 ^{c}	6.132 ^{a}	33.87 ^{b}
	$SEM_{treatment}$	0.583	0.151	0.653
Boiling time	fresh yolk	43.36 ^{d}	3.134 ^{a}	15.318 ^{d}
	10 min boiling time	77.12 ^{c}	1.969 ^{b}	28.205 ^{c}
	20 min boiling time	99.83 ^{b}	1.279 ^{c}	37.897 ^{b}
	30 min boiling time	121.53 ^{a}	−0.331 ^{d}	41.457 ^{a}
	$SEM_{boiling\ time}$	0.682	0.177	0.764
p-Value				
Treatment		<0.0001	<0.0001	<0.0001
Boiling time		<0.0001	<0.0001	<0.0001
Treatment × Boiling time		0.293	<0.0001	<0.0001

Where: CON, conventional diet; CV2%, conventional diet supplemented with 2% chlorella powder; SP 2%, conventional diet supplemented with 2% spirulina powder; SEM, standard error of the mean. $^{a–i}$ Mean values within a column with different letters are significantly different at $p \leq 0.05$.

4. Discussion

From a nutritional point of view, the two microalgae (chlorella and spirulina) are considered food additives with high biological value due to their nutrient concentration. The results of our study analyses strongly indicate that spirulina shows higher antioxidant properties, carotenoid levels, polyphenols, and a superior DPPH inhibition, when compared to chlorella, which suggests that spirulina has a greater capacity to combat oxidative stress. On the other hand, the microalgae proximal composition showed that chlorella had a higher concentration of PUFA, particularly omega-3 fatty acids and a higher omega-3 content, and lower ∑ PUFA n-6/∑ PUFA n-3 ratio compared to spirulina.

Other authors confirm that the microalgae contain the highest protein value with an excellent essential amino acid profile [33,34], bioactive compounds, PUFA fatty acids, polysaccharides, volatile and phenolic compounds, vitamins, sterols, and natural pigments [35]. The high levels of carotenoids and fatty acids, especially α-linolenic, are associated with health benefits and nutrition [36]. The microalgae utilization in animal feed improves productive performance, the immune system, antioxidant activity, and tissue regeneration [35]. Other authors [37] found a concentration of 3.291 mg/L chlorophyll *a*, 1.174 mg/L chlorophyll *b*, 4.466 mg/L total chlorophyll, and 0.919 mg/L carotenoids in blue-green algae spirulina. Abou-El-Souod et al. [38] stated that chlorella possesses chloroplasts that contain green photosynthetic pigments called chlorophylls *a* and *b*. Utilizing the process of photosynthesis, it exhibits rapid growth and multiplication by utilizing carbon dioxide, water, sunlight, and a minimal amount of minerals. Similar findings to our results have been reported in other studies investigating the antioxidant activity and fatty acid composition of spirulina and chlorella. Khan et al. [39] found that spirulina exhibited significantly higher antioxidant activity compared to the control group. The presence of active compounds such as phycocyanin and beta-carotene in spirulina contributed to its strong antioxidant potential. In a study, Stunda-Zujeva et al. [40] stated that phycocyanin is the main antioxidant of spirulina, offering various uses for health benefits, although care should be taken in terms of the antioxidant activity, which fluctuates. Numerous studies have highlighted the higher antioxidant capacity and beneficial fatty acid profiles, including higher concentrations of omega-3 polyunsaturated fatty acids, in both microalgae species compared to control groups. These fatty acids are known for their beneficial effects on human health, including cardiovascular health and anti-inflammatory properties. Another study by [41] investigated the fatty acid profiles of microalgae species and found that both spirulina and chlorella exhibited higher concentrations of omega-3 fatty acids, particularly ALA, compared to the control group. They also noted that these microalgae species had lower levels of saturated fatty acids, contributing to a more desirable fatty acid composition. Other researchers [42] evaluated the fatty acid composition of spirulina and highlighted its high content of gamma-linolenic acid (GLA), an omega-6 fatty acid with anti-inflammatory properties.

Our research revealed that adding chlorella and spirulina to the laying hens' diet at a 2% inclusion rate did not have a significant impact on initial or final body weight. Nevertheless, the group supplemented with spirulina demonstrated enhanced feed conversion efficiency, larger eggs, and higher rates of egg production compared to the control and chlorella groups. This suggests that dietary supplementation with spirulina could have more pronounced positive effects on egg production efficiency and size uniformity, with practical benefits for egg producers and consumer health. These findings are similar to those of other studies which studied different microalgae sources and inclusion levels and noticed an improved production parameter when including microalgae in poultry diets due to the high protein content, essential amino acids, vitamins, and minerals present in spirulina and chlorella. Additionally, the presence of certain bioactive compounds and antioxidants in microalgae may have positive effects on production performance. Mariey et al. [43] included four levels of spirulina powder (0, 0.10, 0.15, or 0.20%) in laying hens' diet and registered an improved egg production rate, daily egg mass, and feed conversion ratio compared to those of the control group. Shanmugapriya and Saravanababu [44] tested spirulina on broilers and found a significant increase in body weight.

Other studies have [45] supplemented the basal diet of laying hens raised under a chronic hot ambient temperature with spirulina powder (0.15 mg/kg diet) and seleno-methionine (0.10 mg/kg diet). The obtained results indicated that dietary spirulina and organic selenium showed improved productive performance under heat stress. In contrast, the chlorella supplementation at varying dosages of 2.5 g, 5.0 g, or 7.5 g per kg feed, in both spray-dried and bullet-milled/spray-dried forms, did not result in any impact on laying intensity, egg weight, daily egg mass production, or feed conversion. However, it was observed that the treatment groups exhibited an increase in yolk weight and an improvement in egg quality [46].

In a study conducted by Omri et al. [47], laying hens at 44 weeks of age were fed with diets containing 1.5% and 2.5% spirulina for a period of 6 weeks. The results indicated that the inclusion of 2.5% spirulina in the diet significantly increased egg weight. However, no significant effects were observed on other productive parameters, including dietary treatment, duration of the diet, or their interaction.

Concerning the antioxidant enzyme activity, the results obtained in our study showed that chlorella and spirulina dietary addition exhibited significant improvements in blood antioxidant enzyme activities (SOD, CAT, GSH) and total antioxidant capacity (TAC). Moreover, the increased serum levels of GSH and TAC in both experimental diets demonstrate and support the idea that the microalgae-supplemented diets positively influenced the hens' antioxidant status compared to the control group. The main antioxidant enzymes, such as SOD, CAT, and GSH, protect the organism against oxidative stress [48], improving the poultry immune system [49]. CAT is one of the most important antioxidant enzymes which mitigates oxidative stress via the catalysis of hydrogen peroxide [50]. Park et al. [51] obtained the same linearly increased GPx and SOD enzymes in broilers fed with spirulina and explained that this was due to the fact that spirulina contains antioxidants such a β-carotene, tocopherol, selenium, polypeptide pigment, or phenolic acids. Wu et al. [52] suggested that spirulina has stronger antioxidant capabilities than chlorella, which is probably due to the higher content of phenolic compounds.

Utilization of microalgae in laying hens' diet had no effect concerning the apparent digestibility coefficients. Our results are similar to those of [53], who reported that the incorporation of green seaweed (*Ulwa spp*.) meal between 20 and 35 g/kg in Boschveld hens' diets did not alter apparent nutrient digestibility.

Additional research [19] indicated that the inclusion of brown seaweed meal derived from (*Ecklonia maxima*) into the diet of Boschveld cockerels did not have a significant impact on the digestibility of dry matter, organic matter, crude protein, and fiber. This result was observed despite the seaweed inclusion rate ranging from 2 to 8 g/kg.

In our experiment, we obtained a high β-carotene content and increased antioxidant capacity of the yolk, which represents indicators of an improved egg quality, with potential health-promoting effects for consumers. Omri et al. [47] observed no effect ($p > 0.05$) on total cholesterol concentration when using spirulina (1.5% and 2.5%) in laying hen diets.

The dietary microalgae supplementation had no influence on egg quality parameters (egg weight and its components). Similar results on egg weight were observed by [46] using chlorella supplementation in laying hens (26-week-old) diets. Other authors, such as [54], used chlorella supplementation in Hy-Line brown laying hens, aged 70 weeks, without any effects on egg weight, but registered the highest Haugh units when supplementing diets with 2.4% liquid chlorella in their study.

Our data indicate that the dietary treatments of chlorella and spirulina influenced the yolk coloration, spirulina having a more pronounced effect on enhancing red color. Additionally, longer boiling times result in darker and lower/more negative values for a* (greenish-gray ring) and higher/more positive values for b*.

In other studies [34,46,54], both chlorella- and spirulina-supplemented diets were confirmed to increase the color of yolk by lutein dosing.

The intensity of yolk color can vary depending on the types and concentrations of carotenoids consumed by the laying hens. Englmaierová et al. [55], using chlorella at

12.5 g/kg, noticed a significant intensification of the yellowness of fresh yolk. In the case of boiled eggs, a statistically significant increase in redness was observed. Conversely, an extension of the boiling duration to 10 min resulted in an increase in lightness and a concomitant reduction in yolk coloration.

The L* value for fresh yolk indicates that the color of the fresh yolk has a moderately bright appearance. As the boiling time increased, the L* values also increased. The L* value for the 10 min. boiling indicated that the boiled yolk became significantly brighter ($p \leq 0.0001$) compared to the fresh yolk. The L* value increased progressively for the 20 min. and 30 min. boiling times, respectively; yolks became lighter as they were boiled for longer durations. The differences in L* values between the boiling time highlight the effect of heat exposure on the lightness of the yolks. This change in lightness can be attributed to structural and chemical transformations that occur during the cooking process, causing the denaturation of the proteins and altering the protein molecules. As a result, the yolks appear brighter or lighter in color [56]. According to Muñoz-Miranda and Iñiguez-Moreno [57], marine biopigments can be categorized into three main groups: chlorophylls, carotenoids, and phycobiliproteins. The rich carotenoid concentration of the pigments zeaxanthin, xanthophylls, and β-carotene offer different greenish, green, golden, red, and brown colors of algae [58]. Other authors [59] tested, in a short-term study, the effects of 1% and 3% spirulina supplementation on color, nutritional value, and stability of yolk. A decreased luminosity and increased redness ($p = 0.0001$) and yellowness ($p = 0.0103$) were observed for 1% supplementation, after only 15 experimental days, meaning that the high carotenoid levels present in spirulina are efficiently absorbed by the laying hens' gastrointestinal tract [60].

Dietary supplementation with chlorella at 1% and 2% levels on Hisex Brown laying hens aged 56 weeks revealed a significant increase in total carotenoid deposition by 46% and 119% for the 1% and 2% chlorella groups, respectively. This increase was accompanied by a significant improvement in the yolk egg color, as evidenced by the Roche Fan Yolk Color grade, which registered 5.0 and 6.1 for the 1% and 2% chlorella groups, respectively, compared to 4% for the control group ($p < 0.001$). These findings suggest that chlorella dietary supplementation can enhance the carotenoid content and improve the color of yolks in laying hens.

Omri et al. [47] obtained increases in egg yolk redness from 1.33 (C) to 12.67 (1.5% spirulina) and 16.19 (2.5% spirulina), and a significant yellowness (b*) reduction parameter from 62.1 (C) to 58.17 (1.5% spirulina) and 55.87 (2.5% spirulina). Overall, the yolks from the experimental diets were highly significantly ($p < 0.0001$) darker, exhibited a stronger red color, and had reduced yellowness compared to the yolks from the control group. Other studies [43] tested 0.1%, 0.15%, and 0.2% spirulina in laying hens' diet and observed increasing yolk color scores (RYCF) of 6.3, 6.7, and 7.6, respectively. Similarly, supplementation levels of 1.5%, 2%, and 2.5% of spirulina were tested by [61] and obtained significant yolk intensifications of 10.55, 11.43, and 11.66, respectively, compared to the control.

The present study also investigated the effectiveness of microalgae in enhancing the fatty acid composition of eggs, specifically through the increased presence of docosahexaenoic acid (DHA). The process of enriching eggs with omega-3 polyunsaturated fatty acids (n-3 PUFA) from dietary sources is gradual and requires time. However, achieving sufficient enrichment of eggs with these beneficial fatty acids is economically significant for the industry. The n-6/n-3 PUFA ratio reflected diet composition, with the ratio being lower for the eggs of the hens fed microalgae. Some studies consider that many salt and fresh-water microalgae, including spirulina, contain high concentrations of n3-long-chain polyunsaturated fatty acids (PUFA) (25–38%), including α-linoleic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), which are anti-inflammatory and cardiovascular- and brain-protective [39,62]. Microalgae, due to their high concentrations of n-3 PUFA, present an exceptional n-6/n-3 PUFA ratio [39,62]. Studies have demonstrated that laying hens fed with microalgae-enriched diets produce DHA-enriched eggs [63–65].

5. Conclusions

In conclusion, the supplementation of laying hens' diet with chlorella and spirulina at a concentration of 2% each has demonstrated several positive effects on egg quality and nutritional content. This study has revealed significant improvements in egg weight, size, yolk intensity color, beta carotene content, and antioxidant capacity. Furthermore, the incorporation of chlorella has led to a noteworthy increase in omega-3 polyunsaturated fatty acids, resulting in a substantial reduction in the omega-6/omega-3 ratio. As a lower omega-6/omega-3 ratio is widely recognized for its potential benefits to human health and overall well-being, these findings have important implications beyond poultry production. While these results showcase the promising potential of chlorella and spirulina as valuable dietary supplements for laying hens, it is important to acknowledge that further research is necessary to comprehensively evaluate their capabilities in partially substituting costly protein sources in laying hens' diets. By doing so, these microalgae could contribute to more sustainable and economically viable feed ingredients within poultry production systems.

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Abbreviations

∑ PUFA n-3	sum of polyunsaturated fatty acids with omega-3 double bond
∑ PUFA n-6	sum of polyunsaturated fatty acids with omega-6 double bond
a*	red-green intensity (in egg color determination)
ALA	alpha-linolenic acid
b*	yellow-blue intensity (in egg color determination)
C*a*	chlorophyll *a*
CAT	catalase
C*b*	chlorophyll *b*
Cc	total carotenoids
CF	crude fiber
CON	conventional diet
CP	crude protein
CV2%	diet with 2% *Chlorella vulgaris*
DCP	in vitro digestibility of protein
DDM	in vitro digestibility of dry matter
DEE	in vitro digestibility of ether extract
DF	dilution factor
DFI	daily feed intake (g/day/layer)
DHA	docosahexaenoic acid
DM	dry matter

DNFE	in vitro digestibility of non-fermentable extractive substance
DOM	in vitro digestibility of organic matter
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5′-dithio-bis-[2-nitrobenzoic acid] (Ellman's reagent)
EDTA	ethylendiaminotetraacetic acid
EE	ether extract
EPA	eicosapentaenoic acid
EW	egg weight (g)
FAME	fatty acid methyl esters (for fatty acid chromatography)
FCR	feed conversion ratio (g feed/g egg)
GAE	gallic acid equivalents (for polyphenols)
GLA	gamma-linolenic acid
GSH	reduced glutathione (peroxidase)
HDEP	hen day egg production (%)
L*	lightness (in egg color determination)
ME	metabolizable energy
MUFA	total monounsaturated fatty acids
NBT	nitro blue tetrazolium
NFE	non-fermentable extractive substance
OM	organic matter
PUFA	total polyunsaturated fatty acids
SFA	total saturated fatty acids
SOD	superoxide dismutase
SP2%	diet with *2% Spirulina platensis*
TAC	total antioxidant capacity
TNB	5-thio-2-nitrobenzoic acid

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Effect of Dietary Salicin Standardized Extract from *Salix alba* Bark on Oxidative Stress Biomarkers and Intestinal Microflora of Broiler Chickens Exposed to Heat Stress

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Abstract: The implication of heat stress (HS) in the oxidative stress phenomenon and its related diseases in chickens has been widely reported. Salicin is a precursor for the synthesis of salicylic acid and aspirin obtained from the bark of *Salix alba*, with an undeniable anti-inflammatory effect. However, little attention has been paid to salicin's antioxidant/oxidative stress-reducing effect compared to its well-known anti-inflammatory effect. The purpose of the study was to investigate the effect of dietary salicin standardized extract from *Salix alba* bark (SAB) on oxidative stress biomarkers and intestinal microflora of broiler chickens exposed to heat stress. In our study, chickens (14 days) were randomly allocated to three treatment groups (SAB0; SAB25; SAB50), each of which included five replicates with eight birds per replicate. Broilers were exposed to heat stress (32 ± 2 °C) between 14 and 42 days. The liver tissues were collected to analyze oxidative stress biomarkers (total antioxidant capacity, glutathione, catalase, superoxide dismutase). The intestinal content was collected and measurements of the intestinal microbial population were performed (*E. coli*, staphylococci, lactobacilli). Results indicated that liver malondialdehyde and protein carbonyls activity decreased ($p < 0.05$) in SAB50 treatment concomitantly with linearly increased total antioxidant capacity, and glutathione concentration. Dietary supplementation with SAB reduced ($p < 0.05$) the abundance of staphylococci and increased the number of lactobacilli. Taken together, SAB possesses an advantageous effect on liver oxidative status and the balance of intestinal microflora in broilers exposed to heat stress. These findings provide new insight into the potential use of salicin standardized extract from *Salix alba* bark for liver damage prevention and dysbiosis related to heat stress.

Keywords: broiler chicken; salicin; *Salix alba* bark; heat stress; oxidative stress; microbiota

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1. Introduction

Heat stress (HS) is an environmental factor that impacts broiler chickens' health and production. As global warming intensifies and breeds become more sensitive (due to genetic improvement to respond to the demands for meat quantity), economic damage related to heat stress in poultry increase repeatedly [1,2]. When broilers are exposed to heat stress, the production of reactive oxygen species (ROS) increases, while the activities of antioxidant enzymes and the capacity to scavenge the free radicals decrease, a phenomenon called oxidative stress. Oxidative stress is associated with damage to demanding lipids, proteins, and DNA, and disrupts redox homeostasis, leading to decreased meat quality and increased tissue damage [3,4]. Heat stress has been observed to decrease endogenous

enzymatic (superoxide dismutase, SOD; catalase, CAT) and non-enzymatic antioxidant (glutathione, GSH) levels and increase lipid and protein oxidation markers in liver tissue and meat [5]. As metabolically active organs, liver and intestinal tissues are very sensitive to HS, such disruption is directly conducive to decreased performance and augments the proneness to disease [5,6]. In the intestine of broiler chickens, an imbalance between harmful and commensal bacteria and a disturbance of the intestinal barrier (a critical element of the gut-liver axis) were observed during heat stress [6]. The link between liver and gut bacteria is acknowledged by several studies [7,8] and continues to evolve. It has been reported that alteration of the gut microbiota leads to increased gut permeability, which causes the activation of various inflammatory pathways in the liver [7]. Due to the negative effects of HS, exploring nutritional solutions to reduce heat stress has become an important research goal.

Endogenous antioxidants (e.g., superoxide dismutase, SOD, catalase, CAT, glutathione peroxidase, GPx) represent the beginning point of antioxidant protection, but during HS their levels are insufficient; therefore, supplementation with exogenous antioxidants is required. Supplementation of chicken feed with exogenous antioxidants, such as vitamins [9], minerals [10], herbal extracts [11,12], and other bioactive compounds [13], is an encouraging solution to mitigate the detrimental effects of HS. Natural antioxidants obtained from leaves, shrubs, and barks could be a safe and easy substitute for chemical additives. Salicin is among the natural compounds extracted from plants. Salicin is a precursor for the synthesis of salicylic acid and aspirin with undeniable anti-inflammatory effects. Recently, however, other biological properties of salicin have been investigated [14]. However, compared to its known anti-inflammatory activity, little attention has been paid to the antioxidant/oxidative stress-reducing effects of salicin. Salicin is the active constituent obtained from the bark of *Salix* species (about 400 species) that can be used in medicine. The addition of *Salix* sp. bark to broiler diets has been shown to support the balance of gut microflora [15], improve performance, and decrease the panting rate [16]. However, to the best of our knowledge, no study has investigated the effect of salicin from the bark of *Salix alba* on the oxidative status of broiler chickens under heat stress. The purpose of the study was to investigate the effect of dietary salicin standardized extract from *Salix alba* bark on liver oxidative stress biomarkers and intestinal microflora of broiler chickens subjected to heat stress.

2. Materials and Methods

2.1. Birds, Diets, and Experimental Design

The procedures concerning animal care, handling, and sampling were conducted under the approval (No. 52/30.07.2014) of the Ethics Committee of the National Research and Development Institute of Animal Biology and Nutrition, Romania. A total of one hundred and twenty 1-day-old Cobb broilers were housed in digestibility cages. Broilers were fed a commercial diet based on corn and soybean meal until 14 days of age. After 2 weeks of acclimation, chicks were randomly assigned into 3 treatment groups (5 replicates/each group with 8 birds per replicate). Three treatment groups were designated as follows: the control group (SAB0), in which birds were fed a basal diet; the experimental groups, in which birds were fed a basal diet supplemented with 25 g *Salix alba* bark extract (SAB)/100 kg diet containing 0.006% salicin (SAB25); and a basal diet supplemented with 50 g *Salix alba* bark extract/100 kg diet (SAB50) containing 0.012% salicin (Table 1). *Salix alba* bark extract was purchased as a powder from a company in China (Changsha Vigorous-Tech Co., Ltd., Changsha, China) containing salicin as an active principle (25% salicin). During the entire experimental period (d 14–d 42), broilers were exposed to heat stress (32 $\pm$ 2 °C). The environmental control systems automatically controlled the temperature and relative humidity (Viper Touch computer). A lighting schedule of 23 h light/day was followed. Water and feed were offered ad libitum.

Table 1. Diet structure of experimental diets (%).

Ingredients (%)	Grower Phase (14–35 Days)			Finisher Phase (36–42 Days)		
	SAB0	**SAB25**	**SAB50**	**SAB0**	**SAB25**	**SAB50**
Corn	62.00	61.97	61.95	60.50	60.47	60.45
Soybean meal	26.58	26.58	26.58	25.46	25.46	25.46
Gluten	4.00	4.00	4.00	6.00	6.00	6.00
Oil	2.50	2.50	2.50	3.75	3.75	3.75
Salix alba bark extract (25% salicin)	-	0.025	0.050	-	0.025	0.050
Calcium carbonate	1.40	1.40	1.40	1.33	1.33	1.33
Monocalcium phosphate	1.36	1.36	1.36	1.13	1.13	1.13
Salt	0.37	0.37	0.37	0.33	0.33	0.33
Methionine	0.26	0.26	0.26	0.25	0.25	0.25
Lysine	0.48	0.48	0.48	0.20	0.20	0.20
Choline	0.05	0.05	0.05	0.05	0.05	0.05
Premix	1.00	1.00	1.00	1.00	1.00	1.00
Total ingredients	100	100	100	100	100	100
Chemical analysis						
Total polyphenols, mg/g GAE	1.70	1.92	2.27	1.69	1.81	2.24

Diet and premix structure published by [17].

2.2. Sample Collection

To evaluate the antioxidant effect, samples of *Salix alba* bark were used to test its effect on in vitro-induced lipid peroxidation (LPO) compared with a synthetic antioxidant, vitamin E. At 42 days, broilers were sacrificed by dislocating of the cervical spine. After exsanguination and evisceration, the whole gastrointestinal tract was rapidly removed. Samples of the intestinal content were collected and kept at −20 °C for microflora measurement. Liver tissues were sampled and stored frozen at −80 °C until used for the analysis of biomarkers of oxidative stress (total antioxidant capacity, TAC; glutathione, GSH; catalase, CAT; superoxide dismutase, SOD; thiobarbituric acid reactive substances, TBARS; and protein carbonyls, PCOs).

2.3. Chemical Analysis

Iron-induced lipid oxidation—Iron-induced lipid oxidation was performed according to the method described previously by [18,19]. Meat samples collected from broilers fed a conventional diet were used to obtain a homogenate as described previously [18]. In three 10 mL plastic tubes, 2 mL of homogenate was transferred. Then, 1000 mg L^{-1} methanolic extract of SAB (tube 2) and 500 µM vitamin E (tube 3) were added to two of them in a total volume of 4 mL. After this, 0.4 mL of peroxidation mix was added to each of the three tubes. The peroxidation solutions consisted of 0.2 mL FeCl2 (100 µM) and 0.2 mL ascorbic acid (500 µM). The mixture was incubated at 37 °C for 60 min and used to quantify thiobarbituric acid reactive substances (TBARS) expressed as mg/kg malondialdehyde (MDA). The inhibition of in vitro-induced lipid peroxidation (LPO) was calculated using the following formula:

$$\% \textbf{ Inhibition LPO} = \left(conc\ MDA_{\ peroxidized\ meat} - conc\ MDA_{peroxidized\ meat+SAB/Vitamin\ E}\right) * 100) / \text{ conc MDA}_{peroxidized\ meat}$$

2.4. Oxidative Stress Biomarkers in Liver

Preparation of liver homogenate—To obtain the liver homogenate, 1 g of liver tissue was weighted and homogenized with 10 mL of potassium phosphate buffer (66 mM, pH 7.2) including 1 mM EDTA. After centrifugation (10,000× *g* for 15 min at 4 °C), the supernatant was collected and used for the analysis of total antioxidant capacity (TAC), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), thiobarbituric acid reactive substance (TBARS), and protein carbonyls (PCOs).

Total antioxidant activity (TAC) analysis—The assay of TAC was performed based on the capacity of buffered aqueous extracts of the liver in scavenging 2,2- diphenyl-1-picrylhydrazyl free radical (DPPH) [20]. Specifically, 20 µL of the liver extract was homogenized with 480 µL of potassium phosphate buffer (22 mM, pH 7.4). To the resulting mixture was added 500 µL of DPPH solution (0.1 mM). The mixture was stored in a dark room for 30 min and centrifuged (3 min, 10,000× *g*). The absorbance of the mixture was recorded at 520 nm and was converted to mmol/L 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from a Trolox standard curve.

Glutathione (GSH) assay—GSH was determined using a spectrophotometric method according to [21]. An aliquot volume of 20 µL of the liver extract was removed to a plastic tube and mixed well with 5% trichloroacetic acid (TCA). Then, 650 µL potassium phosphate buffer (pH 8.0) and 330 µL of 5,5 dithiobis-2-nitrobenzoate (DTNB) were added. The samples were stored for 45 min in a dark room. The absorbance of solutions was recorded at 412 nm and the GSH concentration was obtained using a calibration curve. The results were expressed as µmol/g tissue.

Catalase activity (CAT) assay—CAT activity was performed as according to [22]. A volume of 10 µL diluted liver extract was mixed with 2980 µL of potassium phosphate buffer (pH 7.4). The solutions were stored for 10 min at 37 °C and then 10 µL of hydrogen peroxide was added. The absorbance was recorded at 240 nm for 3 min. Catalase activity was calculated using the molar extinction coefficient of H_2O_2 and was expressed as U/mg tissue.

Superoxide dismutase activity (SOD)—Superoxide dismutase (SOD) was assayed using a commercial enzymatic kit (FlukaTM, Charlotte, CA, USA) following the manufacturer's protocol. SOD activity was expressed as U/g tissue.

Thiobarbituric acid reactive substance assay (TBARS)—Thiobarbituric acid reactive substances (TBARS) were evaluated by a spectrophotometric method using malondialdehyde (MDA) as standard [23]. The absorbance of samples was read using a spectrophotometer (Jasco V-530, Japan Servo Co. Ltd., Tokyo, Japan) at 532 nm and the results were expressed as mg MDA/kg tissue (liver).

Protein carbonyls (PCOs)—PCOs were assayed by the DNPH-based method [24]. A volume of 50 µL liver extract was mixed with 50 µL TCA (20%) and kept in the water-ice bath for 15 min. The mixture was centrifuged at 15,000× *g* for 10 min at 4 °C. The supernatant obtained was mixed with 500 µL of 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.5 N HCl and maintained at room temperature for 1 h. After incubation, the mixture was homogenized with a vortex and centrifuged (15,000× *g* for 5 min, 4 °C). The supernatant was mixed with 1 mL TCA, then vortexed and centrifuged. The supernatant was transferred and mixed with 1 mL of ethanol-ethyl acetate (1:1, *v*/*v*); the solution was vortexed, and centrifuged. This washing step was repeated twice. The resulting supernatant was homogenized with 1 mL of 5 M urea (pH 2.3) and maintained at 37 °C for 15 min. Consequently, the solutions were centrifuged and the absorbance was read at 375 nm. The calculation of PCOs concentration was based on the molar extinction coefficient. The results were expressed as nmol DNPH/mg protein. The protein concentration was calculated by reading the absorption at 280 mm and using the bovine serum albumin (BSA) as standard.

2.5. Intestinal Microbial Population Measurements

The intestinal content collected was used to determine the intestinal microbial population. The populations of *Escherichia coli*, staphylococci, and lactobacilli were determined as described previously [12,25]. The colonies were counted with a Scan 300 colony counter (Interscience, Paris, France). The results were indicated as log base 10 colony-forming units (CFU)/gram of intestinal contents.

2.6. Statistical Analysis

The analyses were performed using Addinsoft statistical software [26] (version 2022.3.1). The effect of dietary treatments on tested parameters was determined using one-way analysis of variance (ANOVA) followed by Tukey's multiple-range test. Graphs were drawn

using Prism-GraphPad software v. 9.03 (San Diego, CA, USA). Statistical significance was considered as $p < 0.05$. Correlations between antioxidant capacity, lipid and protein oxidation, and intestinal microflora were performed using Pearson's correlation coefficient analysis. The significant correlations are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3. Results

3.1. Effect of Salicin Standardized Extract from SAB on In Vitro Induced Lipid Peroxidation

Table 2 shows the effect of *Salix alba* bark on in vitro-induced lipid peroxidation (LPO) of meat compared to vitamin E. Vitamin E is a well-known antioxidant, being used as a standard for several analytical methods to highlight the antioxidant capacity. As expected, peroxidized meat treated with synthetic vitamin E had the highest percentage of LPO inhibition, vitamin E being a more effective antioxidant than *Salix alba* bark in retarding lipid peroxidation.

Table 2. Effect of dietary salicin standardized extract from SAB on in vitro-induced lipid peroxidation of meat compared to vitamin E.

Item	TBARS (mg/kg)	% Inhibition of LPO
Meat with induced LPO	1.58 ^{a}	-
Meat with induced LPO and SAB (1000 mg/kg)	1.39 ^{b}	11.60 ^{b}
Meat with induced LPO and vitamin E (500 µM)	1.19 ^{c}	24.21 ^{a}
SEM	0.547	0.813
p-value	<0.0001	<0.0001

$^{a–c}$ Means within a column with no common superscript differ ($p < 0.05$). LPO—lipid peroxidation.

3.2. Oxidative Stress Biomarkers

To evaluate the effect of dietary salicin standardized extract from SAB on the oxidative status of the liver in heat-stressed broilers, we determined the total antioxidant capacity (TAC), the activities of CAT, SOD, and GSH levels (Table 3).

Table 3. Effect of dietary salicin standardized extract from SAB on liver oxidative stress biomarkers of broiler chickens.

Item	Groups			SEM	*p*-Value
	SAB0	SAB25	SAB50		
TAC (mmol Trolox/L)	0.86 ^{a}	0.98 ^{a}	1.12 ^{b}	0.027	0.001
CAT (U/mL)	1848.60	1788.38	1596.81	0.438	0.4346
GSH (µmol/g tissue)	3.138 ^{a}	3.66 ^{ab}	4.05 ^{b}	0.179	0.012
SOD (U/g tissue)	2085.8	2187.3	2208.7	0.137	0.8161

^{a,b} Means within a column with no common superscript differ ($p < 0.05$). Abbreviations: TAC—total antioxidant capacity; CAT—catalase; GSH—glutathione; SOD—superoxide dismutase.

Compared with the SAB0 group, TAC activity and GSH concentration were increased in the liver of heat-stressed broilers fed SAB50 (Table 3). Liver CAT and SOD activity did not differ significantly among the groups.

Figure 1 shows the effects of SAB on liver concentrations of TBARS and PCOs. Liver malondialdehyde was decreased ($p < 0.05$) both in SAB25 and SAB50 treatment compared to SAB0. Protein carbonyls (PCOs) decreased only in the SAB50 group. Notably, the higher level of SAB50 significantly decreased the PCOs activity compared to the lower one.

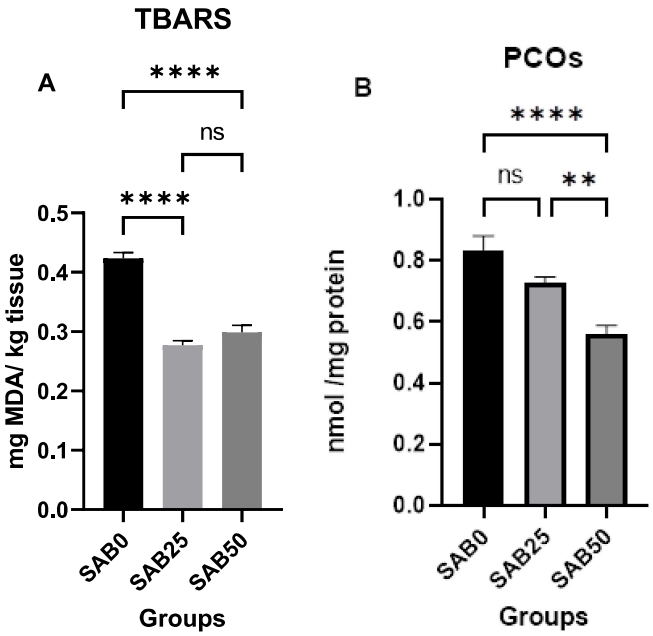

Figure 1. Effects of dietary salicin standardized extract from SAB on liver concentrations of (**A**) thiobarbituric acid reactive substances (TBARS), and (**B**) protein carbonyls (PCOs) in broiler chickens. Bars represent means ± SEM. Abbreviations: SAB0—basal diet, without SAB; SAB25—basal diet + 25 g SAB/100 g diet; SAB50—basal diet + 50 g SAB/100 g diet; ** $p < 0.01$; **** $p < 0.0001$.

3.3. Intestinal Microbial Population Measurements

Figure 2 shows the effects of dietary salicin standardized extract from SAB on intestinal microflora in broiler chickens. *E. coli* did not show any difference between treatments. The number of staphylococci was significantly lower in SAB25 and SAB50 compared to SAB0. Lactobacilli were significantly higher in groups fed supplements with SAB compared to those fed a non-supplemented diet (SAB0). *E. coli*:lactobacilli was significantly lower in SAB 25 and SAB50 compared to SAB0.

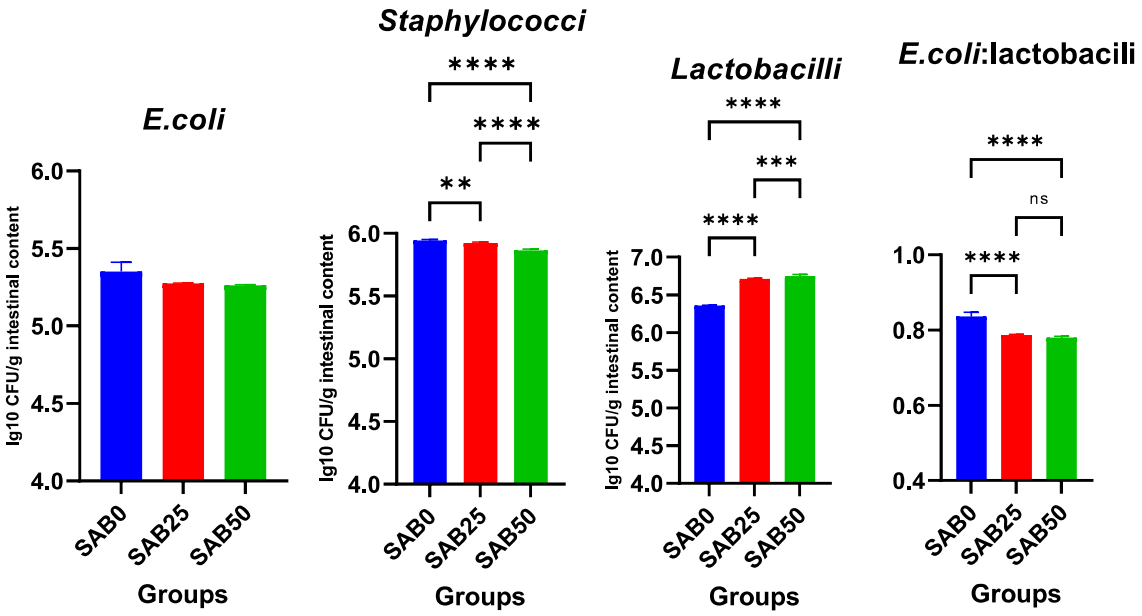

Figure 2. Effects of dietary salicin standardized extract from SAB on intestinal microflora in broiler chickens. Bars represent means ± SEM. Abbreviations: SAB0—basal diet, without SAB; SAB25—basal diet + 25 g SAB/100 g diet; SAB50—basal diet + 50 g SAB/100 g diet; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3.4. Correlation between Antioxidant Capacity, Lipid and Protein Oxidation Biomarkers, and Intestinal Microflora

TAC showed positive correlation with lactobacilli and negative correlation with TBARS, PCOs, and staphylococci. According to Figure 3, TBARS was negatively correlated with TAC and lactobacilli and positively correlated with PCOs and staphylococci. Pearson's correlation showed a positive correlation of PCOs with TBARS and staphylococci and a negative correlation with TAC and lactobacilli. *E. coli* was found to be negatively correlated with lactobacilli. Regarding the staphylococci content, a positive correlation was found with TBARS and PCOs and a negative correlation with TAC and lactobacilli. In addition, lactobacilli were negatively correlated with TBARS, PCOSs, *E. coli*, and staphylococci.

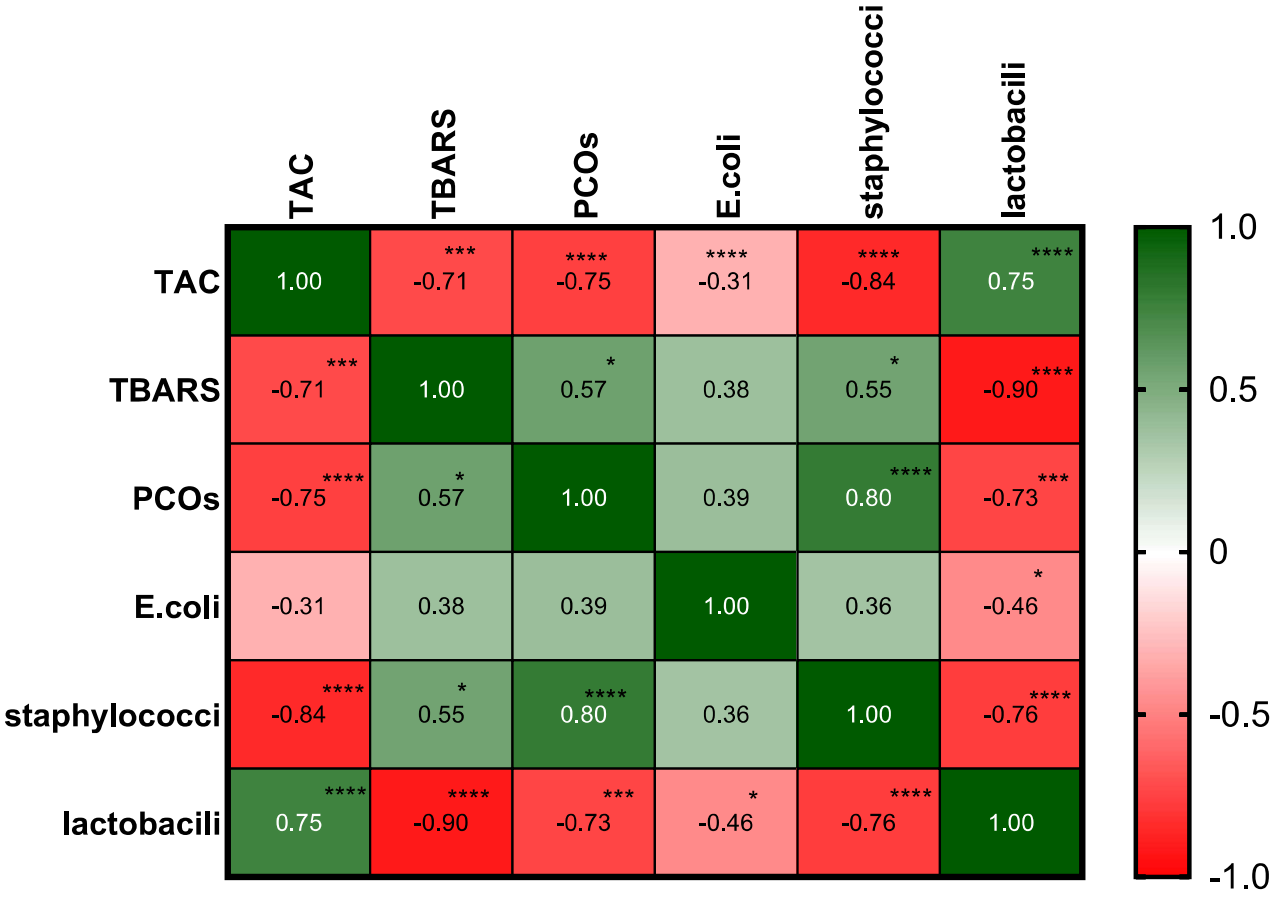

Figure 3. Pearson's correlations between antioxidant capacity, lipid and protein oxidation biomarkers in liver and intestinal microflora. Each cell contains the correlation coefficient (r^2) and statistical level of significance (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$). The negative correlations are highlighted with green and the positive correlations are highlighted with red. The darker the color is, the higher the correlation between variables. Abbreviations: TAC—total antioxidant capacity; TBARS—thiobarbituric reactive species; PCOs—protein carbonyls.

4. Discussion

4.1. Effect of Salicin Standardized Extract from Salix Alba Bark on In Vitro-Induced Lipid Peroxidation

The bark of *Salix alba* is known for its content of salicin, a β-glucoside compound with anti-inflammatory properties that have been demonstrated in in vitro and in vivo studies. However, in the present study, we have shown that the bark of *Salix alba* possesses antioxidant activity in vitro, exhibiting an LPO inhibitory effect of 11.60%, which is almost half that of vitamin E. However, considering that it is an antioxidant standard and SAB is a natural product, this percentage proves that SAB has a significant antioxidant capacity.

These findings are in agreement with several studies [27–29]. Moreover, recent evidence has demonstrated that the biological effects of willow bark extract are not exclusively due to salicylates, but also to the synergism between different bioactive compounds such as chlorogenic acid and salicin derivatives [29].

4.2. Oxidative Stress Biomarkers

The endogenous antioxidant system is the first line of defense against oxidation in the body. In general, under HS conditions, an excess of RS is formed, which decreases the activity of antioxidants (CAT, GSH, SOD) and, accordingly, leads to a disturbance of the equilibrium between antioxidant and oxidative systems in broiler chickens. In this study, feeding with SAB50 increased the concentration of GSH, a tripeptide produced by the body that participates in the body's defenses to combat oxidative stress. GSH may exhibit antioxidant properties in several ways: it detoxifies H_2O_2 and lipid peroxides with the help of glutathione peroxidase (GSH-Px); provides an electron to H_2O_2 for conversion to H_2O and O_2; and protects lipid membranes from oxidative stress by transferring protons [30,31]. In this study, the increase in GSH concentration means that the antioxidant capacity of the body has increased, which was statistically demonstrated by the increase in TAC. These results support the beneficial effects of SAB on the liver of heat-stressed broiler chickens. Overall, this evidence showed for the first time that dietary supplementation with SAB50 can enhance the antioxidant status of broiler chickens by increasing GSH and TAC levels in the liver and protecting heat-stressed chickens from oxidative stress. These results are consistent with those of other studies [17] when broilers raised under thermoneutral conditions were supplemented with 0.05% *S. alba* bark. However, the specific antioxidant mechanism of SAB needs further research. Thus, supplementation of broiler diets with salicin from *Salix alba* bark could explain the increase in GSH levels in the liver with increasing SAB dose. According to some authors [32], an extract of *Salix alba* bark containing 2% salicin increased antioxidant status and inhibited lipid peroxidation in rats. In another study on rats with rheumatoid arthritis, salicin exposure (240 mg) could improve the level of GSH, SOD, and CAT in joint tissues [33]. Although *Salix alba* extracts are commonly standardized to salicin, the same authors showed that those effects are due to both salicin and other compounds in the extracts such as salicylates, polyphenols, and flavonoids, and their interaction may also contribute to the overall antioxidant effect. Polyphenols such as flavonoids improved the expression of γ-glutamylcysteine synthetase and increased the intracellular GSH concentration in muscle [34]. However, the increase only of GSH activity in the liver must be further studied. A possible explanation for the fact that the activity of the other enzymes did not increase may be that the body did not have to call on all the resources to fight the stress because there were enough endogenous and exogenous antioxidants.

As a result of an overwhelmed antioxidant defense system, rises in the levels of MDA and PCOs were recorded in the liver of broiler chickens reared under heat-stress [35,36]. The explanation is that the presence of ROS leads to both protein and lipid oxidation. In heat stress, the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) causes protein side chain oxidation that gives rise to reactive aldehydes and ketones known as protein carbonyls (PCOs) [37]. Protein carbonyls are the most commonly used biomarker for protein oxidative damage in tissues since they highlight cellular damage caused by different forms of ROS [38]. As mentioned above, the presence of RS also leads to lipid oxidation. Malondialdehyde (MDA) is the end-product derived from the cleavage of polyunsaturated fatty acids by ROS processes in cells [39]. Interestingly, in our study, SAB administration reduced TBARS and PCOs levels in the liver of broiler chickens reared under heat-stress. The observation made was probably due to the capacity of metabolites of SAB as agonists to induce the synthesis of antioxidants such as GSH (electron donor in peroxides reduction) in the present study, improving oxidative status, and, correspondingly, retarding the process of lipid and protein oxidation in the liver tissue. On the mechanism, SAB has been shown to activate the Nrf2 pathways, which induces the transcription of antioxidant

genes [40–42]. Through those mechanisms, *Salix alba* bark can manage oxidative stress, which altogether may decrease the generation of ROS, plausibly explaining the lower levels of TBARS and PCOs. Studies have shown that the treatment with 50 and 100 mM salicin downregulated oxidative stress induced by advanced glycation end-products (AGEs) in SW1353 human chondrocytes, significantly reducing the ROS level in a dose-dependent manner [43]. Others [33] showed that salicin (240 mg) stabilizes the oxidative stress in rats with rheumatoid arthritis by increasing nuclear factor (erythroid-derived 2)-like 2 (Nrf2) translocation and increasing the expression of heme oxygenase-1 (HO-1). Moreover, the activation of NRf2 plays an important antioxidant role in reducing oxidative stress and inflammation in organs such as the liver, kidneys, and intestine [44,45]. To the best of our knowledge, no studies have investigated the effect of a dietary salicin standardized extract from *Salix alba* bark on the oxidative status of broilers under heat stress. Thus, the present findings show for the first time that, in addition to its beneficial anti-inflammatory effect, salicin from a standardized *Salix alba* bark extract may improve oxidative status in heat-stressed broilers.

4.3. Intestinal Microbial Population Measurements

Oxidative stress related to HS affects gut microbiota composition and causes barrier disruption and dysbiosis in broilers [46,47]. In fact, the gut barrier is strengthened by the commensal bacteria existing in the gut (e.g., *Lactobacillus, Bifidobacterium*) which prevents the colonization of opportunistic pathogens (e.g., *E. coli*, staphylococci, coliforms, and *Clostridium*). These actions are efficient to protect against pathogens [48]. On the other hand, *E. coli* and staphylococci are continuously reported as the main pathogens involved in many intestinal and extra-intestinal disease conditions in poultry. It is a Gram-negative Bacillus which triggers serious health problems including yolk sac infection, respiratory tract infection, etc., and is responsible for considerable economic losses in the poultry industry [49]. *Staphylococcus* genus included bacteria that colonize the surface of the skin and mucous membranes of poultry leading to different diseases, which usually occurs when the natural immunity is destabilized (e.g., stress factors) [50]. Such diseases lead to economic losses resulting from decreased weight gain, mortality, and condemnation at slaughter.

In the present study, dietary administration of SAB positively changed the gut microbiota of heat-stressed broiler chickens, reducing the number of colony units of staphylococci and increasing the abundance of commensal bacteria such as lactobacilli. The natural supplement, SAB, can be metabolized by gut microbiota in new and active small molecules, which possess active pharmacological effects. For instance, the pharmacokinetics of SAB implies a hydrolyzation of salicin to salicyl alcohol, which is further oxidized to salicylic acid (a major metabolite of salicin). The latter, salicylic acid, is converted to salicylic acid and gentisic acid, which are excreted as glucuronides [51]. Some authors [52] identified mono- and oligosaccharides (sucrose, raffinose, and stachyose) and aromatic phytochemicals (triandrin, catechin, salicin, and picein) as antibacterial metabolites of SAB, which showed a high antibacterial effect of *Salix alba* bark water extract on the growth of *S. aureus*. Other authors [53] showed that possibly salicin and salicylic acid have a main contribution to the antimicrobial effect against *Staphylococcus aureus* of the extracts. Studies showed that oral administration of salicin from *Salix alba* bark (100 and 200 mg per body weight, administered daily through oral gavage, 7 days) had an anti-inflammatory effect in DSS-treated mice, improving the recovery in *Lactobacillus* and *Bifidobacterium* populations during the short period of treatment [54]. The mechanism by which the metabolites of SAB act positively on the gut microflora can be by favoring the growth of beneficial bacteria and inhibiting pathogenic bacteria, thereby playing a dual regulatory role in gut microbiota composition. Many herbal medicines were studied to increase beneficial bacteria and reduce harmful bacteria levels, thereby playing a dual regulatory role [55].

4.4. Correlation between Liver Oxidative Biomarkers and Intestinal Microflora

Increasing evidence indicates a crucial role for the gut microbiota in maintaining liver function through modulation of the gut–liver axis [56]. The gut–liver axis defines the mutual interaction between the gut and its microbiota on one hand and the liver on the other. Indeed, bile acids formed in the liver control the microbiota, and intestinal products manage bile acid synthesis, and glucose and lipid metabolism in the liver. A variety of liver diseases are characterized by alterations in the intestinal flora, and some of the altered species have been considered predictive of liver disease outcomes. In addition, it is increasingly evident that oxidative stress leads to disruption of the gut–liver axis (dysbiosis leads to the activation of various inflammatory pathways in the liver) and further to chronic liver disease [7,57]. Interestingly, the present study demonstrated the close link between gut microbiota and liver oxidative status. Present findings showed a strong positive correlation between lactobacilli and TAC and showed that the increase in lactobacilli in the intestine improved the antioxidant status of the liver. Improvement of the intestinal microbiota (decreasing staphylococci and increasing lactobacilli abundance) was attributed to the increase in TAC and the reduction of MDA and PCOs levels in the liver. Little evidence was found regarding the correlation between the gut microbiota and the oxidative state of the liver, if we consider its importance, especially in the context of exposure to a stressor, which in our case would be heat stress. Several studies [58] showed that the redox mechanisms of *Lactobacillus* spp. may take part to the downregulation of these ROS-forming enzymes. Moreover, *Lactobacillus* spp. has been studied to modulate oxidative stress via Nrf-2 and nuclear factor kappa B (NF-kB). In this study, we showed that improving TAC suppresses lipid and protein oxidation of liver tissue, from which we can infer that high hepatic levels of TAC delay oxidative stress in heat-stressed broilers and restores the RS/antioxidant equilibrium. In the case of the TAC–liver oxidative protection relationship, considerable evidence was found [59,60].

5. Conclusions

HS is a serious stressor inflicting oxidative stress and affecting poultry production. In this study, it was confirmed that doses of 25 and 50 mg/100 kg SAB containing 0.006% and 0.012% salicin exerts a positive effect on counteract oxidative stress damage of the liver in broilers exposed to heat stress due to stimulation of GSH synthesis and increased antioxidant capacity. Dietary supplementation with SAB increases beneficial bacteria (lactobacilli) levels and reduces harmful bacteria (staphylococci) abundance in the intestine of heat-stressed broiler chickens, thereby possibly playing a dual regulatory role. The increase in lactobacilli and the decrease in staphylococci due to the supplementation with SAB led to the improvement of the oxidative status of the liver of chickens raised under heat stress. The novel results presented in this study provide new insight into the potential use of salicin standardized extract from *Salix alba* bark for liver damage prevention and dysbiosis related to heat stress.

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Data Availability Statement: All data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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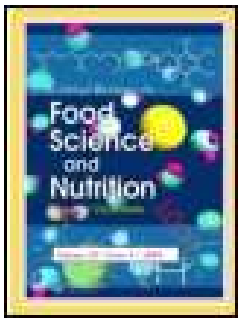

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REVIEW

Plant polyphenols mechanisms of action on insulin resistance and against the loss of pancreatic beta cells

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ABSTRACT

Diabetes mellitus describes a group of metabolic disorders characterized by a prolonged period hyperglycemia with long-lasting detrimental effects on the cardiovascular and nervous systems, kidney, vision, and immunity. Many plant polyphenols are shown to have beneficial activity for the prevention and treatment of diabetes, by different mechanisms. This review article is focused on synthesizing the mechanisms by which polyphenols decrease insulin resistance and inhibit loss of pancreatic islet β-cell mass and function. To achieve the objectives, this review summarizes the results of the researches realized in recent years in clinical trials and in various experimental models, on the effects of foods rich in polyphenols, polyphenolic extracts, and commercially polyphenols on insulin resistance and β-cells death. Dietary polyphenols are able to reduce insulin resistance alleviating the IRS-1/PI3-k/Akt signaling pathway, and to reduce the loss of pancreatic islet β-cell mass and function by several molecular mechanisms, such as protection of the surviving machinery of cells against the oxidative insult; increasing insulin secretion in pancreatic β-cells through activation of the FFAR1; cytoprotective effect on β-cells by activation of autophagy; protection of β-cells to act as activators for anti-apoptotic pathways and inhibitors for apoptotic pathway; stimulating of insulin release, presumably by transient ATP-sensitive K^+ channel inhibition and whole-cell Ca^{2+} stimulation; involvement in insulin release that act on ionic currents and membrane potential as inhibitor of delayed-rectifier K^+ current ($I_{K(DR)}$) and activator of current. dietary polyphenols could be used as potential anti-diabetic agents to prevent and alleviate diabetes and its complications, but further studies are needed.

KEYWORDS

Polyphenols; insulin resistance; β-cells; insulin secretion

Introduction

Diabetes mellitus (DM) describes a group of metabolic disorders characterized by a prolonged period of hyperglycemia. It is caused by an inherited and/or acquired deficiency in pancreas insulin production (type 1 DM), or by the ineffectiveness of the body cells to normally respond to the hormone produced by β-cells of the pancreatic islets, named insulin (type 2 DM). People who suffer from diabetes have an increased risk of developing other diseases (such as cardiovascular disease, stroke, foot ulcers, nephropathy, and retinopathy) that reduce the quality of life and increase the risk of death (Cho et al. 2018). For 2017, the International Diabetes Federation (IDF) estimated 451 million (age 18–99 years) diabetics worldwide, and for 2045 the estimation goes up to 693 million diabetics, representing a 53.65% compared to 2017. Diabetes increasing incidence is a consequence to the eating habits and lifestyle developed worldwide during the last century. People from developed and under development countries have and increasing sedentary lifestyle and are consuming more fat and more sugar than previous generations (Kim, Keogh, and Clifton 2016).

Diabetes prevention and treatment demand a change of the lifestyle, a healthy diet, regular physical exercise, a normal body weight, and avoiding tobacco. A healthy diet involves reducing the consumption of carbohydrates rich foods and saturated fats, and a diet richer in fruits, vegetables, and whole grains, plant polyphenols, terpenoids, alkaloids, sterols, pigments, and unsaturated fatty acids (Alkhatib et al. 2017). Plant phenolics are organic molecules distributed in fruits, grains and vegetables some of them with beneficial effects on human health. They contain one or more benzene rings with one or more hydroxyl substituents, and range from simple phenolic molecules to highly complex molecules. Based on the number of phenol rings and structural elements that bind these rings, plant phenolics are classified into several classes and sub-classes. Lattanzio (2013) identified the following basic skeletons in plant phenolics: C_6, C_6–C_1, C_6–C_2, C_6–C_3, C_6–C_4, C_6–C_1–C_6, C_6–C_2–C_6, C_6–C_3–C_6, $(C_6$–C_3–$C_6)_{2,3}$, $(C_6$–$C_3)_2$, $(C_6$–$C_3)_n$, $(C_6)_n$, and $(C_6$–C_3–$C_6)_n$, and classified them in several classes, showed in Figure 1. The term "polyphenols" is used to define natural phenolic compounds, which contain at least 2 hydroxyl groups on a benzene ring, or at least 2 benzene rings bearing one or more hydroxyl groups. Polyphenols are the most abundant antioxidants in the diet (Denev et al. 2012; Papuc et al. 2017; Huang 2018), and

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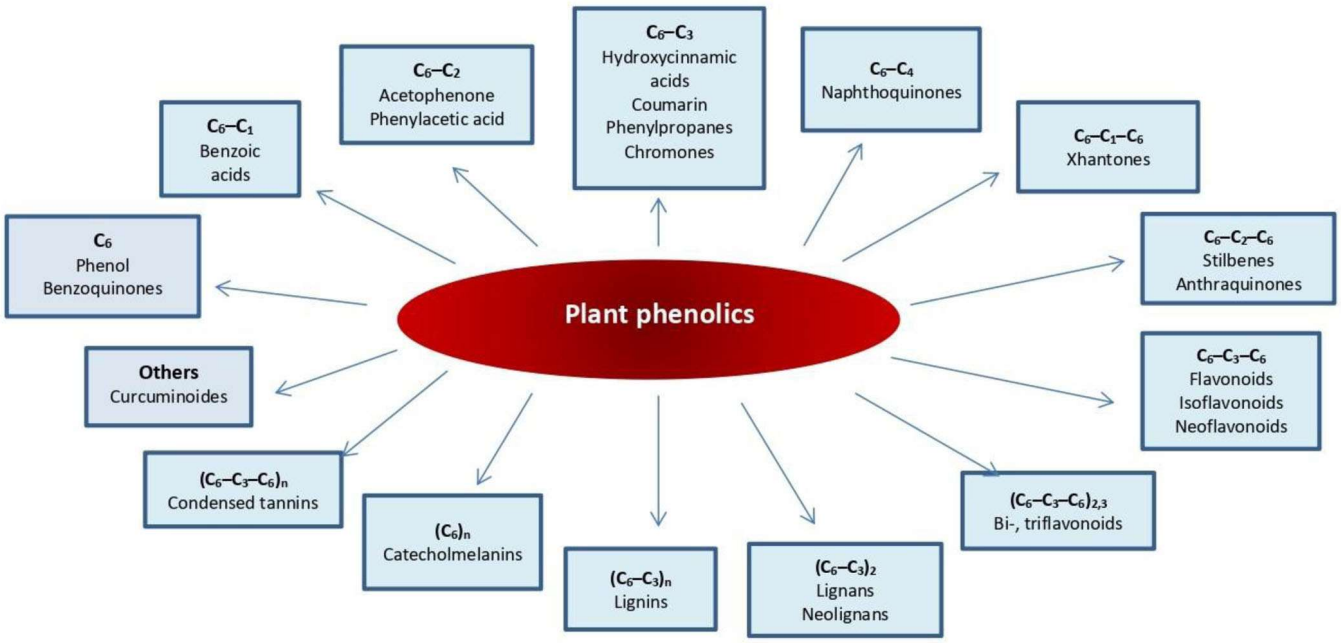

Figure 1. Plant phenolics classes categorized according to their basic skeleton (Lattanzio 2013). C_6 – benzene ring; C_1, C_2, C_3 – side chains with 1, 2, and 3 carbon atoms.

their intake has been associated with a reduced incidence of modern human diseases, such as coronary artery diseases (Cheng et al. 2017; Billingsley and Carbone 2018), cancer (Abdal Dayem et al. 2016; de Cedrón et al. 2018; Kwon 2018), liver diseases (Li et al. 2018), Alzheimer's and Parkinson's diseases (Hügel and Jackson 2015; Desai 2016; Molino et al. 2016), obesity (Wang et al. 2014; Kujawska and Jodynis-Liebert 2018; Wood dos Santos et al. 2018). Also, it is now generally accepted that plant polyphenols may be beneficial for the prevention and treatment of diabetes (Pinent et al. 2008; Cao et al. 2019). Epidemiological studies and associated meta-analyses over the past decades suggested that long term consumption of foods rich in plant polyphenols or commercially polyphenolic suppliments may prevent or alleviate diabetes (Grassi et al. 2005; Song et al. 2005; Jennings et al. 2014; Liu et al. 2014; Gospin et al. 2016; Lefevre et al. 2016; Tresserra-Rimbau et al. 2015; Paquette et al. 2017). In vivo and in vitro studies on various experimental models developed during the past decade hypothesized that polyphenols isolated from plants or plant extracts rich in polyphenols may interfere with cellular mechanisms contributing to reducing of the insulin resistance (Lee, Hsu, et al. 2012; Jimenez-Gomez et al. 2013; Hsu et al. 2014; El-Bassossy et al. 2016; Ahmed et al. 2017; Yoshitomi et al. 2017; Huang, Chang, et al. 2018; Huang, Chang, et al. 2018; Singh et al. 2018; Zhai et al. 2018), loss of pancreatic islet β-cell mass and function (Martín et al. 2013; Martín et al. 2014; Bahar et al. 2017; Adam et al. 2017; Rowley et al. 2017; Xiao et al. 2017; Belhadj et al. 2018; Li et al. 2018a; Li et al. 2018b; Luna-Vital and de Mejia 2018). This review is a critical overview of the published data from clinical trials, observational prospective cohort studies, and experimental model studies, linking dietary polyphenols to pre-diabetes and type 2 diabetes, with a focus on polyphenol-rich foods or individual polyphenols oral administration. The present paper aim is to describe the possible mechanisms by which polyphenols inhibit insulin resistance, and loss of pancreatic islet β-cell mass and function, linked to several studies undertaken in different experimental models in the last 10 years.

Normal insulin signaling

Insulin signaling pathway consists in binding of insulin with the insulin receptor (IR) leading to the activation of glucose transporter 4 (GLUT4), which imports glucose into the cell. IR is a tetrameric membrane protein that is composed of 2 α subunits and 2 β subunits. The α subunits, which form the insulin-binding situs, are totally extracellular and are connected to the extracellular parts of the β subunits. Each β subunit has in its structure an extracellular domain, a membrane-spanning transmembrane domain, and an intracellular kinase domain that can be activated by auto phosphorylation (Lee et al. 2014). Binding of insulin to α subunits of IR activate intracellular tyrosine kinase domains in the β subunits and promotes auto phosphorylation of 3 tyrosine residues (Tyr-1158, Tyr-1162, and Tyr-1163) from the β subunits. One of the phosphorylated tyrosine residues of IR attracts a so-called "docking protein", named insulin receptor substrate (IRS), and phosphorylates 3 tyrosine residues, forming a signaling adapter protein, named insulin receptor substrate 1 (IRS-1).IRS-1 acts as an attachment site for phosphatidylinositol 3-kinase (PI3-k), which produces the phosphorylation of phosphatidylinositols accompanied by the formation of 3-phosphoinositides [phosphatidylinositol-3,4-bisphosphate (PIP2)], and then phosphatidylinositol-3,4,5-trisphosphate (PIP3)]. The cell membrane phospholipid PIP3 serves as docking site for phosphoinositide dependent kinase 1 (PDK1), which then mediates activation of protein kinase B (Akt), also called PKB. This protein is not membrane bound,

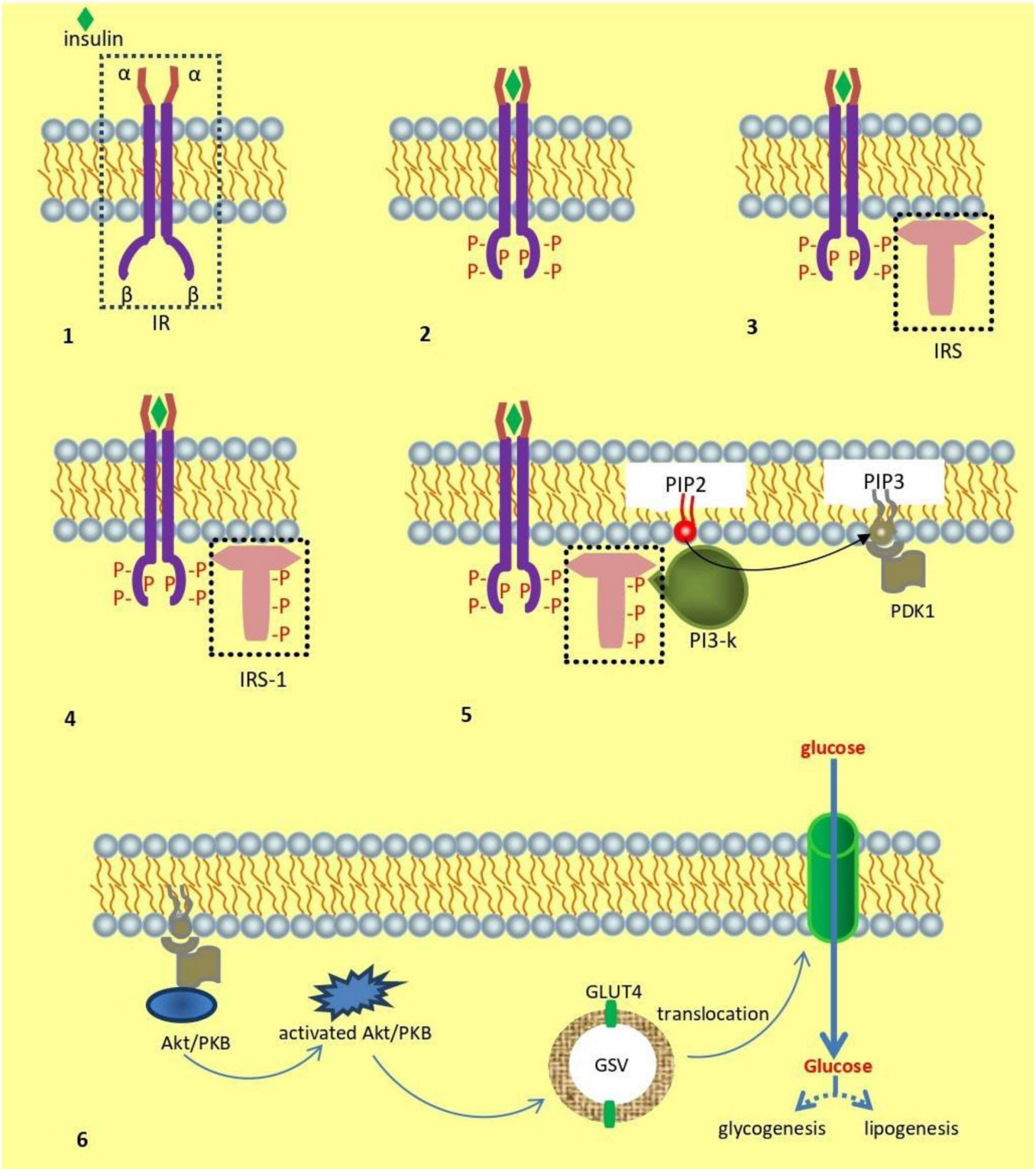

Figure 2. Insulin signaling pathway. 1. Transmembrane IR structure; 2. IR insulin binding and IR autophosphorylation by the tyrosine kinase; 3.IRS protein docking to IR; 4.IRS phosphorylation by the kinase insulin receptor, and IRS-1 forming; 5.Attachment of PI3-k and phosphorylation of PIP2 into PIP3, and PDK1 docking; 6. Activation of Akt/PKB by PDK1, and the diffusion of activated Akt/PKB to stimulate the movement of glucose to cell membrane, mediated by GLUT4.
IR: insulin receptor; IRS: insulin receptor substrate; PI3-k: phosphoinositide 3-kinase; PIP2: phosphatidyl-inositol-3,4-bisphosphate; PIP3: phosphatidyl-inositol-3,4,5-tris-phosphate; PDK1: phosphoinositide dependent kinase 1; Akt/PKB: protein kinase B; GLUT4: glucose transporter 4; GSV: GLUT4 storage vesicle.

and it can diffuse throughout the cell. Activated Akt activates GLUT4, which moves from an intracellular compartment to the plasma membrane, to transport glucose into skeletal muscle and adipose tissue (Martin et al. 2000) and also, can regulate transcription of targetgenes-phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) *via* forkhead box protein O (Foxo-1) (Saini 2010). The normal insulin signaling pathway is presented in Figure 2. Studies conducted in the last decades suggest that the insulin resistance is linked to insulin signaling defects in target

tissues. In this review we'll summarize the current understanding of the nature of the insulin signaling defects described in the last years studies.

Insulin resistance inducing factors

Insulin resistance is well defined as the decreased ability of insulin to regulate glucose metabolism. The key insulin-sensitive tissues are: skeletal muscle (takes 70-80% of glucose), liver and adipose tissue. The healthy individuals are highly sensitive to insulin in all 3 key tissues mentioned, whereas obese individuals and individuals with type 2 diabetes are insulin resistant (Honka et al. 2018). The studies conducted by Honka et al. (2018) on 326 subjects showed that, in the case of insulin-resistant individuals, the skeletal muscle glucose uptake was less than 33 µmol/kg tissue/min, and in subcutaneous adipose tissue was less than 11.5 µmol/kg tissue/min. They suggest that insulin resistance measured by glucose uptake is partially similar in all three insulin-sensitive tissues, and is affected by obesity, aging and gender.

Genetic defects in the insulin receptor

The most severe forms of insulin resistance result from the genetic defects in the insulin receptor. These are relatively rare and cause insulin resistance syndromes as Leprechaunism, Rabson-Mendenhall syndrome, and the type A syndrome of insulin resistance.

The inhibition of signaling downstream of the insulin receptor

Cells exposure to elevated levels of free fatty acids

A very high fat diet, especially saturated fat, decreases insulin sensitivity and thereby might leads to the development of type 2 diabetes (von Frankenberg et al. 2017). Pan et al. (1997) studied the relation between skeletal muscle triglyceride levels and insulin action on animal models, and suggested a relationship of inverse proportionality between skeletal muscle insulin sensitivity and triglyceride levels, as well as by remote depots and circulating lipids. On the other hand, recent studies conducted in 815 Hispanic individuals in the insulin resistance atherosclerosis family, indicated that free fatty acids (FFAs) levels contribute to insulin resistance independent of adiposity (Miller et al. 2012). FFAs are released by subcutaneous fat cell lipolysis, and in particular, it has been shown that the visceral adipose tissue has a high rate of lipolysis (Rydén and Arner 2017). Spontaneous (basal) lipolysis is activated by catecholamines and natriuretic peptides, hormones with a high prolipolytic activity, and inhibited by insulin (Rydén and Arner 2017). Wefers et al. (2018) studied direct effects of circadian misalignment on skeletal muscle insulin sensitivity and the muscle molecular circadian clock in 14 healthy young lean men, and suggested that the circadian misalignment led to higher fasting FFAs levels, and lower triglyceride levels that contribute to significant increasing in muscle insulin resistance. Many researches carried out in recent years have been aimed at deciphering the mechanisms by which FFAs mediates insulin resistance. Randle, Kerbey, and Espinal (1988) demonstrated that fatty acids compete with glucose for substrate oxidation, and supposed that increased fat oxidation causes the insulin resistance associated with obesity. Increasing the concentration of FFAs in plasma produced a dose-dependent decrease in insulin-mediated glucose disposal and inhibition of the insulin signaling cascade that involved decreasing of IR and IRS-1 tyrosine phosphorylation, reducing the attachment of PI3-k to IRS-1, and impairing serine phosphorylation of Akt (Belfort et al. 2005). In many studies conducted over the past 2 decades, it is assumed that FFAs favor serine phosphorylation of IRS proteins inhibiting the ability of IRS proteins to attract PI3-k, which in turn decreases the phosphatidylinositols phosphorylation, thereby impairing of downstream effectors (PDK1, Akt), increasing insulin resistance (Aguirre et al. 2002; Liu et al. 2004; Shaw 2011), and promote degradation of the IRS-1 protein (Shah, Wang, and Hunter 2004; Kim et al. 2012). The effect of dietary fat on insulin resistance varies depending on the type of fatty acid consumed. The long chain saturated fatty acids (SFA) have been consistently associated with insulin resistance. Thus, palmitate (16:0), stearate (18:0), arachidate (20:0), and lignocerate (24:0) inhibited insulin-stimulation of glycogen synthesis, as well as activation of Akt (PKB), and induced the accrual of ceramide and diacylglycerol (DAG), 2 lipid metabolites that have been shown to inhibit insulin signaling in cultured cells, and to accumulate in insulin resistant tissues. Ceramide accumulation or inhibition of Akt activation was not found in case of a diet rich in saturated fatty acids with shorter atom carbons chains, such as laurate (12:0) and myristate (14:0) (Chavez and Summers 2003). Koska et al. (2016) studied a human model of dietary fatty acid induced insulin resistance, and concluded that a short-term SFA-enriched diet induced whole body insulin resistance. They observed persistent insulin resistance overnight after the last SFA meal, and the attenuation of resistance by one day of a healthy diet. Concerning the effect of n-3 and n-6 fatty acids on insulin sensitivity, researchers concluded that both types of PUFAs did not induce insulin resistance. Farsi et al. (2014) studied the role of n-3 PUFAs supplementation on FFAs concentration, insulin sensitivity and resistance in 44 type 2 diabetic patients, and reported the improvement of the insulin sensitivity, and a significant reduction in FFAs. According to Lee et al. (2006), diets rich in n-6 PUFAs (linoleic acid, 18:2) appear to prevent insulin resistance by transformation of fatty acids excess into triacylglycerol (TAG). Contrary, L. Huang, Chang, et al. (2018) reported that the decreasing of the ratio of ω-3 PUFAs to ω-6 PUFAs suggested an increased risk for insulin resistance in the skeletal muscle of Znt7-KO mice. Lalia et al. (2015) evaluated the influence of dietary n-3 PUFAs, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), on insulin sensitivity, insulin secretion, and muscle mitochondrial function in insulin-resistant, non-diabetic humans and reported that EPA + DHA do not appear to be effective in reversing peripheral insulin resistance. On the contrary, Gao et al. (2004) studied the contribution of linoleic acid-

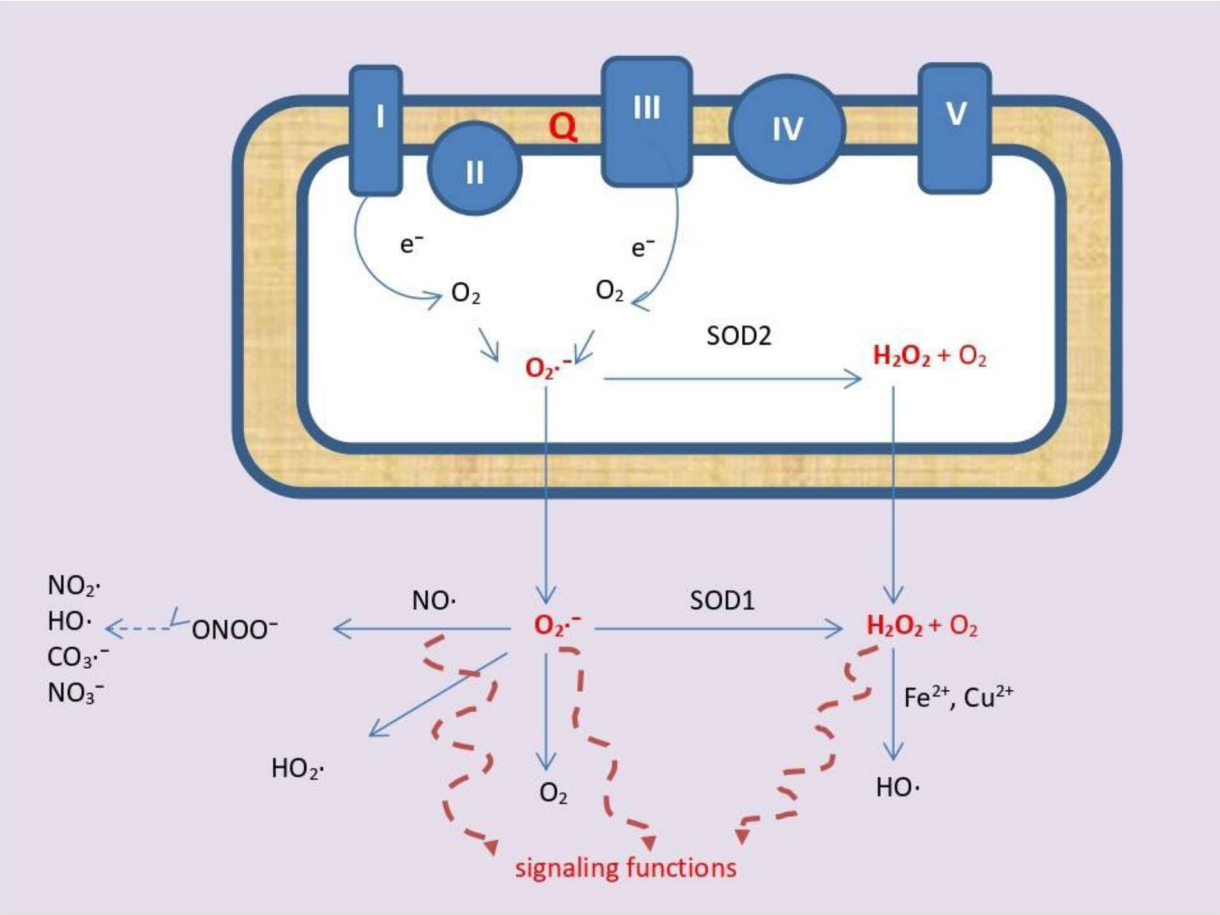

Figure 3. Mitochondrial dysfunctions. The electrons in excess from the mitochondria electro-transport chain are transferred to O_2, which is converted to superoxide anion ($O_2{\cdot}^-$). $O_2{\cdot}^-$ may be converted to H_2O_2, *via* superoxide dismutase (SOD), and then to other ROS and to reactive nitrogen species (RNS).
I: Complex I (NADH: ubiquinone oxidoreductase); II: Complex II (succinate dehydrogenase); III: Complex III (cytochrome bc1 complex); IV: Complex IV (cytochrome c oxidase); Q: ubiquinone; SOD1: Superoxide dismutase [Cu-Zn]; SOD2: Manganese-dependent superoxide dismutase.

signaling pathway to serine phosphorylation and degradation of IRS-1 in adipocytes in dietary obese mice, and reported a reduction in insulin-induced glucose uptake associated with a decrease in IRS-1, and also insulin resistance associated with hyperlipidemia. In lipid insulin sensitivity, peroxisome proliferator-activated receptors (PPARs) play an important role. There are 3 PPAR subtypes, including PPAR-α, PPAR-γ, and PPAR-δ, but from these, PPAR-γ plays a key role. PPAR-γ, present in adipose tissue, but nearly absent in muscle, is involved in the development of insulin resistance as a result of their role in adipogenesis, survival of mature adipocytes, fatty acid uptake, lipid storage, and systemic energy homeostasis (Tyagi et al. 2011).

Mitochondrial dysfunction

The main causes of the mitochondrial dysfunction are the decrease in mitochondrial biogenesis, reduced mitochondrial content, and/or decrease in the expression of mitochondrial oxidative proteins 'per unit of mitochondria' (Montgomery and Turner 2015). The decrease in substrate oxidation (fatty acid oxidation) promotes intracellular lipid accumulation, including deposition of metabolically bioactive lipid mediators such as diacylglycerols (DAG), ceramides (CER), 12,13-dihydroxyocta-9Z-octadecenoic acid (12,13-DiHOME), and 12-hydroxyeicosatetraenoic acid (12-HETE) with a pivotal roles in oxidative stress and inflammation, leading to insulin resistance (Montgomery and Turner 2015; Huang, Chang, et al. 2018; Stanford et al. 2018). Electron microscopy studies have revealed a 38% reduction of muscle mitochondrial density in a lean group insulin resistance offspring of parents with type 2 diabetes. The loss of mitochondrial function was associated with intramyocellular lipid accumulation that lead to the activation of IRS-1 Ser312 and IRS-1 Ser636 phosphorylation, which in turn causes defects in insulin signaling, and insulin resistance in muscle (Morino, Petersen, and Shulman 2006). Mitochondrial dysfunction increases reactive oxygen species (ROS) production whereas mitochondria are the major sites of ROS production. ROS production occurs at the electron transport chain level, located on the inner mitochondrial membrane. The mitochondrial ROS are superoxide anion ($O_2{\cdot}^-$) and hydrogen peroxide (H_2O_2). ROS production is increased when excess electrons are provided to mitochondrial respiratory chain. The excess of electrons is transferred to O_2, which is converted to $O_2{\cdot}^-$. The superoxide anion may be converted to H_2O_2, *via* superoxide dismutase, to other ROS, such as $HO_2{\cdot}$, and to reactive nitrogen species (RNS), such as peroxynitrite anion ($ONOO^-$) (Wang et al. 2018) (Figure 3). ROS and RNS damage various mitochondrial and cellular

components, such as mitochondrial DNA and lipid bilayer, and are suspected to induce mitophagy (removal of damaged mitochondria, and prevention of cell death) or, under high stress levels, apoptosis. The consequence of mitophagy is lowering of the mitochondria number, which leads to the decreasing of the substrate oxidation, further aggravating lipid accumulation and insulin resistance (Montgomery and Turner 2015).

The highest rate of ROS production occurs when the proton gradient is high and oxygen consumption (ATP requirement) is low. Excessive calories ingestion and low energy consumption can lead to high proton-motive force and less ATP request. Consequently, the most electron carriers will be saturated with electrons, and the electrons excess will be transferred to O_2 to form $O_2 \cdot^-$, without ATP generation (Kim, Wei, and Sowers 2008). ROS excess is noxious to cellular physiology whereas disorders of the proteins, DNA, and lipid components of membranes result in mitochondrial dysfunction. The consequences of mitochondrial dysfunction are the accumulation of fatty acid metabolites and allosteric activators of serine kinases that phosphorylate IRS proteins, leading to insulin resistance. Also, ROS stimulate proinflammatory signaling *via* tumor necrosis factor α (TNFα) and interleukin 1β (IL1β) that can contribute to insulin resistance (Kim, Wei, and Sowers 2008; Copps and White 2012). The effects of ROS on insulin signaling pathway were demonstrated in various experimental models with the aid of different prooxidant agents. ROS can effectively block the activity of phosphatases involved in the dephosphorylation of the insulin receptor and its phosphorylated cellular substrates, and can reverse their inhibitory effects on insulin signaling (Afanas'Ev 2010).

Excessive exposure of cells to inflammatory cytokines

Adipose tissue acts as an endocrine organ responsible for the synthesis and secretion of several cell signaling proteins named adipokines or adipocytokines, such as, TNF-α, interleukin-6 (IL-6), leptin, adiponectin, and resistin, which modulate glucose homeostasis. Inflammatory cytokines inhibit the specific pathway for activating insulin receptor-mediated signaling, thereby inhibiting insulin action (Jang, Ridgeway, and Kim 2013). Weisberg et al. (2006) postulated that macrophages, which are present in larger quantities in visceral adipose tissue than in subcutaneous adipose tissue, are the primary source of adipose derived TNF-α and IL-6. TNF-α is an inflammatory cytokine considered a molecular link between obesity and insulin resistance (Wellen and Hotamisligil 2005). Studies performed on different cell types showed that TNF-α is able to inhibit the insulin-stimulated autophosphorylation of IR, and subsequent inhibition of IRS-1 phosphorylation affects the downstream effectors and promotes insulin resistance (Feinstein et al. 1993; Hotamisligil et al. 1994; Uysal, Wiesbrock, and Hotamisligil 1998). Recent studies undertaken on 9 healthy men confirm that excessive concentrations of TNF-α negatively regulate insulin signaling by increasing phosphorylation of p70 S6 kinase (S6K), ERK-1/2, and JNK in skeletal muscle, concomitant with increasing serine phosphorylation of IRS-1 at site 312 (Plomgaard et al. 2005). As TNF-α, IL-6 is an inflammatory citokine that can inhibit the insulin signaling pathway by impairing IRS-1 phosphorylation in adipocytes and hepatocytes. (Coelho, Oliveira, and Fernandes 2013; Akbari and Hassan-Zadeh 2018).

Vitamin D deficiency

In the last years, several prospective studies identified an inverse relationship between vitamin D_3 deficiency and insulin resistance in obese individuals (von Hurst, Stonehouse, and Coad 2010; Hoseini et al. 2013; Durmaz et al. 2017). von Hurst, Stonehouse, and Coad (2010) suggested that improving vitamin D status in insulin resistant subjects has led to improving insulin resistance and sensitivity, but has not led to changing in insulin secretion. Investigations performed on 170 insulin resistant individuals (146 females and 24 males) by Durmaz et al. (2017) showed that exist a positive correlation between insulin resistance and glycosylated hemoglobin, and a negative correlation between vitamin D level and insulin resistance. Benetti et al. (2018) investigated the effect of vitamin D supplementation in a murine model of a diet-induced insulin resistance, and concluded that vitamin D restored the impaired muscle insulin signaling. The biological mechanisms of vitamin D involving in glycemic control has not yet been well understood, but was proposed an association with inherited gene polymorphisms including vitamin D-binding protein (DBP), vitamin D receptor (VDR), and vitamin D 1-alpha-hydroxylase (CYP1alpha) gene. Also, it was suggested the crucial role of the vitamin D insufficiency in the development of autoimmune response against β-cells (Sung et al. 2012) and oxidative stress (Tao et al. 2017). The main factors that induce insulin resistance are summarized in Figure 4.

The effects of polyphenols on insulin resistance

The effects of polyphenols on insulin resistance in clinical trials

The most investigated markers of insulin resistance are homeostatic model assessment of insulin resistance (HOMA-IR), insulin sensitivity check index (QUICKI), and insulin sensitivity index (ISI) calculated using glucose level and insulin level during oral glucose tolerance test (OGTT). The most investigated markers for inflammation, associated with insulin resistance, are plasminogen activator inhibitor-1 (PAI-1) and high-sensitivity C-reactive protein (hsCRP), IL-6, TNFα, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), secretory phospholipase A2 (sPLA2), soluble E-selectin (SE-selectin), soluble intracellular adhesion molecule-1 (ICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), and von Willebrand factor (vWF) levels. Other marker is adiponectin level. Adiponectin is negatively correlated in vivo with inflammation markers (Krakoff et al. 2003) and insulin resistance (Yadav et al. 2013), and may be the link between adiposity, inflammation, and type 2 diabetes (Krakoff et al. 2003; Toulis et al. 2018).

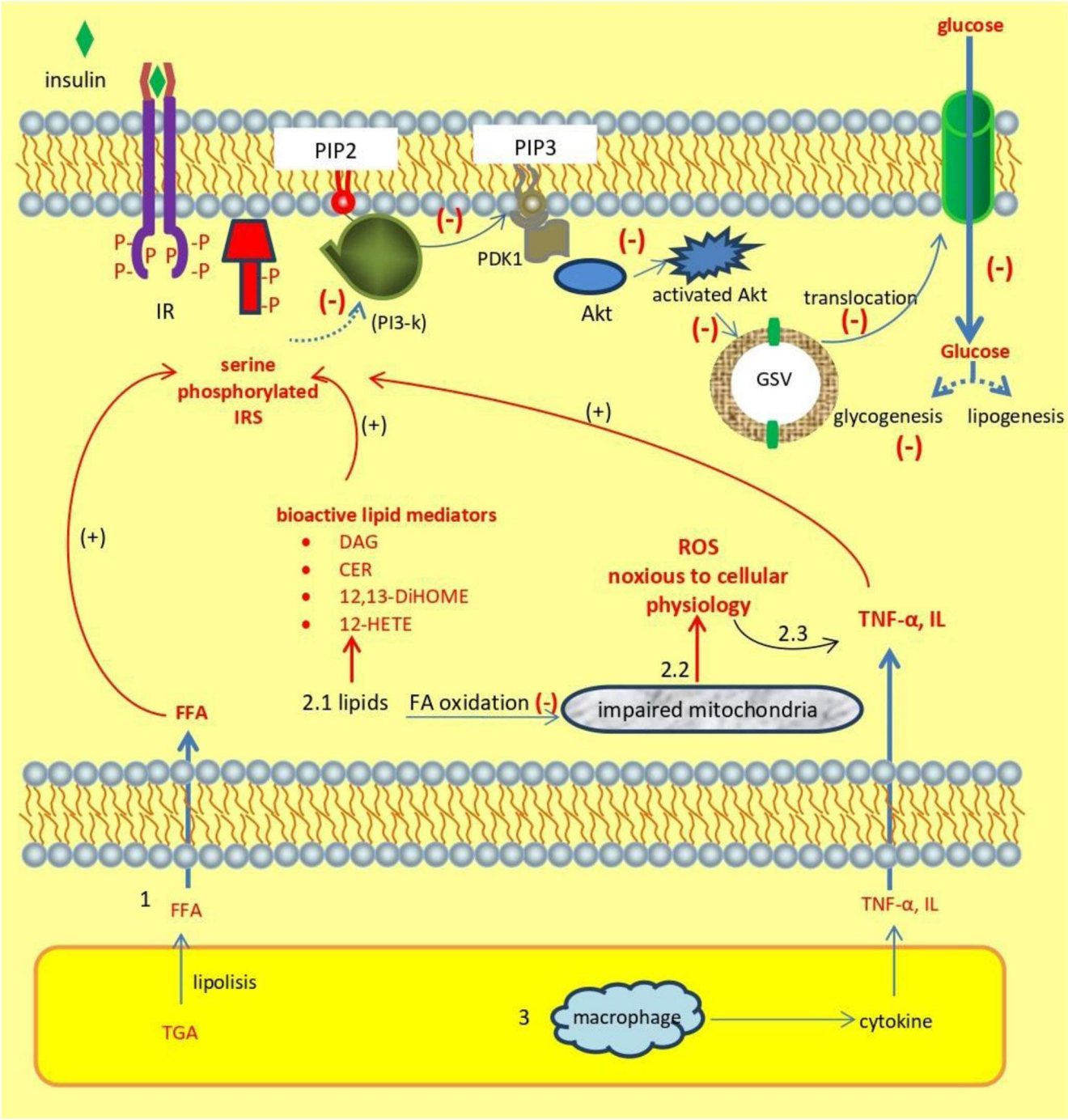

Figure 4. The inhibition of signaling downstream of the insulin receptor. 1. FFAs released by subcutaneous fat cell lipolysis cross the membrane of the muscle cell and favor serine phosphorylation of IRS proteins inhibitingthe insulin signaling cascade. 2. Mitochondrial dysfunction. 2.1. The decrease in FA oxidation promotes intracellular lipid accumulation, including deposition of metabolically bioactive lipid mediators such as DAG, CER, 12,13- DiHOME, 12-HETE that favor serine phosphorylation of IRS proteins. 2.2. Excessive ROS production is noxious for cellular physiology. 2.3. ROS stimulate proinflammatory signaling *via* TNF-α and IL1β; 3. Macrophages from adipose tissue release adipokines that cross the membrane of the muscle cell and favor serine phosphorylation of IRS proteins.
FFAs: free fatty acids; FA: fatty acids; DAG: diacylglycerols TGA: triacylglycerols; CER: ceramides; 12,13-DiHOME: 12,13-dihydroxyocta-9Z-octadecenoic acid; 12-HETE: 12-hydroxyeicosatetraenoic acid; ROS: reactive oxygen species; TNF-α: tumor necrosis factor α, IL: interleukins.

In this review are presented some suggestions drawn from studies that show the positive effects of polyphenols on improving insulin sensitivity, as well as studies that suggest that these biomolecules are not involved in mechanisms that help alleviate insulin resistance. Results of researches conducted over the past 2 decades showed that polyphenols alleviate insulin resistance by several mechanisms. They can be inhibitors for serine IRS proteins phosphorylation, improvers for protein kinase B (Akt) phosphorylation, promoters for GLUT4 translocation, and powerful scavengers of the ROS superoxide. Also, has been demonstrated that some polyphenols can improve gene expression of proteins involved in insulin signaling. Studies on the effect of polyphenols on insulin resistance were performed on cohorts/patients predisposed to type 2 diabetes or diagnosed with type 2 diabetes who took polyphenols from food, but also

on animals/cell culture models treated with extracts rich in polyphenols obtained from different plants or with commercial polyphenols.

Grassi et al. (2005) studied the effect of flavanol-rich dark chocolate, comparatively with white chocolate, on insulin resistance in 20 patients (10 males and 10 females) with never-treated essential hypertension. The patients received daily 100 g dark chocolate bars, containing catechin, epicatechin, quercetin, kaempferol, and isorhamnetin, or 90 g flavanol-free white chocolate bars without any flavonoids over a period of 15 days. 7 days after the end of the treatment, they found that only dark chocolate decreased significantly HOMA-IR, improved QUICKI and ISI, and suggested that dark chocolate ameliorated insulin sensitivity in hypertensive patients. Whereas, it has been shown that insulin sensitivity is partly dependent on insulin-mediated NO release, Grassi et al. (2005) suggested that flavanols and other dietary antioxidants may decrease insulin resistance in hypertensives by ameliorating NO bioavailability. Stull et al. (2010) reassessed insulin sensitivity and inflammatory biomarkers on 32 obese, non-diabetic, and insulin-resistant subjects who had in the diet either a smoothie containing blueberry bioactive polyphenols (anthocyanins) or a smoothie of equal nutritional value without added blueberry, twice daily for 6 weeks. They reported an insulin sensitivity improvement, without significant changes in inflammation biomarkers, in blueberry bioactive polyphenols diet group. Jennings et al. (2014) investigated the association between a gamma of flavonoid subclasses, as well as their main dietary sources, and insulin resistance markers. They monitored a cohort of healthy women aged between 18 and 76 years with a diet rich in one of the following flavonoid subclasses: flavanones (eriodictyol, hesperetin, and naringenin), anthocyanins (cyanidin, delphinidin, malvidin, pelargonidin, petunidin, and peonidin), flavan-3-ols (catechins and epicatechins), flavonols (quercetin, kaempferol, myricetin, and isohamnetin), flavones (luteolin and apigenin), and polymers (including proanthocyanidins, theaflavins, and thearubigins). Jennings et al. (2014) found that higher anthocyanins intake was associated with significantly lower peripheral insulin resistance, lower hsCRP concentration, associated with lower insulin and inflammation levels, while higher flavones intake was associated with significantly lower peripheral insulin resistance and the improvement of the adiponectin concentrations. They reported no significant associations with total or other flavonoid subclasses, and suggested that more dose–response intervention trials on anthocyanins/flavones and anthocyanin/flavones-rich foods for the prevention and management of type 2 diabetes are needed. A recent cohort analysis of the non-diabetic participants in the PREDIMED (Prevención con Dieta Mediterránea) had as its objective the examination of the associations between the intake of total polyphenols and different classes/subclasses of polyphenols [flavonoids (flavanones, and dihydroflavonols), phenolic acids, stilbenes, lignans, and others] on the risk of incident diabetes trial (Tresserra-Rimbau et al. 2015). To achieve this research goal, they evaluated the effects of either a Mediterranean diet, supplemented with extra-virgin olive oil or nuts, or advised them to adhere to a low-fat control diet on cardiovascular outcomes in elderly men and women at high cardiovascular disease risk. They reported inversely association between the intake of polyphenols and subclasses of polyphenols and diabetes risk. Also, after multivariable adjustment, they observed a 28% reduction in new-onset diabetes in the highest tertile compared with the lowest tertile of total polyphenol intake. Based from these observations Tresserra-Rimbau et al. (2015) suggested that a high intake of total polyphenols can reduce the risk of diabetes in elderly persons at high risk of cardiovascular disease. In insulin resistant overweight or obese non-diabetic adults who consumed a strawberry and cranberry beverage, rich in proanthocyanidins and phenolic acids (especially p-coumaric acid, m-coumaric acid, and coumaroyl glucoside), 333 mg polyphenols daily for 6 weeks, the insulin sensitivity was improved and the increasing in compensatory insulin secretion was prevented, without affecting plasma lipids, hsCRP, pro-inflammatory cytokines, and antioxidant capacity (Paquette et al. 2017). Contrary, some result on studies undertaken on human subjects showed that a diet rich in some classes of polyphenols did not influence the markers of insulin resistance and the marker for diseases associated with type 2 diabetes. Thus, studies conducted to determine the effect of green tea, plant rich in flavonoids, consumption on insulin resistance, and on other parameters associated with diabetes, such as inflammatory markers, showed that after consuming 9 g of green tea/day for 4 weeks, were registered no significant changes in investigated parameters. Ryu et al. (2006) concluded that green tea consumption does not influence insulin resistance, adiponectin, hsCRP, and IL-6 levels, in type 2 diabetes patients. Song et al. (2005) calculated relative risks (RRs) of incident type 2 diabetes on 38,018 women, aged over 45, and free of cardiovascular disease, cancer and diabetes, according to dietary intake of total or individual flavonols and flavones, and flavonoid-rich foods (tea, apples, broccoli, onions, and tofu) concluded that the hypothesis that high intake of flavonols and flavones protects against the development of type 2 diabetes has not been demonstrated. 29 overweight/obese (16 males and 13 females) provided 2 well-controlled diets that were identical in macronutrient content (protein; fat and carbohydrate), but differed markedly in flavonoid content (low flavonoid diet, 10 mg/1000 kcals; high flavonoid diet, 340 mg/1000 kcals) for 6 weeks. Surprisingly, markers of insulin resistance (fasting insulin, glucose, HOMA-IR) were modestly, but significantly higher on the high flavonoid diet relative to the low flavonoid diet. Moreover, the high flavonoid diet had no significant effects on blood pressure and plasma lipids (triglycerides, LDL-C, HDL-C), and tended to increase (all non-significant) markers of inflammation (hsCRP, soluble TNFα receptor-1, soluble TNFα receptor-2, IL-6) (Lefevre et al. 2016). Oral supplementation with genistein for 8 weeks in nonalcoholic fatty liver patients has led to lowering of insulin resistance, lipid peroxidation and inflammation, reflected in low levels of serum insulin, HOMA-IR, serum malondialdehyde (MDA), TNF-α and IL-6 (Amanat et al. 2018). Many studies evaluated the effect of resveratrol

administration on insulin sensitivity in controlled trials. In a meta-analysis undertaken on 388 subjects Liu et al. (2014) showed that resveratrol reduced insulin resistance in persons with diabetes, but does not affect glycemic measures in healthy persons. Improving of insulin sensitivity by resveratrol, in human model was also reported by Gospin et al. (2016). They reported that the glucose uptake was accompanied by the decrease of the pro-inflammatory cytokines TNFα and IL6, and the increase of the adiponectin expression in the adipose tissue. Méndez-del Villar et al. (2014) conducted a clinical trial in patients with diagnosis of metabolic syndrome and suggested that *trans*-resveratrol decrease total insulin secretion and increase insulin sensitivity. Effects of polyphenols rich foods and individually polyphenols on insulin resistance in some clinical trials are summarized in Table 1.

The effects of polyphenols on insulin resistance in different experimental models

The effects of polyphenols on decreasing of insulin resistance had also been demonstrated on different models using rich-polyphenols extracts obtained from various sources and commercially polyphenols. In Table 2 are summarized the plant extracts rich in polyphenols/commercially polyphenols effects on insulin resistance in different experimental models presented in this review. Intensively studied were the extracts rich in anthocyanins. Thus, it was demonstrated that a chokeberries extract decreases the insulin resistance and inflammation markers levels after treatment of the experimental animals (Qin and Anderson 2012). The extract obtained from *Sophora davidii* (Franch.) skeels, rich in the flavonoids apigenin, maackiain, leachianone A, and leachianone B, was used for in vitro and in vivo studies to elucidate the antidiabetic activity mechanism. In L6 cells the results indicated that the extract increased glucose uptake by enhanced GLUT4 expression and translocation to the plasma membrane *via* the AMPK pathway. In vivo studies, using a spontaneously type 2 diabetes model (KK-Ay mice), demonstrated the capacity of the extract to improve oral glucose tolerance, to reduce serum insulin level and HOMA-IR value confirming the same mechanism responsible for *Sophora davidii* (Franch.) skeels extract in improving insulin resistance (Huang, Chang, et al. 2018). *Cyclocarya paliurus* leaves ethanolic extract, rich in chlorogenic acid, cryptochlorogenic acid and quercetin-3-O-β-D-glucuronide, increased glucose uptake in C2C12 cells by the following suggested pathways: stimulation of GLUT4 translocation to the plasma membrane; enhancing of tyrosine phosphorylation of IRS, and activation of phosphatidylinositol 3-kinase and protein kinase B (Akt) *via* sirtuin1 (Yoshitomi et al. 2017). In diabetic mice model, *Cyclocarya paliurus* extract increased the expression of skeletal muscle membrane GLUT4, and enhanced the phosphorylation of Akt (Yoshitomi et al. 2017). Zhai et al. (2018) suggested that in diabetic mice, a *Cyclocarya paliurus* leaves ethanolic extract (rich in flavonoids and phenolic acids) has beneficial effects, possibly related with PI3-k signaling and mitogen-activated protein kinase (MAPK) signaling pathways. A navel orange hydroethanolic extract rich in naringin and naringenin, naringin, and naringenin administrated on nicotineamide (NA)/streptozotocin (STZ)-induced type2 diabetic rats, enhanced the mRNA expression of insulin receptor β-subunit, GLUT4, and adiponectin in adipose tissue. For these reasons, Ahmed et al. (2017) suggested that the antidiabetic effects of the extract and the commercially flavonoids are induced *via* 2 pathways: insulinotropic effects and improved insulin signaling action. Naringenin and mangiferin isolated from *Salacia oblonga* exhibited antidiabetic activity on streptozotocin-induced diabetic rats. The real-time reverse transcription polymerase chain reaction and western blot analyses revealed that the antidiabetic properties of these polyphenols are the results of gradual activation of peroxisome proliferator-activated receptor-gamma (PPARγ)/GLUT4 signaling pathways and oxidative stress reduction activity (Singh et al. 2018). Also, it was suggested that naringenin and mangiferin had a strong binding affinity toward PPARγ and GLUT4 (Singh et al. 2018). In differentiated myotubes, and in mice model of insulin resistance and type 2 diabetes, it has been shown thatquercetin-3-O-rutinoside (rutin), isolated from *Toona sinensis* Roem enhanced insulin receptor kinase (IRK) activity, thereby improving the insulin signaling pathway resulting enhanced GLUT4 translocation, and increased glucose uptake (Hsu et al. 2014). An ethanol extract of *Fagopyrum tataricum* (buckwheat) and the polyphenols found in this extract, rutin, quercetin, and rutin + quercetin, were used in a cells model and an animal model to elucidate anti-insulin resistance mechanism. *Fagopyrum tataricum* extract, rutin, quercetin, and rutin + quercetin attenuated insulin resistance *via* activation of Akt phosphorylation and prevention of PPARγ degradation, caused by high-glucose induction for 48 hs in FL83B hepatocytes. Also, *Fagopyrum tataricum* ethanol extract inhibited the increase of blood glucose and insulin levels in high fructose diet-induced diabetic mice (Lee, Hsu, et al. 2012). In the last years, was evaluated the effect of resveratrol administration on insulin sensitivity in animal and cell culture models. After 2 years of resveratrol administration in Rhesus monkeys with high-fat and high-sugar diet, Jimenez-Gomez et al. (2013), found that resveratrol decreased adipocyte size, increased sirtuin 1 expression, decreased nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation and improved insulin sensitivity in visceral, but not in subcutaneous white adipose tissue. Also, they suggested that resveratrol modulates the expression of insulin-signaling markers in white adipose tissue whereas increasing of IRS 1 protein levels, decreasing of Akt serine 473 phosphorylation, and increasing the content of insulin-responsive GLUT4 in visceral fat depot were observed. Patel, Gupta, and Dey (2011) demonstrated, using a specific pharmacological inhibitor that in Neuro-2A (N2A) cells, resveratrol stimulates insulin signaling *via* improving phosphorylation of protein kinase B (Akt) and glycogen synthase kinase-3β (GSK-3β), and glucose uptake *via* activation 5′-adenosine monophosphate-activated protein kinase (AMPK). The peripheral insulin resistance was improved by resveratrol in 2 diabetic models, Irs2-deficient [Irs2(-/-)] mice and streptozotocin (STZ)-injected mice (González-Rodríguez et al. 2015). Yan et al. (2012) used obese KK-ay mice, high-fat diet-induced obese rats, and induced insulin resistant 3T3-L1 adipocytes as models to study the effects of (–)-epigallocatechin gallate

Table 1. Effects of polyphenols rich foods and individually polyphenols on insulin resistance in some clinical trials.

Polyphenols (sources/ subclass/name)	Clinical trial/observational prospective cohort studies	Insulin resistance markers	Conclusions	References
Flavanols rich dark chocolate (catechin, epicatechin, quercetin, kaempferol, and isorhamnetin)	20 patients treated 15 days with dark chocolate or flavanol-free white chocolate	HOMA-IR↘, QUICKI↗, ISI↗	Flavanols from cocoa decrease insulin resistance by ameliorating NO bioavailability	Grassi et al. (2005)
Smoothie containing blueberry bioactive polyphenols (anthocyanins)	32 obese, nondiabetic, and insulin-resistant subjects (men and women)	ISI↗	The bioactives from blueberries enhance insulin sensitivity	Stull et al. (2010)
Diet rich in one of the following flavonoid subclasses:	1997 healthy women aged between 18 and 76 years		Dose–response trials are required to ascertain optimal intakes for the potential reduction of type 2 diabetes risk	Jennings et al. (2014)
• flavanones (eriodictyol, hesperetin, and naringenin)		HOMA-IR and ADPN→		
• anthocyanins (cyanidin, delphinidin, malvidin, pelargonidin, petunidin, and peonidin)		HOMA-IR↘, ADPN↗		
• flavan-3-ols (catechins and epicatechins)		HOMA-IR and ADPN→		
• flavonols (quercetin, kaempferol, myricetin, and isohamnetin)		HOMA-IR and ADPN→		
• flavones (luteolin and apigenin) and polymers (including proanthocyanidins, theaflavins, and thearubigins)		HOMA-IR↘, ADPN↗		
• total flavonoids subclasses		HOMA-IR and ADPN→		
Mediterranean diet (MedDiet) supplemented with extra-virgin olive oil or nuts or advice to adhere to a low-fat control diet	3430 individuals free of diabetes at baseline (October 2003 to December 2010)	Glucose concentration↘	A high intake of total polyphenols and total flavonoids is associated with a reduced risk of diabetes in elderly persons	Tresserra-Rimbau et al. (2015)
Strawberry and cranberry beverage, rich in proanthocyanidins and phenolic acids	46 overweight or obese and insulin resistant non-diabetic adults who consumed daily 333 mg polyphenols for 6 weeks from beverages	ISI↗	Strawberry and cranberry polyphenols improve insulin sensitivity in insulin resistance	Paquette et al. (2017)
Green tea	55 type 2 diabetes patients consumed 900 mL water containing 9 g of green tea daily 4 weeks	HOMA-IR→, ADPN→	Green tea consumption does not influence insulin resistance	Ryu et al. (2006)
Flavonoid-rich foods (apple tea, broccoli, onions, tofu), individual flavonols and flavones including quercetin, kaempferol, myricetin, apigenin, and luteolin	38,018 women, aged over 45 and free of cardiovascular disease, cancer and diabetes	HOMA-IR↗	High intake of flavonols and flavones protection against the development of type 2 diabetes have not been demonstrated by the study	Song et al. (2005)
Flavonoid-rich diet	29 overweight/obese patients	HOMA-IR↗	A high flavonoid diet is not improving markers of insulin resistance	Lefevre et al. (2016)
Genistein	41 patients with nonalcoholic fatty liver disease daily supplemented with 250 mg genistein for 8-weeks	HOMAR-IR↘	Genistein reduce insulin resistance	Amanat et al. (2018)
Resveratrol	388 subjects ingested resveratrol for ≥2 wk	HOMA-IR↘	Resveratrol significantly improves insulin sensitivity in persons with diabetes	Liu et al. (2014)
Resveratrol	21 non-diabetic subjects ingested 2 g resveratrol/ day, 28 days	HOMA-IR↘ ADPN expression ↗	Resveratrol improves insulin sensitivity in humans	Gospin et al. (2016)
trans-Resveratrol	24 patients with diagnosis of metabolic syndrome ingested 500 mg *trans*-resveratrol 3 times *per* day before meals for 90 days	ISI↗	Resveratrol improves insulin sensitivity	Méndez-del Villar et al. (2014)

↗ – significant increase; ↘ – significant decrease; → – insignificant changes.
ADPN – adiponutrin; HOMA-IR – homeostatic model assessment of insulin resistance; ISI – insulin sensitivity index; QUICKI – insulin sensitivity check index.

Table 2. Action mechanism of some plant extracts rich in polyphenols/commercially polyphenols on insulin resistance in different experimental models.

Plant extract/polyphenols	Experimental model	Action mechanism	Conclusions	References
Chokeberries extract	Rats fed a fructose-rich diet	Modulates multiple pathways associated with insulin signaling	Decreases risk factors related to insulin resistance	Qin and Anderson (2012)
Sophora davidii (Franch.) skeels extract rich in the flavonoids apigenin, maackiain, leachianone A and leachianone B	L6 cells Spontaneously type 2 diabetic models (KK-A^y mice)	Enhances GLUT4 expression and translocation to the plasma membrane *via* the AMPK pathway, stimulating glucose uptake	Improves insulin resistance	Huang, Hao, et al. (2018)
Cyclocarya paliurus leaves ethanolic extract rich in chlorogenic acid, cryptochlorogenic acid and quercetin-3-O-β-D-glucuronide	C2C12 cells	Stimulates the translocation of GLUT4 to the plasma membrane Enhances tyrosine phosphorylation of IRS and activates PKB (Akt) *via* SIRT1	Exerts antidiabetic effects similar to those of insulin	Yoshitomi et al. (2017)
	STZ-induced type 2 diabetic rats	Increases of the expression of skeletal muscle membrane GLUT4 and enhances the phosphorylation of Akt		
Cyclocarya paliurus leaves ethanolic extract	High fat diet and STZ-induced diabetic mice	Interacts with the molecules involved in PI3-k and MAPK signaling pathways	Improves diabetes complications	Zhai et al. (2018)
Navel orange peel hydroethanolic extract, naringin and naringenin	NA/STZ-induced type 2 diabetic rats	Enhances the mRNA expression of insulin receptor β-subunit, GLUT4 and adiponectin expression in adipose tissue Has antioxidant activity	The extract, naringin and naringenin have insulinotropic effects and improve insulin action	Ahmed et al. (2017)
Naringenin and mangiferin isolated from *Salacia oblonga* leaves	STZ-induced diabetic rats	Activate PPARγ/GLUT4 signaling pathways Reduce oxidative stress	Naringenin and mangiferin have antidiabetic properties	Singh et al. (2018)
Rutin isolated from *Toona sinensis* Roem	Differentiated myotubes	Enhances insulin receptor kinase (IRK) activity Increases GLUT4 translocation	Rutin may be a potential agent for glycemic control	Hsu et al. (2014)
	S961-treated C57BL/6 mice	Normoglycemic effect in the OGTT		
Fagopyrum tataricum (buckwheat) extract, rutin, rutin and quercetin	FL83B hepatocytes High fructose diet-induced diabetic mice	Activate Akt phosphorylation and prevent PPARγ degradation ROS scavengers Promotors for antioxidant enzymes	*F. tataricum* extract, rutin, quercetin, rutin and quercetin may reduce insulin resistance by improving insulin signaling	Lee, Hsu, et al. (2012)
Resveratrol	Rhesus monkeys with high-fat and high-sugar diet	Increases of IRS-1 protein levels, decreases of Akt serine 473 phosphorylation and increases in the content of insulin-responsive GLUT4 in visceral fat depot	Modulates the expression of insulin-signaling protein markers in visceral white adipose tissue	Jimenez-Gomez et al. (2013)
Resveratrol	Neuro-2A (N2A) cells	Improves phosphorylation of Akt and GSK-3β *via* AMPK	Potentiates insulin signaling	Patel, Gupta, and Dey (2011)
Resveratrol	Irs2-deficient (Irs2(-/-)) mice STZ-injected mice	Restores IRS1-mediated insulin signaling	Improves systemic insulin sensitivity	Gonzalez-Rodriguez et al. (2015)
(–)-epigallocatechin gallate (EGCG) Green tea catechins (GTCs)	KK-A^y mice, high-fat diet-induced obese rats Induced insulin resistant 3T3-L1 adipocytes	EGCG decreases JNK phosphorylation and promotes GLUT-4 translocation; attenuates ROS generation; GTCs reduces ROS content in animals and adipocytes	EGCG and GTCs reduce adipose insulin resistance	Yan et al. (2012)
EGCG	High-fat diet induced insulin resistance in male C57BL/6J mice	Restores insulin-stimulated phosphorylation of eNOS, IRS-1, and Akt	Improves insulin sensitivity	Jang, Ridgeway, and Kim (2013)
Gallic acid from *Cyamopsis tetragonoloba*	High-fat diet fed-STZ-induced experimental type 2 diabetic rats	Interactions with GLUT4, GLUT1, PI3-k, activates Akt and PPARγ	May improve adipose tissue insulin sensitivity	Gandhi et al. (2014)
Gallic acid	High-fat diet fed-STZ-induced experimental type 2 diabetic rats	Activates the AMPK/Sirt1/PGC-1α pathway	Gallic acid or its derivatives may be a potential therapeutic intervention for insulin resistance	Doan et al. (2015)
Gallic acid	High-fructose diet-induced diabetic rats	Restores the expression of insulin signaling-related proteins, such as IR, PKC-ζ,	Is a potent polyphenol in preventing the progression of diabetes mellitus (DM) complications	Huang, Chang, et al. (2018)

(*continued*)

Table 2. Continued.

Plant extract/polyphenols	Experimental model	Action mechanism	Conclusions	References
		and GLUT4 in the perirenal adipose tissues		
Apigenin	High-fat diet -induced obese mice	Decreases insulin resistance *via* metabolic and transcriptional modulations in the liver	Ameliorates insulin resistance	Jung, Cho, and Choi (2016)
Ferulic acid	High-fructose diet-induced diabetic rats	Restores normal induction of NO	Alleviates insulin resistance	El-Bassossy et al. (2016)
Ferulic acid	High-fat and fructose-induced type 2 diabetic adult male rats	Reduces the negative regulators of insulin signaling	Improves insulin sensitivity	Narasimhan, Chinnaiyan, and Karundevi (2015)
Caffeic, ferulic, gallic and protocatechuic acids	High-fructose diet-induced metabolic syndrome in rats	Restore, in normal levels metabolic hormones (insulin, leptin, and adiponectin), the activity of the antioxidant enzymes and the oxidative stress	Phenolic acids reverse insulin resistance	Ibitoye and Ajiboye (2018)
Ferulic acid	High-fat diet (HFD)-induced obese mice	Up-regulates the expressions of IRS-1, PI3-k, and Akt	Has anti-insulin resistance effect	Naowaboot et al. (2018)
Ferulic acid and γ-Oryzanol	High-fat and high-fructose rodent diet	Inhibit intracellular triglyceride accumulation; decrease inflammation; increase ADPN level	Ferulic acid and γ-Oryzanol reduce insulin resistance	Wang et al. (2015)
Naringenin and hesperidin	high fat-fed/STZ-induced type 2 diabetic rats	Increase in adipose tissue GLUT4 mRNA expressions	Naringenin and hesperidin have potent antihyperglycemic activity *via* insulin signaling pathway	Mahmoud et al. (2015)
Naringenin	High fructose diet rats	Enhances insulin-stimulated tyrosine phosphorylation	Improves insulin signaling and sensitivity	Kannappan and Anuradha (2010)
Genistein	High fructose induced insulin resistance in rodent	Antioxidant activity; decreases inflammation	Reduces insulin resistance	Incir et al. (2016)
Chicoric acid	Glucosamine in HepG2 cells	Increases the phosphorylation of the AMPK, down-regulates IRS-1 serine phosphorylation, and activates the PI3-k/Akt, stimulates translocation of GLUT2, reduces oxidative stress	Attenuates insulin resistance	Zhu et al. (2015)
Chicoric acid	Palmitate-induced insulin-resistant C2C12 myotubes High-fat-fed mice	Enhances mitochondrial membrane potential and oxygen consumption Increases the expression of genes related to mitochondrial biogenesis and oxidative phosphorylation in the liver and skeletal muscle	Attenuates insulin resistance and promotes insulin sensitivity by enhancing mitochondrial function	Kim et al. (2018)
Nobiletin	Core clock gene Bmal1	Activates IRS-1/Akt insulin signaling pathway; attenuates palmitate-stimulated excessive secretions of ROS; restores the depletion of mitochondrial membrane potential	May serve as a nutritional preventive strategy in recovering metabolic disorders	Qi et al. (2018)
Quercetin	Fluorescence titration experiment, STD-NMR experiments	Interaction between quercetin and the VDR at the molecular level may serve as a scaffold for the development of VDR modulators	Has selective biological activities	Benetti et al. (2018)

ADPN – adiponutrin; Akt/PKB – protein kinase B; AMPK – 5′ adenosine monophosphate-activated protein kinase; eNOS – endothelial nitric oxide synthase; GLUT1 – glucose transporter 1; GLUT2 – glucose transporter 2; GLUT4 – glucose transporter 4; GSK-3β – glycogen synthase kinase-3β; IRS – insulin receptor substrate; JNK – Jun kinase; MAPK – mitogen-activated protein kinase; NA – nicotineamide; OGTT – oral glucose tolerance test; PGC-1α - peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PI3-k – phosphoinositide 3-kinase; PKC-ζ – protein kinase C-zeta; PPARγ – peroxisome proliferator-activated receptor-gamma; ROS – reactive oxygen species; SIRT1 – sirtuin 1; STD – saturation transfer difference; STZ – streptozotocin; VDR – vitamin D receptor.

(EGCG) and green tea catechins (GTCs) on insulin signals, and found that EGCG had mitigated insulin resistance, and TNF-α promoted ROS generation, increased glucose uptake ability, and promoted GLUT4 translocation. Research findings have led to the conclusion that EGCG and green tea catechins (GTCs) could reduce adipose insulin resistance, due to their ROS scavenging activity. Similar results were reported by Jang, Ridgeway, and Kim (2013). They showed that in mice model

fed a high-fat diet with EGCG supplement that insulin-stimulated phosphorylation of endothelial nitric oxide synthase (eNOS), insulin receptor substrate-1 (IRS-1), and protein kinase B (Akt) were restored leading to the improving of insulin sensitivity, and endothelial function. Gallic acid, compound with 3 phenolic OH, given to high-fat diet fed-streptozotocin-induced experimental type 2 diabetic rats, improved adipose tissue insulin sensitivity, and enhanced glucose uptake. Gandhi et al. (2014) explained these results by suggesting the docking of gallic acid with peroxisome proliferator-activated receptor gamma (PPARγ), and the exhibiting of promising interactions with the GLUT4, GLUT1, PI3-k, and activated (phosphorylated) Akt. Doan et al. (2015) suggested that gallic acid might be a potential therapeutic intervention for insulin resistance because this compound regulated body weight and glucose homeostasis trough AMP-activated protein kinase (AMPK) activation in gallic acid-treated high-fat diet fed-STZ-induced experimental type 2 diabetic rats. Doan et al. (2015) also suggested that this phenolic compound regulates mitochondrial function *via* the activation of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α). The possible implication of gallic acid in the phosphatidylinositol 3-kinase (PI3-k)/phosphorylated protein kinase B (Akt) dependent pathway was reported by Huang, Chang, et al. (2018). They reported that gallic acid restored expression of insulin signaling-related proteins, such as insulin receptor (IR), protein kinase C-zeta (PKC-ζ), and GLUT4 in the perirenal adipose tissues of high-fructose diet-induced diabetic rats. The ability to decrease insulin resistance has also been suggested in animal studies for apigenin. C57BL/6J mice fed with apigenin-supplemented high-fat diet showed lower plasma levels of pro-inflammatory mediators and fasting blood glucose (Jung, Cho, and Choi 2016). In the last 5 years cinnamic acid derivatives (ferulic acid, caffeic acid, sinapic acid, p-methoxycinnamic acid, isoferulic acid), and other phenolic acids have been extensively studied due to their antidiabetic properties, and it was found that they alleviate insulin resistance (Wang et al. 2015; El-Bassossy et al. 2016; Narasimhan, Chinnaiyan, and Karundevi 2015; Naowaboot et al. 2018). Ferulic acid, a hydroxycinnamic acid, alleviated insulin resistance in fructose-fed rats (El-Bassossy et al. 2016; Narasimhan, Chinnaiyan, and Karundevi 2015). Ibitoye and Ajiboye (2018) investigated the influence of caffeic, ferulic, gallic, and protocatechuic acids on high-fructose diet-induced metabolic syndrome in rats, and found that the mentioned phenolic acids reverse insulin resistance, and other related diseases such as, inflammation and oxidative stress. Obese mice treated with ferulic acid improved the insulin sensitivity in hypothalamus, and up-regulated the expressions of insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3-k), and phosphorylated-protein kinase B (Akt) (Naowaboot et al. 2018). Ferulic acid or a stearilferulate, named γ-Oryzanol, supplemented high-fat and high-fructose rodent diet for 13 weeks. Ferulic acid and γ-Oryzanol exhibited similar effects in alleviating HFFD-induced insulin resistance, but γ-Oryzanol significantly decreased serum levels of CRP and IL-6, and increased serum concentration of adiponectin (Wang et al. 2015). Commercially flavonoids, naringin, naringenin, hesperidin, and mangiferin, were demonstrate to have potential health benefits for the prevention and the treatment of type 2 diabetes. So, in high fat-fed/STZ-induced type 2 diabetic rats' model, Mahmoud et al. (2015) showed that hesperidin and naringin have potent antihyperglycemic activity *via* insulin signaling pathway. They suggested that the administration of both mentioned phenolics in diabetic rats increased GLUT4 mRNA expressions in adipose tissue. Naringenin, was orally administered to high fructose diet rats for 45 days. The degree of protein tyrosine phosphorylation in response to insulin signaling was determined by assaying protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) in the liver. If in control group PTP activity was higher, while PTK activity was lower, in the group treated with naringenin reversed this alteration suggesting the improvement of the insulin signaling and sensitivity, *via* enhancing insulin-stimulated tyrosine phosphorylation (Kannappan and Anuradha 2010). The isoflavone genistein administrated for 6-8 weeks in fructose induced insulin resistance rodent model caused a significant decrease in HOMA-IR (Incir et al. 2016). Also, Incir et al. (2016) showed that genistein administration in rats fed with fructose lowered oxidative stress, and inflammations, confirmed by serum 8-isoprostane, liver TNF-α, serum and liver IL-6, and serum visfatin levels lower.

Zhu et al. (2015) investigated the phosphorylation of IRS-1 and Akt in HepG2 cells to determine if chicoric acid could be directly concerned with the regulation of the insulin signaling pathway. Chicoric acid ameliorated insulin resistance by increasing the phosphorylation of the AMP-activated protein kinase (AMPK). It also down-regulated IRS-1 serine phosphorylation and activated the PI3-k/Akt pathway to reverse the insulin resistance *via* activation of the antioxidant response. Also, chicoric acid down-regulated the expression of PTP1B and IRS-1 serine phosphorylation, activated the PI3-k/Akt pathway to reverse the insulin resistance *via* suppression of oxidative stress, and stimulated the translocation of GLUT2. Investigations carried out to determine the effect of chicoric acid on insulin resistance and mitochondrial dysfunction in diet-induced obese mice model showed that chicoric acid treatment significantly increased the mitochondrial DNA content, citrate synthase, and ATP content, as well as the expression of genes related to mitochondrial biogenesis and oxidative phosphorylation in the liver and skeletal muscle. These results led Kim et al. (2018) to suggest that in diet-induced insulin resistance chicoric acid attenuated insulin resistance by partly modulation of mitochondrial function. Similar results were found for the flavonoid nobiletin, which increased activated IRS-1/Akt insulin signaling pathway, attenuated palmitate-stimulated excessive secretions of ROS, and restored the depletions of mitochondrial membrane potential (Qi et al. 2018).

Lee et al. (2016), used fluorescence titration and saturation transfer difference (STD) NMR experiments to study the interaction of quercetin with vitamin D receptor (VDR), and reported an interaction between the VDR and quercetin at the molecular level, revealing that VDR activation by quercetin is exerted by direct interaction, triggering subsequent downstream signaling events. General action mechanisms of polyphenols on insulin signaling pathways are summarized in Figure 5.

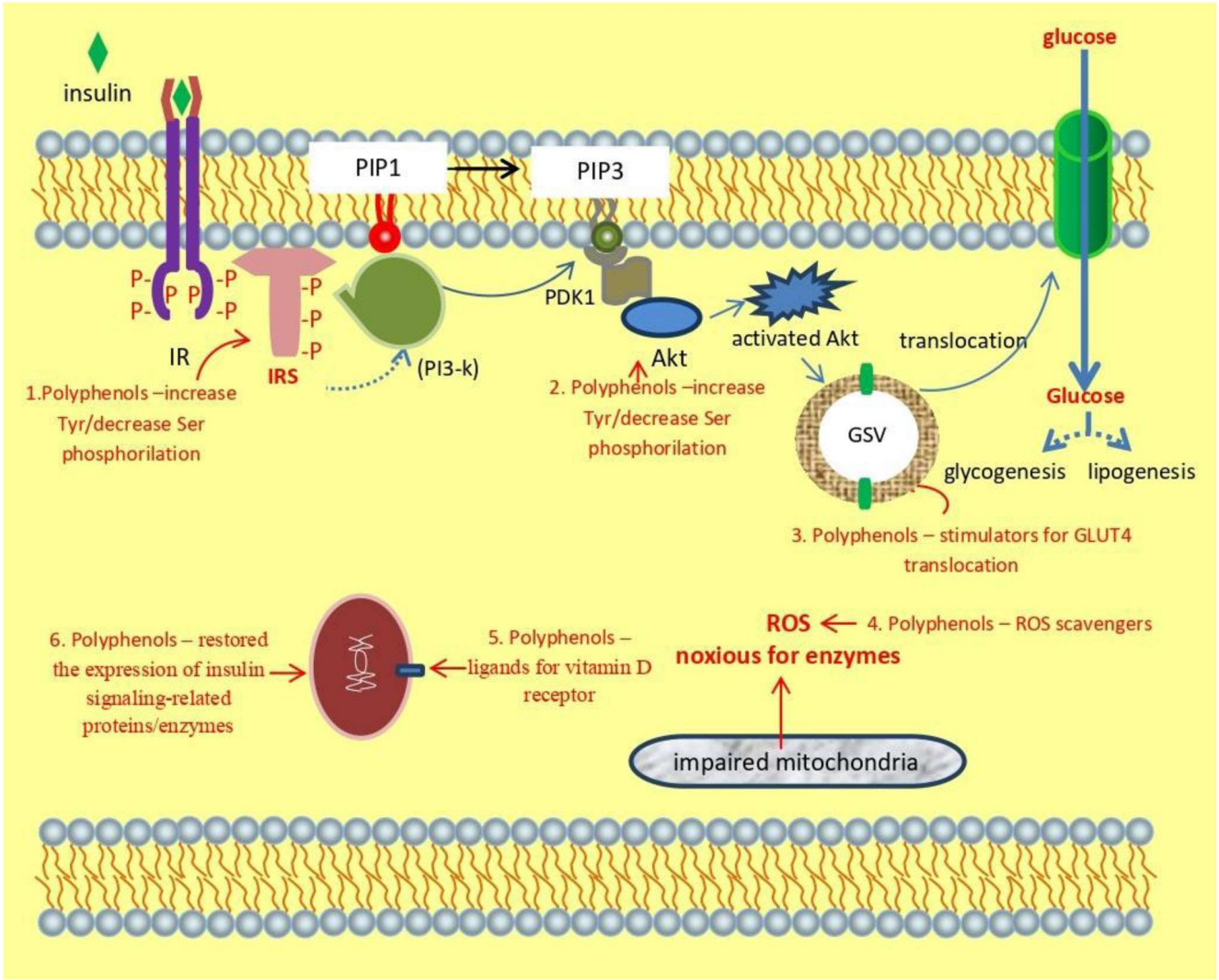

Figure 5. The effects of polyphenols in insulin signaling pathway. 1. EGCG, chlorogenic acid, cryptochlorogenic acid and quercetin-3-O-β-D-glucuronide, resveratrol, chicoric acid, nobiletin increase Tyr/decrease Ser phosphorylation of IRS. 2. EGCG, gallic acid, chlorogenic acid, cryptochlorogenic acid and quercetin-3-O-β-D-glucuronide, resveratrol, chicoric acid, nobiletin increase Tyr/decrease Ser phosphorylation of Akt. 3. Apigenin, maackiain, leachianone A, leachianone B, chlorogenic acid, cryptochlorogenic acid, quercetin-3-O-β-D-glucuronide, rutin, EGCG and catechins stimulate GLUT4 translocation.4. Caffeic, ferulic,protocatechuic acids, nobiletin decrease the oxidative stress. 5. Quercetin binds to the ligand binding site of the VDR. 6. Apigenin, maackiain, leachianone A, leachianone B, chlorogenic acid, cryptochlorogenic acid, quercetin-3-O-β-D-glucuronide, naringin, narincenin restored the expression of insulin signaling-related proteins/enzymes.
*IR: insulin receptor; IRS: insulin receptor substrate; PI3-k: phosphoinositide 3-kinase; PIP2: phosphatidyl-inositol-3,4-bisphosphate; PIP3: phosphatidyl-inositol-3,4,5-tris-phosphate; PDK1: phosphoinositide dependent kinase 1; Akt: protein kinase B; GLUT4: glucose transporter 4; GSV: GLUT4 storage vesicle.

Loss of pancreatic islet β-cell mass and function

Insulin resistance leads to increased metabolic demand for insulin. In the first step, the pancreas compensates for insulin resistance by hypersecretion, thus managing to maintain blood glucose within physiological ranges or close to physiological ranges. In the second phase, pancreatic compensation is followed by the loss of pancreatic β-cells responsible for insulin secretion, in which the pancreas fails to secrete enough insulin, and diabetes occurs.

Insulin secretion in islet β-cells

Insulin is the peptide hormone essential for maintaining glucose homeostasis. This hormone is produced in β-cells of the pancreatic islets, and then is released into the blood. Insulin biosynthesis is controlled by multiple factors, but glucose metabolism is the most important factor that stimulates insulin gene transcription, and mRNA translation (Poitout et al. 2006). Insulin circulates in the bloodstream, and acts at skeletal myocytes and adipocytes levels to facilitate the passage of glucose through the membrane, and is mediated by the insulin-sensitive glucose transporter GLUT4 (Amisten et al. 2013; Satoh 2014). GLUT4 is a transmembrane protein that facilitates the diffusion of peripheral blood glucose into the cell across the plasma membrane, down the concentration gradient, without consumption of ATP (Satoh 2014). The stimulus for insulin secretion is glucose level. Glucose uptake leads to accelerated glucose metabolism and increased ATP. The increasing of ATP concentration leads to closure of ATP-sensitive K^+ channels, membrane depolarization, opening of the voltage-dependent Ca^{2+} channels, increasing of Ca^{2+} influx, and triggering of the insulin secretion in β-cells (Fridlyand, Jacobson, and

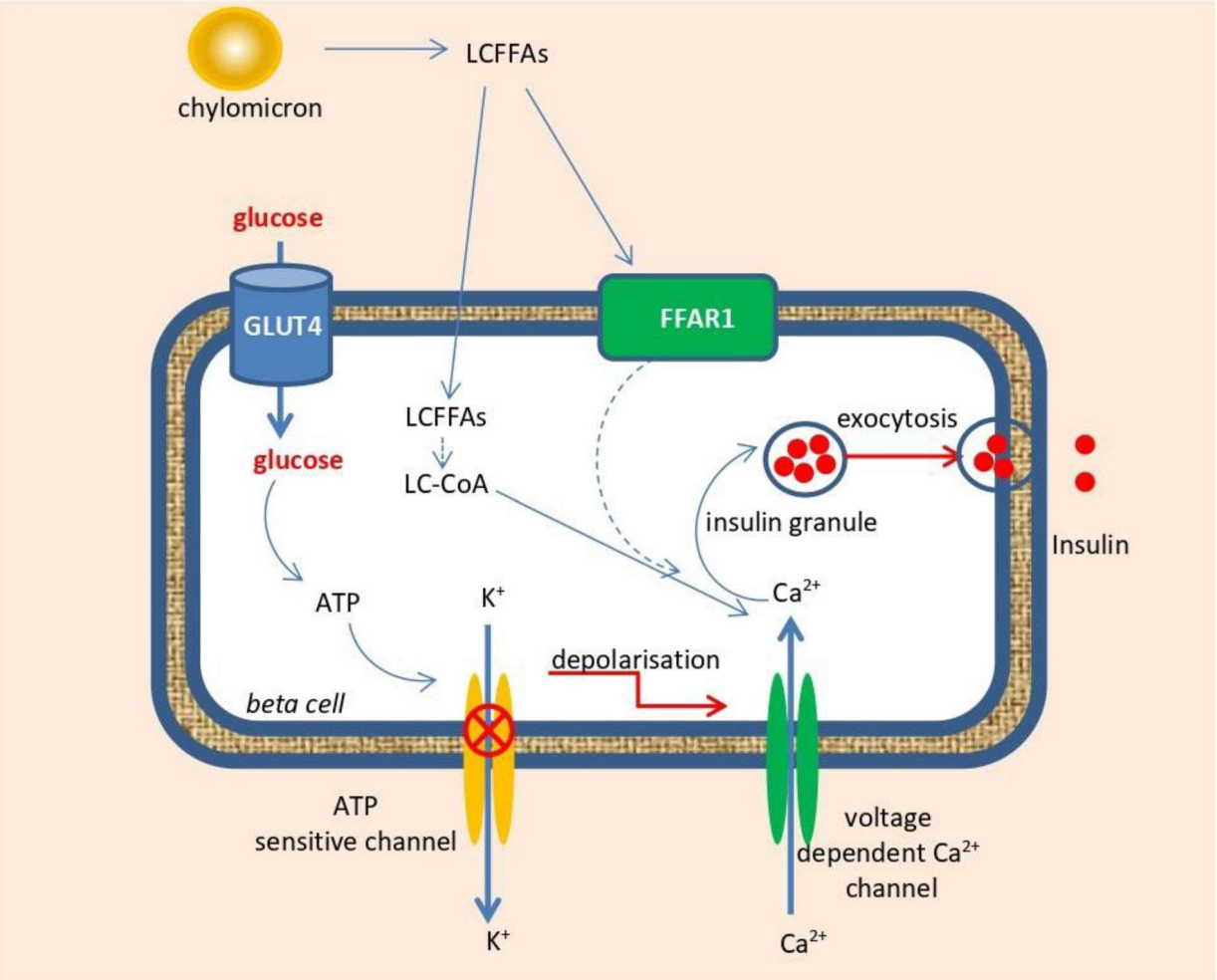

Figure 6. Glucose-stimulated insulin secretion activated by long chain FFAs.
GLUT2: Glucose transporter 2; FFAR1: free fatty acid receptor 1; LCFFAs: long-chain free fatty acid; LC-CoA: long-chain acyl-CoA.

Philipson 2013). In unstimulated cells, GLUT4 is sequestered into a specialized intracellular compartment termed GLUT4 storage vesicles (GSVs) (Satoh 2014). Glucose-stimulated insulin secretion (GSIS) is amplified by long-chain free fatty acids (LCFFAs) found in the vicinity of the β-cells after a meal, as result of local lipoprotein lipase activity, which releases the fatty acids from chylomicrons. LCFFAs potentiate GSIS by 2 distinct mechanisms. In the first mechanism, LCFFAs can be transported into the β-cell across the plasma membrane, and then are activated into long-chain acyl-CoA (LC-CoA), which can directly modulate ion channel activity. In the second mechanism, LCFFAs bind to free fatty acid receptor 1 (FAR1) and promote Ca^{2+} efflux from the endoplasmic reticulum after a cascade of reactions (Poitout 2018). In Figure 6 is presented glucose-stimulated insulin secretion activation by long chain FFAs.

Effects of hyperglycemia on loss of pancreatic islet β-cell mass and functions

The loss of β-cell mass and function is crucial for the development of both type 1 diabetes and type 2 diabetes. Chronic exposure to hyperglycemia produces oxidative stress, inflammation, dysregulated autophagy, changes in the regulation of gene expression that converge on impaired insulin secretion, and pancreatic β-cell mass death.

Oxidative stress. Formation of ROS in β-cells is stimulated mainly by glucose and free fatty acids catabolism. In β-cells, glycolytic flux is tightly coupled to increased mitochondrial oxidative activity, which leads to increased ROS production. Also, high concentrations of FFAs are associated with increased oxidative phosphorylation, and increased ROS production. Due to their very low antioxidant capacity, β-cells are extremely sensitive toward oxidative stress induced by excess of ROS and RNS (Drews, Krippeit-Drews, and Düfer 2010), with potential pathological consequences. High glucose and FFAs levels determine high ROS levels, and implicitly exaggerated and persistent oxidative stress that is not alleviated by the endogenous antioxidants. The most important targets for reactive species are cell metabolism and ATP-sensitive K^+ channels (Drews, Krippeit-Drews, and Düfer 2010). The persistent oxidative stress can lead to β-cell damage and death (Gerber and Rutter 2017) as a result of deteriorations caused by ROS in organelles, particularly the mitochondria, and cellular proteins, lipids, and nucleic acids (Simmons 2007). Damaged organelles and cytoplasmic proteins may be eliminated by autophagy, to maintain

cellular homeostasis (Stienstra et al. 2014), but in chronic hyperglycemia dysregulated autophagy in β-cells occurs.

Dysregulated autophagy. Autophagy is a regulated cellular mechanism of degradation and recycling of cytoplasmic proteins and damaged organelles by the lysosomal machinery (Watada and Fujitani 2015). Hyperglycemia as result of insulin resistance produces in the pancreatic β-cells oxidative stress and reticulum endoplasmic stress, caused by the increased insulin requirement. Marasco and Linnemann (2018) suggested that endoplasmic reticulum stress results from the accumulation of unfolded and misfolded proteins, and can initiate apoptosis if the stress conditions are not resolved. Bachar-Wikstrom et al. (2013) supposed that autophagy is a key adaptive mechanism in β-cell stress, *via* clearance of misfolded proinsulin. During insulin resistance, autophagic activity appears to be dysregulated to adapt to the dynamic changes occurring in β-cells. Thus, in the β-cells of insulin-resistant mice, induced by a high-fat diet, was reported either enhanced autophagic flux (Bachar-Wikstrom et al. 2013) or the inhibition of autophagic degradation (Quan et al. 2012). Dysregulated autophagy is involved in various neurodegenerative diseases, including type 2 diabetes, inasmuch as impaired of autophagy is also involved in pancreatic β-cells death (Chen et al. 2011; Yang et al. 2017). Several studies highlight that autophagy is necessary to maintain integrity and function of pancreatic β-cells (Jung et al. 2008; Fujitani, Kawamori, and Watada 2009; Sheng et al. 2017). Fujitani, Kawamori, and Watada (2009) found low-level constitutive autophagy in β-cells of C57BL/6 mice fed a standard diet; however, autophagy was markedly upregulated in mice fed a high-fat diet, and concluded that autophagy serves to maintain the integrity and functions of β-cells, and in the same time is a key factor of stress responses to protect β-cells under insulin-resistant states. To show that autophagy is necessary to maintain structure, mass and function of pancreatic β-cells, Jung et al. (2008) used in the experiment mice with β-cell-specific deletion of Atg7 (autophagy-related 7). Atg7 mutant mice showed impaired glucose tolerance and low serum insulin level. They suggested that β-cell mass and pancreatic insulin content were reduced due to increased apoptosis and decreased proliferation of β-cells. Also, Atg7 mutant mice showed reduced basal and glucose-stimulated insulin secretion and impaired glucose-induced cytosolic Ca^{2+} transients in autophagy-deficient β-cells, accumulation of ubiquitinated protein aggregates colocalized with nucleoporin p62, accompanied by mitochondrial swelling, endoplasmic reticulum distension, and vacuolar changes in β-cells. Recent studies have shown that autophagy deficiency led to deteriorated β-cell mass and function after high-fat and high-glucose feeding, and points out that autophagy is a key regulator of pancreatic β-cell homeostasis in response to high-fat and high-glucose diets (Sheng et al. 2017).

Changes in the regulation of gene expression. High glucose levels affect β-cells gene expression and several of these genes also exhibit epigenetic changes contributed to the impaired insulin secretion. Studies undertaken in different experimental models, suggested that high glucose levels altered expression of genes involved in inflammation, glucose metabolism, and ion channels (Fridlyand, Jacobson, and Philipson 2013; Taneera et al. 2015; Davegårdh et al. 2018). Hyperglycemia may also affect the genome-wide DNA methylation pattern in pancreatic islets. Dayeh et al. (2014) reported altered DNA methylation patterns in pancreatic islets in subjects with type 2 diabetes compared with non-diabetic controls, with positively correlations between HbA1c level and DNA methylation. Hall et al. (2018) assayed the effects of elevated glucose level on global gene expression in combination with DNA methylation patterns, and reported gene-specific changes in mRNA expression and DNA methylation, as well as enrichment of pathways due to exposure to high glucose levels. They suggested that these changes contributed to impaired insulin secretion. The studies conducted in human pancreatic islets and in a β-cells line by Yang et al. (2012) showed that hyperglycemia increased DNA methylation and decreased expression of pancreatic duodenal homeobox 1 (PDX-1) that plays a key role in pancreas development and function. Yang et al. (2012) suggested that impaired insulin expression and secretion in human diabetic islets is the result of the epigenetic modifications of PDX-1. Ottosson-Laakso et al. (2017) found that chronic hyperglycemia induced gene expression changes in human pancreatic islets. Thus, they proposed that the decrease of the expression of TMEM132D and MBP, both after acute and chronic hyperglycemia, and positively correlation with insulin secretion, suggest that their down-regulation could be involved in impaired insulin secretion. Ishikawa et al. (2015) cultured INS-1 cells in high glucose conditions, and reported that high-glucose levels significantly suppressed insulin mRNA and increased DNA methylation of the rat insulin 1 gene (*Ins1*) promoter depending of the exposure time and glucose concentration. Also, Ishikawa et al. (2015) suggested that long-term environmental exposure to high glucose levels would be responsible for epigenetic changes and irreversible injury in β-cells.

Pancreatic β-cell mass death. At the molecular level, pancreatic β-cell loss by apoptosis seems to be a key factor in the development of insulin deficiency, and the onset and progression of the disease. Several authors have showed that loss of pancreatic islet β-cell mass and functions involve an imbalance of β-cell replication and apoptosis. Chronic hyperglycemia and dyslipidemia are the most supposable causes of β-cell death acceleration, which may induce the reduction of β-cell mass in type 2 diabetes (Shimabukuro et al. 1998; Donath et al. 1999; Cnop et al. 2005; Tomita 2016).

The mechanisms by which hyperglycemia deteriorates functional β-cell mass have been extensively studied in recent years but, due to their complexity, not yet have been fully decrypted. Studies on Sprague-Dawley rats submitted to 85–95% pancreatectomy or sham-pancreatectomy showed that chronic hyperglycemia leads to β-cell hypertrophy and loss of β-cell differentiation that is correlated with changes in the gene c-Myc and other key transcription factors (Jonas et al. 1999). Donath et al. (1999) studied the effect of hyperglycemia on β-cell apoptosis in pancreatic islets of

Psammomys obesus during development of diabetes, and reported that exposure of islets from diabetes-prone *Psammomys obesus* to high glucose levels lead in a dose-dependent increase in beta-cell DNA fragmentation and inhibition of β-cell proliferation. Another mechanism of chronic toxicity of hyperglycemia to pancreatic β-cells was studied using a mouse pancreatic β-cell line (MIN6N8 cells) by Kim et al. (2005). They reported that high glucose level increases β-cell death mediated by Bax oligomerization, cytochrome c release, and caspase-3 activation. Beta cells apoptosis was accompanied by glucokinase (GCK) expression decreases, concomitant with a decrease in cellular ATP production and insulin secretion. Several studies suggested that chronic hyperglycemia leads over time to the formation of ROS in excess and cause chronic oxidative stress, which in turn causes defective insulin gene expression and insulin secretion, as well as increased apoptosis (Robertson 2004). Studies conducted on INS-1 cells showed that fluctuation hyperglycemia increased significantly more apoptosis and decreased the insulin secretory capacity than the chronic hyperglycemia, and proposed that the anti-oxidative enzyme Mn-SOD and the anti-apoptotic signal Bcl-2 can be the mediators of the processes (Kim et al. 2010). Recent studies undertaken on INS-1 cells exposed to intermittent high glucose, constant high glucose, and normal glucose showed that intermittent high glucose induced the higher apoptosis, and intracellular ROS and xanthine oxidase (XOD) levels. Also, in the INS-1 cells exposed to intermittent high glucose the levels of cell cycle related proteins (cyclinD1 and Skp2) significantly decreased, while the expressions of the genes p27 and p21 significantly increased. Due to these results, Zhang et al. (2014) suggested that the mechanism by which intermittent high glucose induces damages in INH-1cells is excessive activation of cellular stress and regulation of cyclins. Advanced glycation end-products (AGEs) are suspected to be cytotoxic to pancreatic β-cells. So, glycated albumin (GA), an early precursor of AGEs, have been demonstrated to induce β-cell apoptosis by inhibiting autophagy induction and flux by 2 ways: (1) increasing susceptibility to endoplasmic reticulum stress and high level ROS production, and (2) increasing expression of SQSTM1/p62, both accompanied by increased levels of nuclear factor-κB and inducible nitric oxide synthase (iNOS) (Song et al. 2013). The effects of chronic dyslipidemia were intensely studied in the last decade. High levels of FFAs in healthy individuals have stimulatory effects on insulin secretion, but may contribute to progressive β-cell death in individuals with a genetic predisposition to diabetes. Chronic saturated fatty acids (SFA) high levels in plasma contribute to beta-cell dysfunction and apoptosis (Oh et al. 2018). For example, the deleterious effects of palmitic acid, the most abundant SFA in human plasma, are mediated *via* ceramide-mitochondrial apoptotic pathways Liang et al. 2011; Oh et al. 2018). Very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) were reported to be pro-apoptotic due to the fact that they reduce insulin mRNA levels and β-cell proliferation (Kruit et al. 2010). Oxidized LDL reduces preproinsulin expression levels in isolated β-cells and requires the endoplasmic reticulum stress activation involving oxidative stress (Plaisance et al. 2016).

The effects of polyphenols on loss of pancreatic islet β-cell mass and function

Food rich in plant polyphenols/supplements with polyphenols with antioxidant properties have been used to decrease oxidative stress and improved pancreatic β-cell proliferation and function in type 2 diabetes. In Table 3 are summarized the action mechanism of some plant extracts rich in polyphenols/commercially polyphenols on insulin secreting β-cells in different experimental models. A number of polyphenols isolated and partial purified from plants, including quercetin, (–)-epigallocatechin gallate (EGCG), epicatechin, cyanidin and morin, exhibited great antioxidant properties with beneficial effects on enhancement of β-cell proliferation and functions. The studies on human Ins-1E cells showed that Cocoa phenolic extract protect beta cells on oxidative stress. In Ins-1E cells pretreated with Cocoa phenolic extract, the oxidative damages induced by the powerful oxidant *i*-BOOH were significantly reduced. So, Cocoa extract prevented cell death, ROS production, reduced glutathione (GSH) depletion and completely recovered the activity of the antioxidant enzymes glutathione peroxidase (GPx) and glutathione reductase (GR) to pre-stress values (Martín et al. 2013). Cocoa polyphenol epicatechin was assayed in Ins-1E cells for evaluation of the effects on cell viability, oxidative status, phosphorylated Jun kinase (p-JNK) expression, and insulin secretion by Martín et al. (2014). Since pretreatment of cells with epicatechin prevented the t-BOOH-induced ROS, carbonyl groups, p-JNK expression and cell death, and recovered insulin secretion, Martín et al. (2014) suggested that cocoa polyphenol epicatechin protect the integrity of secreting and surviving machineries in insulin-producing Ins-1E cells. Cocoa extracts were used to study the beneficial effects of cocoa flavanols on INS-1 832/13-derived β-cells, and primary rat islets cultured. The results of the study showed that the catechin-rich fraction improved β-cells functions by enhancing mitochondrial respiration, alleviating cellular redox state, and increasing glutathione concentration (Rowley et al. 2017). Bahar et al. (2017) used an extract rich in polyphenols (gallic acid and quercetin), obtained from *Crassocephalum crepidioides*, to study its effects on β-cells in a rat pancreatic cell line (INS-1) exposed to alloxan and in alloxan-induced diabetes rats. In vitro studies showed that in the INS-1 cells treated with alloxan and *Crassocephalum crepidioides* extract the intracellular ROS level and apoptosis decreased significantly, comparatively with cells treated only with alloxan. Comparative morphological studies conducted in alloxan-induced diabetes rats treated with *Crassocephalum crepidioides* extract and in untreated mice revealed that the percent of β-cells was higher in the rodents treated with the extract. An aqueous extract of *Marontades pumilum* administrated in streptozotocin-nicotinamide induced male diabetic rats decreased the level of the lipid peroxidation products and increased the levels of antioxidative enzymes (SOD, CAT and GPx) in the pancreas. Also, in rat pancreas, *Marontades pumilum* extract reduced

Table 3. Action mechanism of some plant extracts rich in polyphenols/commercially polyphenols on insulin secreting β-cells in different experimental models.

Polyphenols/plant extract rich in polyphenols	Experimental model	Effects on β-cells	General conclusions	References
Cocoa phenolic extract	Ins-1E cells - oxidative stress induced by tert-butylhydroperoxide (t-BOOH)	Prevents the t-BOOH-induced ROS and carbonyl groups, and returns antioxidant defense to adequate levels	Protects the surviving machineries of cells against the oxidative insult	Martín et al. (2013)
Cocoa epicatechin	Ins-1E cells- oxidative stress induced by tert-butylhydroperoxide (t-BOOH)	Prevents the t-BOOH-induced ROS, carbonyl groups, p-JNK expression, and cell death	Protects β-cells against the oxidative insult	Martín et al. (2014)
Cocoa phenolic extracts	INS-1 cells Primary rat islets cultured	Catechin-rich fraction enhances mitochondrial respiration, improves cellular redox state, and increases glutathione concentration	Improves β-cell function	Rowley et al. (2017)
Crassocephalum crepidioides methanol extract	Alloxan induced apoptosis in INS-1 cells	Protects pancreatic β-cells from oxidative damage by regulating ROS production Acts by an anti-apoptotic signaling mechanism	May be a natural remedy for diabetes treatment	Bahar et al. (2017)
	Alloxan induced diabetes in rat	Leads to increase of the pancreatic islets number		
Marontades pumilum aqueous extract	STZ-NA induced male diabetic rats	Decreases oxidative stress in the pancreas Decreases apoptosis in the pancreas	Has beneficial effects in treating diabetes	Adam et al. (2017)
	3T3-L1 adipocyte cells	Enhances glucose uptake		
Cyclocarya paliurus tea leaves aqueous extract	High-fat diet-low dose STZ-induced diabetic mice	Suppresses pancreatic β-cell apoptosis	*Cyclocarya paliurus* tea leaves may be used as a new remedy for diabetes	Xiao et al. (2017)
	STZ-induced apoptosis in NIT-1 cells	Suppresses pancreatic β-cell apoptosis by modulating MAPK and Akt pathways		
Cyclocarya paliurus aqueous extract	High fat diabetic rats	Reduces the excessive oxidative stress improved β-cell survival and sustains insulin secretion	Has protective effects on β-cell	Li et al. (2018a)
Cyclocarya paliurus, *Dendrobium*, *Morus alba L.*, and *Pericarpium Citri Reticulatae* (4:2:2:1) aqueous extract	high-fat/ dexamethasone diabetic mice	Protects the pancreas by inhibiting inflammation and apoptosis pathways Protects the pancreas against oxidative stress damage	May be a potential anti-diabetic agent to prevent and treat diabetes, and its complications	Li et al. (2018b)
Jojoba seeds aqueous extract	RINm5f cells	Decreases ROS level and improves antioxidant defense	Is a powerful agent to prevent the destruction of RINm5f beta cells induced by hyperglycemia	Belhadj et al. (2018)
Quercetin and EGCG	INS-1 cells	Antioxidant activity Anti-apoptotic activity	Have protective effect on INS-1 cells	Kim et al. (2010)
Cyaniding-3-glucoside isolated from mulberry fruit	MIN6 pancreatic β-cells	Reduces the oxidative stress-induced cell apoptosis Regulates the intrinsic apoptotic pathway-associated proteins	May be used for preventing oxidative stress-induced β-cell apoptosis	Lee et al. (2015)
	Secoisolariciresinol diglucoside	STZ-induced diabetic rats	Improves antioxidant defense Regeneration of β-cell	Maintain tissue function which result in improving the sensitivity and response of target cells in

				STZ-induced diabetic rats to insulin
Moree et al. (2013)				
Kaempferol	Palmitic acid-stressed RIN-5F cells	Kaempferol up/down-regulates AMPK/mTOR phosphorylation	Cytoprotective effect on β-cells by activation of autophagy	Varshney, Gupta, and Roy (2017)
Purple corn aqueous extract and pure anthocyanins cyanidin-3-O-glucoside, peonidin-3-O-glucoside, pelargonidin-3-O-glucoside, and delphinidin-3-O-glucoside	iNS-1E cells	Increase insulin secretion in pancreatic β-cells *in vitro* potentially through activation of FFAR1	The purple corn extract supplementation may protect the diabetes associated consequences	Luna-Vital and Gonzalez de Mejia (2018)
	HepG2 cells	Increase glucose uptake		
Mulberry polyphenols: 1-deoxynojrimycin, cyanidin-3-glucoside, cyanidin-3-rutinoside, resveratrol and oxyresveratrol	H_2O_2 induced oxidative stress in MIN 6 cells	1-Deoxynojrimycin, cyanidin-3-glucoside, and cyanidin-3-rutinoside inhibit ROS production, decrease apoptosis rate, lead to intracellular ATP accumulation and insulin secretion. Resveratrol and oxyresveratrol accelerate the apoptosis at high dose (50 μM).	Potential application in the intervention against hyperglycemia	Zheng et al. (2016)
Lithospermic acid B	Cytokine-induced apoptosis in INS-1 cells	Alleviates apoptotic pathways Activates anti-apoptotic pathways of Nrf2-HO-1 and Sirt1	Cytoprotective effects in pancreas	Lee et al. (2011)
	OLETF rats	Improves glucose uptake		
Cyanidin	INS-1 cells	Stimulates insulin secretion and increases intracellular Ca^{2+} signals	May be used as a promising agent to stimulate insulin secretion	Suantawee et al. (2017)
Morin	MIN6 cell line	Increases intracellular Ca^{2+} content	May be used as a promising agent to stimulate insulin secretion	Lin et al. (2017)
Quercetin	INS-1 cells Rat isolated pancreatic islets	Increases Ca^{2+} influx through an interaction with L-type Ca^{2+} channels	Stimulates insulin secretion	Bardy et al. (2013)
Quercetin	INS-1 cells	Stimulates insulin release, presumably by transient ATP-sensitive K^+ channel inhibition, and whole-cell Ca^{2+} stimulation Long term application inhibits cell proliferation, and induces apoptosis	Stimulates insulin secretion, and reduces the viability of beta-cells in long term application	Kittl et al. (2016)
Curcumin Demethoxycurcumin Bisdemethoxycurcumin	Rat insulin-secreting (INS-1) insulinoma cells	Curcumin acts on ionic currents and membrane potential	Curcumin has insulin-releasing effect	Kuo et al. (2018)

STZ – streptozotocin; NA –nicotineamide.

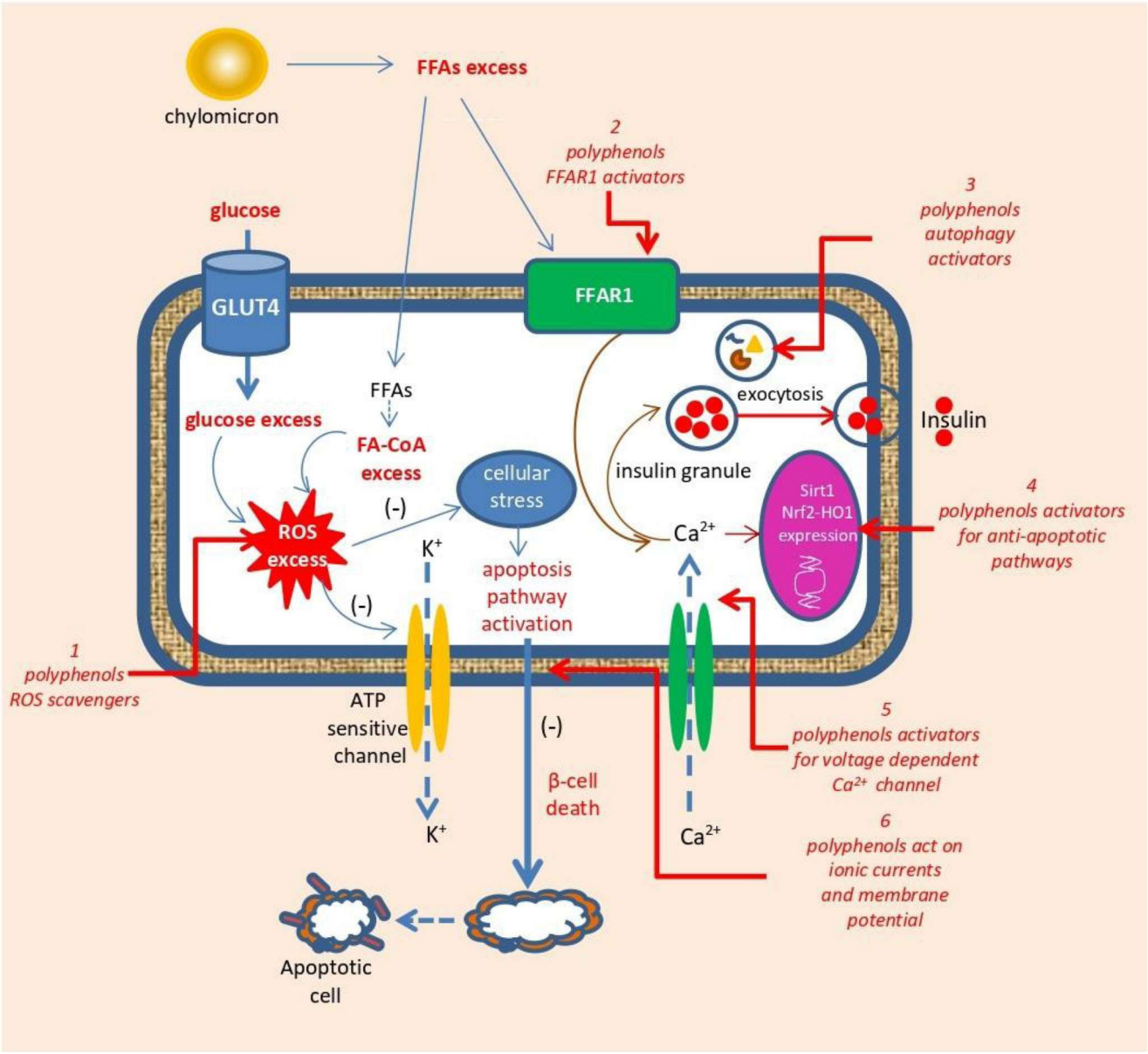

Figure 7. Polyphenols inhibition of β-cell death and functions loss. 1. Polyphenols ROS scavengers. Glucose and fatty acids excess generate ROS excess, which target molecular metabolism and ATP sensitive K^+ channals causing β-cell death. ROS excess induce cellular stress that produces apoptosis pathway activation trough Bax oligomerization, cytochrome C release, and caspase-3 activation. Polyphenols/plant extracts rich in polyphenols are antioxidant activity, decrease ROS level and improve antioxidant defence. 2. Polyphenols activators for FFAR-1. Anthocyanins cyanidin-3-O-glucoside, peonidin-3-O-glucoside, pelargonidin-3-O-glucoside and delphinidin-3-O-glucoside activate FFAR1 and increase insulin secretion. 3. Polyphenols autophagy activators. Kaempferol activates the autophagy *via* AMPK/mTOR pathway. 4. Polyphenols activators for anti-apoptotic pathways. Lithospermic acid B activates Nrf2-HO-1 and Sirt1 expresion. 5. Polyphenols activators for voltage-dependent Ca^{2+} channels. Flavonols morin and quercetin favor Ca^{2+} stimulated insulin secretion and the expression of genes involved in this process. 6. Polyphenols act on ionic currents and membrane potential. Curcumin suppressed the amplitude of delayed-rectifier K^+ current.
ROS: reactive oxygen species; GLUT2: glucose transporter 2; FFAs: free fatty acids; FA-CoA: fatty acyl coenzyme A; FFAR1: free fatty acid receptor 1; AMPK: 5′ adenosine monophosphate-activated protein kinase; mTOR: mammalian target of rapamycin; Nrf2: heterodimers of NF-E2-related factors 2; HO-1: heme oxygenase 1; sitr1: sirtuin 1.

apoptosis, reflected by caspase-9 and Bax levels, and increased the anti-apoptosis marker Bcl-2 levels (Adam et al. 2017). Studies conducted in cell lines and in different animal models showed that *Cyclocarya paliurus* tea leaves extracts, rich in polyphenols, prevented loss of pancreatic islet β-cell mass and function. Thus, in diabetic mice and in NIT-1 cells was demonstrated that the *Cyclocarya paliurus* tea leaves extract, containing 1-caffeoylquinic acid, 5-caffeoylquinic acid, chlorogenic acid, isoquercitrin, kaempferol-3-glucoside, kaempferol 3-rhamnoside, and quercetin, suppresses pancreatic β-cell apoptosis (Xiao et al. 2017). Xiao et al. (2017) suggested that *Cyclocarya paliurus* tea inhibited pancreatic β-cell apoptosis by suppressing the expression of caspase 8, caspase 9, and cleaved caspase-3, as well as Bax/Bcl-2 ratio, down-regulating p38, ERK, and JNK phosphorylation, and up-regulating Akt phosphorylation. The aqueous extract obtained from *Cyclocarya paliurus* reduced excessive oxidative stress and inflammation in the pancreas. Also, the transcriptome profiling analysis and regulatory network analysis suggested that *Cyclocarya paliurus* extract ameliorated diabetes through improving β-cell survival and sustaining insulin secretion in the pancreas (Li et al. 2018a). The aqueous

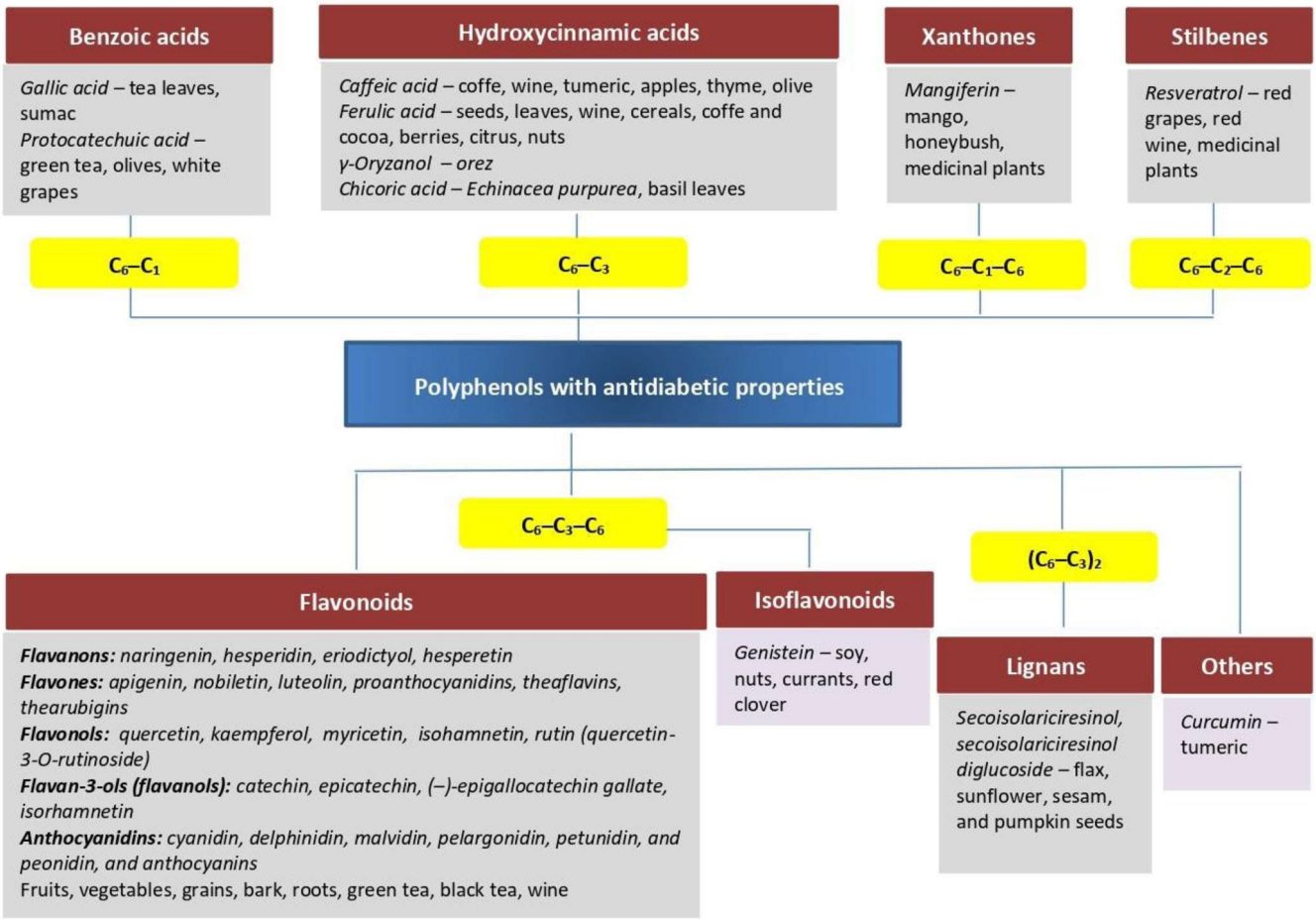

Figure 8. Polyphenols with antidiabetic properties. C_6 – benzene ring; C_1, C_2, C_3– sidechains with 1, 2 and 3 carbon atoms.

extract obtained from *Cyclocarya paliurus*, *Dendrobium*, *Morus alba L.*, and *Pericarpium Citri Reticulatae* (4:2:2:1, w/w/w/w, dry weight) protects the pancreas in diabetic mice by inhibiting inflammation, apoptosis pathways and oxidative stress (Li et al. 2018b). Similar results were showed for jojoba seeds aqueous extract. Thus, the extract decreased ROS and caspase-3 activation and improved antioxidant defense, inhibiting p22phox, and increasing nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Belhadj et al. 2018). Kim et al. (2010) investigated the effect of quercetin and EGCG on cell death induced by oxidative stress and their mechanisms in insulin-producing INS-1 cells. They found that both compounds protect INS-1 cells against oxidative stress and restored insulin secretion, and suggested a dual action *via* antioxidant defense and *via* anti-apoptosis pathway. Thus, they showed that both compounds significantly increased MnSOD protein and glutathione peroxidase activity, but only quercetin significantly increased catalase proteins.

Also, it was found that quercetin and EGCG were involved in PI3-k and Akt phosphorylation, and restoring of glucose stimulated insulin secretion (GSIS) for 5 mM, and even for 25 mM glucose concentration. Cyaniding-3-glucoside isolated from mulberry fruit also protects pancreatic β-cells against oxidative stress induced apoptosis (Lee et al. 2015). Synthetic secoisolariciresinol diglucoside, a lignan generally found in flax, sunflower, sesame, and pumpkin seeds, administrated to diabetic rats improved antioxidant defense, regenerated β-cells and retarded diabetes complications (Moree, Kavishankar, and Rajesha 2013). Varshney, Gupta, and Roy (2017) studied the effects of kaempferol in palmitic acid-stressed RIN-5F pancreatic β-cells, and hypothesized that kaempferol had cytoprotective role against lipotoxicity by activation of autophagy *via* AMPK/mTOR pathway.

Free fatty acid receptor 1 (FFAR1, also known as GPR40) has attracted the interest of researchers in the study of diabetes in the last years. Activation of FFA1 results in an increase of cytosolic Ca^{2+} concentration in β-cells. This process is followed by the initiation of a signal cascade, resulting in the secretion of insulin. Luna-Vital and Gonzalez de Mejia (2018) suggested that the anthocyanins present in purple corn and pure anthocyanins (cyanidin-3-O-glucoside, peonidin-3-O-glucoside, and pelargonidin-3-O-glucoside) improved the glucose-stimulated insulin secretion and glucose absorption in INS-1E *via* FFAR1-dependent insulin secretory pathway. Zheng et al. (2016) investigated the effects of 5 polyphenols found in mulberry to decipher the regulation mechanism of insulin secretion under stress conditions. In this assay, 1-deoxynojrimycin, cyanidin-3-glucoside, and cyanidin-3-rutinoside inhibited ROS production, decreased apoptosis rate, leaded to intracellular ATP accumulation, and insulin secretion. Surprisingly, resveratrol and oxyresveratrol accelerated the apoptosis at high dose (50 μM). Lithospermic acid B, polycyclic phenolic carboxylic

acid isolated from plants of the genus *Lithospermum*, had cytoprotective effects on pancreatic β-cells by 2paths: alleviating apoptotic pathways, and activating anti-apoptotic pathways of Nrf2-HO-1 and Sirt1 (Lee et al. 2011). Suantawee et al. (2017) suggested that cyanidin stimulates insulin secretion and pancreatic β-cell gene expression trough activation of L-type voltage-dependent Ca^{2+} channels. The increasing of intracellular Ca^{2+} level in β-cells, accompanied by potentiation of GSSI was shown also for morin (Lin et al. 2017), flavonoid found in guava leaves. Bardy et al. (2013) assayed the effects of quercetin on insulin secretion and on the intracellular calcium concentration in INS-1 β-cells. They showed that quercetin increased insulin secretion and elevated Ca^{2+} level concentration-dependent, and suggested that quercetin stimulates insulin secretion by increasing Ca^{2+} influx through an interaction with L-type Ca^{2+} channels. Recent studies, completed by Kittl et al. (2016), showed that quercetin (50 μM) significantly depolarized membrane potentials, stimulated whole-cell Ca^{2+}, and caused a 50% inhibition of ATP-sensitive K^+ channels, stimulating insulin releasing. Surprising, this study suggested that long term application of quercetin inhibits cell proliferation and induces β-cells apoptosis *via* PI3-k/Akt signaling. Kuo et al. (2018), studied the effects of curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) on ionic currents and membrane potential in rat insulin-secreting (INS-1) insulinoma cells, and assumed that only curcumin-mediated decrease delayed-rectifier K^+ current ($I_{K(DR)}$), and increased the current inactivation, with impact on insulin releasing, and demethoxycurcumin and bisdemethoxycurcumin did not evidently increase current inactivation. The most important action mechanisms on protective effects of polyphenols on pancreatic β-cells mass and functions are shown in Figure 7. In Figure 8 are presented the polyphenols with antidiabetic properties described in this review.

Conclusions

In the last decade have been made different epidemiological studies associated with meta-analyses, and also many in vivo and in vitro studies on different experimental models to decipher the mechanism by which plant polyphenols prevent/alleviate diabetes. From this review result that dietary polyphenols reduce insulin resistance and loss of pancreatic islet β-cell mass and function. They reduced the risk of diabetes in elderly and obese persons, and alleviated diabetes by reducing insulin resistance. Studies undertaken on different animal models and different cells line show that impaired insulin activation of the IRS-1/PI3-k/Akt signaling pathway is a critical step in insulin resistance induction. These defects may be selectively alleviated by dietary polyphenols that act as insulin-sensitizing agents. Dietary polyphenols are able to reduce the loss of pancreatic islet β-cell mass and function by several molecular mechanisms. In this review the following mechanisms have been identified: protection of the surviving machineries of cells against the oxidative insult; increasing insulin secretion in pancreatic β-cells through activation of the FFAR1; cytoprotective effect on β-cells by activation of autophagy; protection of β-cells to act as activators for anti-apoptotic pathways and inhibitors for apoptotic pathway; stimulating of insulin release, presumably by transient ATP-sensitive K^+ channel inhibition and whole-cell Ca^{2+} stimulation; involvement in insulin release that act on ionic currents and membrane potential as inhibitor of delayed-rectifier K^+ current ($I_{K(DR)}$) and activator of current. Some dietary polyphenols could be used as potential anti-diabetic agents to prevent and alleviate diabetes and its complications, but further studies are needed.

Author contributions

Camelia Papuc conceived of the presented theme, collected the data, drafted the manuscript, and conceived and drafted the figures, Gheorghe V. Goran edited and reviewed the manuscript, and he will be the corresponding author, Corina N. Predescu developed the theory, performed references revision, and edited the figures, Liliana Tudoreanu performed language revision and verified the references, and Georgeta Ștefan performed language and references revision.

Disclosure statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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COMPARATIVE STUDY ON THE VARIATION OF CORTISOL LEVEL IN BLOOD SERUM DEPENDING ON SWINE SLAUGHTERING METHOD

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Abstract

Stress is defined as a complex chain of events, consisting in a stimulus that causes a subsequent reaction in the brain and activates physiological reactions. It is important to adopt good practices during slaughter. In assessing the level of stress, the following variables should be taken into account: the means of transport, the way of slaughter (with or without stunning), accidental fall of animals, refusal of animals to enter the containment box, excessive movement of animals during containment. In the living organism, a series of biochemical and energetic transformations take place, which are in close interdependence, and are subjected to the mechanisms of regulation and metabolic control, which cease with the suppression of animal life, therefore, after slaughtering animals, a series of transformations appear in the muscle tissue. The study was conducted between 2019-2020, on two batches of conventionally slaughtered pigs (with stunning) in slaughterhouses and on a batch of traditionally slaughtered pigs (without stunning). In the slaughterhouses, the technological flow of pigs slaughtering was monitored and blood samples were collected in order to extract the serum and measure the cortisol level. Cortisol was measured in a specialized laboratory by the immunoenzymatic method by chemiluminescence detection. Determination of cortisol levels in blood samples taken from conventional pig slaughter revealed different values, exceeding the established reference values, compared with blood samples collected from households following traditional slaughter, the level of which is lower, sometimes falling within the reference values. The growth and handling of pigs before slaughter induces their stress, so special attention must be paid to the slaughter process in order to minimize stress levels and improve the quality of the meat.

Key words: *cortisol, pigs, stress, stunning.*

INTRODUCTION

Meat has played a crucial role in human evolution and is an important component of a healthy and balanced diet due to its nutritional richness (Savu et al., 2002; Williamson et al, 2005; McNeill & Van Elswyk, 2012; Pereira & Vicente, 2012; Petcu, 2013; Predescu et al, 2018). The nutritional composition of meat varies depending on the animal's breed, age, sex, diet, body weight, fattening status, rational feeding, animal health, animal movement, season, but also on the way of slaughter (with or without stunning) (Williams, 2007; Banu et al., 2009).

In pigs, in particular, there are a number of growth and fattening factors that affect the quality of the meat, such as: type of shelter, shelter size, microclimate in the shelter, animal density, feed, animal sex, age at slaughter, health, genetic factors, stressors and last but not least the weight at slaughter (Banu et al., 2009; Tăpăloagă, 2012).

In the living organism there are a series of biochemical and energetic transformations, which are in close interdependence, as they are subjected to the mechanisms of regulation and metabolic control, mechanisms that end with the suppression of animal life (Ionescu & Diaconescu, 2010).

After the slaughter of the animals, a series of transformations appear in the muscles tissue, as the blood pressure decreases, a peripheral vasoconstriction occurs, the thermoregulatory mechanisms no longer work, disturbances appear at the level of all homeostatic mechanisms and the susceptibility to microbial attacks increases (Ionescu & Diaconescu, 2010; Papuc et al., 2013; Petcu, 2015).

When the blood flow is interrupted, the oxygen supply is suppressed. Tissue respiration continues for a short time, until oxygen depletion. The absence of oxygen leads to the cessation of aerobic processes, and so the formation of lactic acid takes place through the anaerobic degradation of glucose. The accumulation of lactic acid in the muscles has the effect of decreasing the pH value, leading to its acidification (Ionescu & Diaconescu, 2010).

In order to obtain meat with physico-chemical characteristics corresponding to human consumption, animals are slaughtered, by different methods, depending on the species, religious precepts or geographical area. The process of sacrifice entails a series of consequences that have attracted the attention of the scientific world.

Stress is defined as a complex cascade of events, consisting of a stimulus (stressor), which causes a subsequent reaction in the brain (stress perception) and activates physiological reactions (stress response) (Dhabhar & McEwen, 1997; Ciliberti et al., 2017).

A stressor that lasts for a few minutes to hours is defined as acute stress, while a stressor that persists for several hours a day for weeks or months is defined as chronic stress (Dhabhar, 2002; Ciliberti et al., 2017).

Determination of cortisol is one of the most widely used methods of stress assessment in animals, because it provides information about the activity of the hypothalamic-pituitary-adrenal axis. The most frequently collected biological samples for cortisol dosing are: blood (serum, plasma), saliva, urine, feces, milk and hair (Casal et al., 2017).

MATERIALS AND METHODS

The study was conducted in 2019-2020 on three batches of pigs. The pig slaughtering technological flow was monitored in the slaughterhouses and blood samples were collected.

- Batch 1: 8 blood samples collected from a batch of 150 pigs, the Great White breed with an approximate body weight of 110-120 kg and the age of 8-9 months, slaughtered in a slaughterhouse, using stunning.
- Batch 2: 10 blood samples collected from a batch of 390 Metis breed pigs with an approximate body weight of 120-130 kg and the age of 7 months after slaughter in a slaughterhouse, using stunning.
- Batch 3: 12 blood samples collected from traditionally slaughtered pigs in the household of the population, the Metis breed, about one year old.

In the case of slaughter in the slaughterhouse, the pigs enter the adduction corridor and are electrically stunned, by positioning two electrodes at the level of the head. Immediately after stunning, hanging on the airline takes place and the next stage is bleeding.

In the case of traditional slaughtering, the pigs are slaughtered without stunning, by stabbing.

The aim of this study is to perform laboratory tests aimed at dosing cortisol from blood samples collected at the time of bleeding (approximately 9 ml of blood collected in a BD Vacutainer - Clot Activator Tube) (Figure 1). Blood samples were immediately transported to a specialized laboratory, and the cortisol level was dosed by the immunoenzymatic method by chemiluminescence detection.

In order to determine these parameters, specialized training and laboratory equipment, as well as specific materials and reagents are required.

Figure 1. Blood samples

Animal welfare during transport and slaughtering is a matter of concern for consumers. It is necessary to pay attention to animals during transport, before slaughtering and during slaughtering (Petcu, 2015; Small & Hewitt, 2017).

People who understand the behaviour of animals will be able to board them properly into the means of transportation meant to carry them to the slaughterhouses, although stress is inevitable during the transport of animals from farm to slaughterhouse (Ferguson & Warner, 2009). Behavioural principles are recommended for the transport of animals, because this contributes to their welfare (Grandin, 2010; Grandin, 2019).

It is important to adopt good practices during slaughter, including systematic checks to determine when the animal begins to lose consciousness and when it loses it completely (Velarde & Dalmau, 2018).

In assessing the level of stress, the following variables should be taken into account: accidental fall of animals, refusal of animals to enter the containment box, excessive movement of animals during containment (Grandin, 2018).

Animals that become stressed before slaughter will have high levels of blood lactate concentration and will be more likely to have harder muscles. A calm animal that did not became restless and frightened will be more easily manipulated and will also be safer for the slaughterhouse staff (Grandin, 2010; Grandin, 2019).

The slaughtering without stunning is performed mainly for the purpose of religious sacrifice (Halal and Kosher), but also for the traditional sacrifice practiced in Romania for many years. If the animals are conscious during slaughtering, the risk of suffering increases. Immobilization of conscious animals for the purpose of cutting the neck causes stress. The incision made in the neck to cut the blood vessels, involves substantial damage to tissues in areas well represented by nociceptors (activation of the nociceptive system of protection induces suffering, pain in the animal). Death is not immediate and there is a period when the animal is still conscious and can feel anxiety, pain, suffering (Velarde & Dalmau, 2018).

Electrical stunning in pigs

Proper handling of animals during the slaughter process in well-designed units will minimize stress levels, improve efficiency and maintain good meat quality (www.grandin.com).

Handling in the last five to ten minutes before stunning the animals will have a significant effect on the blood lactate concentration. Studies have shown that high levels of lactate are associated with intense handling of animals that leads to stress. Also, improper electrical stunning of pigs and imposition of a second stunning leads to animal stress (Benjamin et al., 2001; Hambrecht et al., 2004; Hambrecht et al., 2005).

Slaughtered animals are stunned in order to enter a state of unconsciousness, insensitivity and immobility before bleeding. This state of unconsciousness should last long enough to ensure that the animal does not feel pain during bleeding (Wormuth et al., 1981; Schutt-Abraham, 1982; Gregory & Wotton, 1990; Hillebrand et al., 1996).

The effectiveness of the stunning process induces a state of instant unconsciousness and insensitivity to pain, which lasts until the death of the animal and has no negative effect on meat quality (Savenije et al., 2002; Joseph et al., 2013).

Animal welfare during slaughter was one of the major criteria that led to the formation of legislative requirements on stunning animals worldwide (Joseph et al., 2013).

Presently, the emphasis is on improving the animal's slaughtering process and new slaughtering procedures are followed, thus implementing various handling, stunning or monitoring techniques. The effectiveness and efficiency of stunning are of the utmost importance in facilitating the slaughter of animals, both for welfare and legislative reasons (Grandin, 2002; Atkinson et al., 2013; Grandin, 2019; Wagner et al., 2019).

Electrical stunning or electronarcosis is the passage through the brain of an electric current with voltage, amperage and frequency related to the species, which causes a disruption of normal brain activity, so that there is an immediate loss of consciousness and sensitivity. The efficiency of electronarcosis results from the interaction that is established between current, application time and the body's resistance. Practically, from the moment the two electrodes (positive and negative) are applied on the surface of the animal's body (Figure 2), the potential difference leads to the appearance of a current flow, with a certain

force, which will be counteracted by the resistance offered by skin (as a first obstacle) and the internal environment of the body (muscles, bones, blood vessels, etc.) (Guide on the protection of animals during slaughter, 2010; Petcu, 2015).

Figure 2. Electrical stunning in pigs

In all cases, the current level must be reached within one second from the start of the stunning and must be maintained for at least 1 to 3 seconds, according to the manufacturer's instructions. The electrodes must be placed so that they enclose the cranial box, allowing current to pass through it. The operator must ensure that there is a good electrical contact. In the case of pigs, the electrodes are located at the base of the ears, between the ears and the eyes. The alternating electric current, with low voltage is applied bilaterally, in the upper region of the skull, with the help of two electrodes of different shapes (Petcu, 2015).
Electrical stunning is based on the short-term action of electric current of a certain intensity and voltage on the central nervous system, causing paresis and loss of consciousness during the time in which the bleeding occurs (Petcu, 2015).
It is undeniable that an insufficient amperage or a current that after touching the animal's head takes it in another direction, without actually crossing the brain, will not induce the necessary state of unconsciousness, but pain caused by electric shock (Petcu, 2015).

RESULTS AND DISCUSSIONS

The period and method of slaughter are very complex and can represent different types of stress for the animal. How animals react to these stressors depends on their individual emotional reactivity (Deiss et al., 2009).
It has been shown that there is a direct correlation between meat quality and how animals are slaughtered (with or without stunning).

Results and discussions about the cortisol level in blood serum

Stress before slaughter has a negative impact on animal welfare and meat quality (D'Eath et al., 2010).
Determination of cortisol level is one of the most widely used methods for assessing stress in animals, as it provides information about the activity of the hypothalamic-pituitary-adrenal axis (Casal et al., 2017).
Deiss et al. has shown that the highest levels of cortisol (measured from blood samples) were observed in isolated animals. In general, young animals showed higher cortisol values (Linares et al., 2008; Deiss et al., 2009).
Determination of cortisol level in blood serum samples collected from conventionally slaughtered pigs revealed different values, exceeding the reference interval set by Jackson et al in 2002.

Study 1 - Determination of cortisol level from blood serum samples harvested from conventionally slaughtered pigs in June 2019

Following the analysis of the cortisol level from the 8 blood serum samples harvested from conventionally slaughtered pigs in June 2019, it was observed that 7 of the total samples had higher values compared to the reference interval (2.6-3.3 μg/dL), a single sample recording an optimal cortisol level, namely 2.80 μg/dL.
The results obtained from the dosing of cortisol level in the samples of group 1 are presented in Table 1.

Table 1. Results of cortisol level dosing in conventionally slaughtered pigs in batch 1

No.	Species	Breed	Age	Sex	Slaughtering date	Method	Cortisol level µg/dL	Reference interval
1.	swine	Large White	8 months	M	06.06.2019	Immunological	2.80 µg/dL	2.6-3.3 µg/dL
2.	swine	Large White	9 months	M	06.06.2019	Immunological	**3.73 µg/dL**	2.6-3.3 µg/dL
3.	swine	Large White	8 months	M	06.06.2019	Immunological	**4.39 µg/dL**	2.6-3.3 µg/dL
4.	swine	Large White	8 months	M	06.06.2019	Immunological	**4.89 µg/dL**	2.6-3.3 µg/dL
5.	swine	Large White	8 months	M	06.06.2019	Immunological	**5.51 µg/dL**	2.6-3.3 µg/dL
6.	swine	Large White	9 months	M	06.06.2019	Immunological	**6.21 µg/dL**	2.6-3.3 µg/dL
7.	swine	Large White	8 months	M	06.06.2019	Immunological	**7.10 µg/dL**	2.6-3.3 µg/dL
8.	swine	Large White	7 months	M	06.06.2019	Immunological	**7.99 µg/dL**	2.6-3.3 µg/dL

Study 2 - Determination of cortisol level from blood serum samples harvested from conventionally slaughtered pigs in November 2019

In November 2019, 10 blood samples collected from conventionally slaughtered pigs in a slaughterhouse were analysed in a specialized laboratory. All cortisol values obtained by analysis using immunological examination exceeded the reference interval. The lowest value recorded was 4.63 µg/dL and the highest value 16.0 µg/dL. The accepted reference interval is 2.6-3.3 µg/dL.

Sample number 7 registered a value 4 times higher compared to the reference interval, and sample number 8 registered a value 5 times higher. The results are presented in Table 2.

Table 2. Results of cortisol level dosing in conventionally slaughtered pigs in batch 2

No.	Species	Breed	Age	Sex	Slaughtering date	Method	Cortisol level µg/dL	Reference interval
1.	swine	half-breed	7 months	M	18.11.2019	Immunological	**7.23 µg/dL**	2.6-3.3 µg/dL
2.	swine	half-breed	7 months	M	18.11.2019	Immunological	**4.63 µg/dL**	2.6-3.3 µg/dL
3.	swine	half-breed	7 months	M	18.11.2019	Immunological	**6.83 µg/dL**	2.6-3.3 µg/dL
4.	swine	half-breed	7 months	M	18.11.2019	Immunological	**7.23 µg/dL**	2.6-3.3 µg/dL
5.	swine	half-breed	7 months	M	18.11.2019	Immunological	**8.15 µg/dL**	2.6-3.3 µg/dL
6.	swine	half-breed	7 months	M	18.11.2019	Immunological	**9.67 µg/dL**	2.6-3.3 µg/dL
7.	swine	half-breed	7 months	M	18.11.2019	Immunological	**12.7 µg/dL**	2.6-3.3 µg/dL
8.	swine	half-breed	7 months	M	18.11.2019	Immunological	**16.0 µg/dL**	2.6-3.3 µg/dL
9.	swine	half-breed	7 months	M	18.11.2019	Immunological	**9.34 µg/dL**	2.6-3.3 µg/dL
10.	swine	half-breed	7 months	M	18.11.2019	Immunological	**7.56 µg/dL**	2.6-3.3 µg/dL

Comparing the two groups analyzed, it can be seen that the highest values of cortisol levels were recorded in the group slaughtered in November, compared to the group slaughtered in June. This most likely correlates with the low temperatures to which the animals were exposed in the cold season, as temperature is, according to numerous studies, an important factor influencing the stress level of animals.

Guerrini and Bertchinger showed that the lowest plasma cortisol values were recorded during exposure of animals in a warm environment, and the highest values were recorded at the time of their exposure in a cool and moist environment. These results suggest that exposure of animals in a moist and low temperature environment causes an increase in cortisol concentration (Guerrini & Bertchinger, 1982).

Study 3 - Determination of cortisol level from blood serum samples harvested from traditionally slaughtered pigs in December 2020

The samples of study 3 were collected, following the traditional slaughter of pigs, in December 2020 in the period before Christmas, from the households of the population from Dâmboviţa county.

12 blood samples were studied, five of them obtaining an optimal cortisol level, and the other seven exceeding the values of the reference interval, but not as much as in the case of the results obtained from pigs slaughtered with stunning in the slaughterhouse, which most likely correlates with the growth method practiced, with the fact that the animals do not suffer from transport

stress and with the fact that the animals do not sit in crowded lots and do not feel the reactions of those slaughtered before them. The results obtained are presented in Table 3.

Table 3. Results of cortisol level dosing in traditionally slaughtered pigs in batch 3

No.	Species	Breed	Sex	Age	Weight	Slaughtering date	Growth system	Method	Cortisol level µg/dL	Reference interval
1.	swine	half-breed	M	12 months	160 kg	12.12.2020	Household	Immunological	3.00 µg/dL	2.6-3.3 µg/dL
2.	swine	half-breed	M	12 months	180 kg	13.12.2020	Household	Immunological	2.49 µg/dL	2.6-3.3 µg/dL
3.	swine	half-breed	M	12 months	200 kg	13.12.2020	Household	Immunological	2.37 µg/dL	2.6-3.3 µg/dL
„4.	swine	half-breed	M	12 months	160 kg	14.12.2020	Household	Immunological	2.12 µg/dL	2.6-3.3 µg/dL
5.	swine	half-breed	M	12 months	190 kg	14.12.2020	Household	Immunological	**6.12 µg/dL**	2.6-3.3 µg/dL
6.	swine	half-breed	M	12 months	160 kg	18.12.2020	Household	Immunological	**5.43 µg/dL**	2.6-3.3 µg/dL
7.	swine	half-breed	F	12 months	130 kg	18.12.2020	Household	Immunological	**6.24 µg/dL**	2.6-3.3 µg/dL
8.	swine	half-breed	M	18 months	350 kg	19.12.2020	Household	Immunological	**3.74 µg/dL**	2.6-3.3 µg/dL
9.	swine	half-breed	M	12 months	140 kg	19.12.2020	Household	Immunological	**5.14 µg/dL**	2.6-3.3 µg/dL
10.	swine	half-breed	M	12 months	220 kg	20.12.2020	Household	Immunological	**7.26 µg/dL**	2.6-3.3 µg/dL
11.	swine	half-breed	M	12 months	160 kg	21.12.2020	Household	Immunological	**4.20 µg/dL**	2.6-3.3 µg/dL
12.	swine	half-breed	M	12 months	200 kg	21.12.2020	Household	Immunological	2.41 µg/dL	2.6-3.3 µg/dL

Śmiecińska et al. in 2011 conducted a study on a batch of 24 pigs slaughtered immediately after transport and a batch of 20 pigs slaughtered after a 24 hour rest period. The cortisol level recorded an average value of 26.54 µg/dL in pigs slaughtered immediately after transport and an average value of 15.44 µg/dL in the group of pigs slaughtered after a rest period of 24 hours.

Batches 1 and 2 of the present study were slaughter after a rest period and had a mean cortisol value of 7.33 µg/dL, which is lower than the results of the above study, but which exceeds the reference interval. of 2.6-3.3 µg/dL.

Batch 3 represented by blood samples from pigs slaughtered in the traditional system, recorded an average cortisol level of 4.21 µg/dL, this being a value close to the maximum limit of the reference interval (2.6-3.3 µg/dL).

Increased cortisol levels are an indicator of the stress response of animals, resulting from the stimulation of the sympathetic and parasympathetic nervous system and the hypothalamic-pituitary-adrenal axis (Śmiecińska et al., 2011).

The above stimulates the adrenergic system to produce catecholamines and improves the secretion of steroid hormones, mainly cortisol, from the adrenal cortex (Zavy et al., 1992). Handling operations before slaughter induce an intense response to stress (Śmiecińska et al., 2011). At the same time, rest before slaughter physiologically balances the body and alleviates the stress induced by pre-slaughter manipulation (Gispert et al. 2000; Fischer, 2001; Śmiecińska et al., 2011).

The results obtained from the summary statistics (mean values and standard deviation) of blood samples collected are shown in Table 4.

Tabel 4. Summary statistics of cortisol level in blood serum samples (mean values and standard deviation) harvested from slaughtered pigs

Batch number	Cortisol (mean values and standard deviation)	Samples number
1	5.3275±1.73425	8
2	8.9340±3.267324	10
3	4.2100±1.785344	12

CONCLUSIONS

In the slaughterhouses from the study, all technological stages of animal slaughter are observed. No accidental fall of the animals on the supply corridor was observed, nor was their refusal to enter the containment box. The stunning method practiced is electric stunning.

Excessive handling of pigs before slaughter induces their stress, therefore special attention must be paid to the slaughter process in order to minimize stress levels and improve meat quality.

Respecting the rest period before slaughter physiologically balances the body and alleviates the stress induced by animals handling.

The highest values of cortisol levels were recorded in the batch slaughtered in November, compared to the batch slaughtered in June, which most likely correlates with the low temperatures to which the animals were exposed, as temperature is an important factor that influences the stress level of the animals.

Samples collected from traditionally slaughtered pigs obtained lower cortisol levels compared to blood samples collected from conventionally slaughtered pigs, which most likely correlated with the way the animals were grown, with the fact that they did not suffer from transport stress and the fact that the animals do not live in crowded batches.

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PHENOLICS CONTENT, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF SOME EXTRACTS OBTAINED FROM ROMANIAN SUMMER SAVORY AND LEBANON WILD THYME

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Abstract

The aim of the present study was to compare the antioxidant and antibacterial properties of summer savory (Satureja hortensis L.) from Muscel County flora (Romania) and wild thyme (Thymus serpyllum) from Lebanon. The aerial parts of plants were harvested in august, dried quickly and alcoholic extracts were prepared. Total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity, ferric reducing antioxidant power (FRAP) value, and antimicrobial properties were determined using the extracts. TPC showed that Lebanon thyme had higher concentration compared with Romanian thyme (13.78 ± 0.95 mg GAE/g DW and 12.14 ± 0.97 mg GAE/g DW, respectively). DPPH• was calculated as IC_{50} and the sample results were compared with gallic acid. FRAP results showed similar values 42.71 ± 4.24 µM Fe^{+2}/g DW (Romanian thyme) and 39.55 ± 4.21 µM Fe^{+2}/g DW (Lebanon thyme). The antibacterial activity of summer savory was found to have maximum effect against Staphylococcus aureus ATCC 9144. Bioactive compounds, measured as total phenolic content, were in higher concentration in both extracts which also relates to their antioxidant and antibacterial activities.

Key words*: Satureja hortensis, Thymus sepyllum, phenolics, flavonoids, antioxidant and antimicrobial activity.*

INTRODUCTION

In the past few years, the food industry producers have tried very hard to change direction toward a clean label. The replacement of synthetic preservatives with phenolic structures, such as butylated hydroxyanisole (BHA), with extracts obtained from aromatic plants rich in natural phenolics, has attracted the attention of the researchers, due to speculation about the possible toxic effects of synthetic antioxidants (Papuc et al., 2010).

Aromatic plants have been studied extensively because they are a rich source of natural antioxidants and antimicrobial substances which can be extracted relatively easily using different solvents. Summer savory and wild thyme, could achieve these demands due to their active compounds found in their extracts (Gedikoğlu et al., 2019). Recent trends for natural food additives made plant extracts with antioxidant and antimicrobial properties an important step to obtain a clean label and proved beneficial food products for the consumers (El-Guendouz et al., 2019; Gonelimali et al., 2018). It was reported that the antioxidant and antimicrobial effects of aromatic plants are closely related to the presence of phenolic compounds (Kulisic et al., 2005). *Satureja hortensis* L. (summer savory, thyme), member of *Lamiaceae* family, is a variety of an annual herbaceous crop species, flowering shrubs, found in many parts of the world, native from the western Mediterranean to southern Europe. Growing up to 30 cm tall, by 40 cm wide, it is a bushy evergreen subshrub with small, aromatic, grey-green leaves and clusters of purple or pink flowers in summer (Fierascu et al., 2018). Summer savory is known especially as aromatic herb and have an intense culinary use. In folk medicine, these herbs are used against headaches, toothaches, colds, asthma, and rheumatism (Gedikoğlu et al., 2019).

Thymus serpyllum is a perennial shrub, known as Breckland thyme, wild thyme, or creeping thyme; however, its specific name "*serpyllum*" is derived from the Greek word meaning "*to creep*", because of wild thyme's trailing habit. It is a species of flowering plant in the mint family *Lamiaceae*, native to regions of Europe, Asia and North Africa. It is a low, subshrub

growing to 2 cm tall with creeping stems up to 10 cm long. The oval evergreen leaves are 3–8 mm long. The strongly scented flowers are either lilac, pink-purple, magenta, or a rare white, all 4-6 mm long and produced in clusters. The hardy plant tolerates some pedestrian traffic and produces odours ranging from heavily herbal to lightly lemon, depending on the variety. It has high tolerance for low water and poor nutrient soils. The increase of pathogenic microorganism's multidrug resistant has led to extensive phytochemical and pharmacological studies of *T. serpyllum* as an important source of medicinal substances with antioxidant and antimicrobial properties (Jarić et al., 2015).

Based on *in vitro* tests, Uysal et al. (2015) reported that their chemical constituents, such as phenols and flavonoids, provide antimicrobial and antioxidant properties.

The ***objective*** of this research was to evaluate the antioxidant and antimicrobial activities of *Satureja hortensis* L. (summer savory), harvested from Muscel County, Romania and *Thymus serpyllum* (wild thyme) growing in Lebanon using a multiple-method approach in relation to their chemical composition, comparatively with synthetic antioxidants used in food industry.

MATERIALS AND METHODS

Reagents and chemicals. Spectrophotometric grade ethanol, 2,4,6 three(2-pyridyl)-S-triazine (TPTZ) reagent $AlCl_3$ anhydrous, sodium nitrite, sodium hydroxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium bicarbonate anhydrous, gallic acid, catechin and Folin - Ciocalteu reagent were supplied by Sigma Aldrich (Germany). Iron (III) chloride hydrate and ferrous sulfate ($FeSO_4 \cdot 7H_2O$) were purchased from Fisher Scientific (UK). Tryptic soy agar, tryptic soy broth, and Muller-Hinton agar were supplied by Merck (Germany). Antimicrobial susceptibility test disks were purchased from Oxoid (UK).

Hydroalcoholic extract preparation. Fresh plant parts (flower, leaves and stems) of both *Satureja hortensis* L. (summer savory) from Muscel County, Romania and *Thymus serpyllum* (wild thyme) growing in Lebanon were collected. Samples were washed, dried and powdered and stored at room temperature, in darkness. For extraction, ten grams of each sample were weighed into a beaker and 100 ml of 60% aqueous ethanol (1:10 ratio of w/v) was added. After 30 min the sample was placed in water bath for 3 hours at 60°C. Next, the homogenates were filtered using Whatman no. 1 filter paper. The two filtrates were placed in 250 ml round-bottom flasks.

Total phenolic content. To determine the total polyphenol content, 0.5 ml of the sample extract and 7 ml of distilled water were mixed with 0.5 ml of Folin - Ciocalteu's reagent. After 5 min, 2 ml of 20% Na_2CO_3 solution was added, and the mixture was incubated for 60 min in the dark. The reaction mixture absorbance was measured at 760 nm, and the reaction mixture without the extract was used as a blank. Gallic acid was used as standard, and a 5 points standard curve was prepared (0-10 mg/dl). The TPC of the plant extract was expressed as gallic acid equivalents/g dry weight plant (mg GAE/g DW) (Singleton and Rosi, 1965).

Total flavonoid content. The total flavonoid content was determined according to the method of Zhishen et al., 1999. 1 ml of extract was placed in a ten ml flask that contained 5 ml distillate water. Then, 0.3 ml of 5% $NaNO_2$ solution were added. After 5 minutes 0.6 ml of 10% $AlCl_3$ were added and after another 5 minutes, 2 ml of 1 M NaOH solution were added and the volume was brought to ten ml with distilled water. The mixture was left 15 minutes at room temperature and the absorbance was read at 510 nm. The results were expressed as mg catechin equivalent/g dry weight plant (mg CAT/g DW).

DPPH radical scavenging activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity was measured according to Blois (1958). Next, 50 µl plant extract (at different concentrations in methanol) was mixed with 5 ml of a 0.004% (w/v) DPPH▪ methanol solution. The reaction was allowed to stand at room temperature for 30 min, and absorbance was read against a blank at 517 nm. The inhibitions of the DPPH radical in percent were calculated as follows:

$$I\,(\%) = \left(\frac{A\ blank\ -\ A\ sample}{A\ blank}\right) \times\ 100$$

Where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts. Extract concentration providing 50% inhibition (IC_{50}) was calculated using the graph ‑ plotted inhibition percentage against the extract concentration. Gallic acid and butylated hydroxy anisole (BHA) were used as positive controls in concentration of 10 mg/100 ml. The IC_{50} value for each sample was determined graphically by plotting the percentage discoloration of DPPH• solution as a function of the sample concentration.

The ferric reducing antioxidant power (FRAP) FRAP assay is based on the ability of phenolics to reduce yellow ferric tripyridyl triazine complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by the action of electron-donating antioxidants (Riahi et al., 2013). The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM TPTZ in 40 mM HCl), and $FeCl_3 \cdot 6H_2O$ (20 mM) in a ratio of 10:1:1 (v/v/v). To carry out the assay, 1.8 mL of FRAP reagent, 180 µl distilled water, and 20 µl of plant extract were mixed. After 15 min at 37°C, the absorbance was measured at 595 nm, using the FRAP solution as a blank. The antioxidant capacity of plant extracts was determined from a standard curve plotted using the $FeSO_4 \cdot 7H_2O$ linear regression. Results were expressed as µmols Fe^{2+}/g DW. BHA and ascorbic acid were used as controls in concentration of 10 mg/100 ml.

Antimicrobial activity. Two Gram-positive and three Gram-negative bacteria were used as test organisms: *Staphylococcus aureus* ATCC 9144, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, and *Salmonella typhimurium* ATCC 14028.

Disk diffusion assay (DDA) (Oke et al., 2009). All the bacterial species were first inoculated into tryptic soy agar and incubated overnight at 37°C. After checking for purity, the bacteria were suspended in a 0.9% NaCl solution. A spectrophotometer was used to adjust the final cell concentration (1.5×10^8 cfu/ml) by reading the DO at 600 nm. Then, 100 µl of the bacterial suspensions was spread on Mueller-Hinton agar. The 6-mm-diameter, sterile, empty disks impregnated with 20 µl of extracts were placed on the inoculated agar. Antibiotic standard disks were used as a control. The inoculated plates were incubated at 37°C for 24 h. As positive controls, ciprofloxacin and Ampicillin (30 µg/disk) were used for bacterial strains. Antibacterial activity was determined by measuring the *zone of inhibition* in mm without including the diameter of the disk (Valgas et al., 2007).

RESULTS AND DISCUSSIONS

Total phenolic and flavonoid content. Plants from *Lamiaceae* family are known to be rich in compounds possessing strong antioxidant activity. Thyme and wild thyme, are found in many parts of the world, especially in the Mediterranean region, are also regarded as medicinal herbs and condiments. Because of the highest concentration in active compounds, as phenols and flavonoids, thyme has positive effect on health of the consumers, when it is used as tea or added in food. In fact, the food products that contain natural antioxidants have a double benefit: better conservation during the viability period and the excess of antioxidants get to the consumers.

Table 1. Total phenolic compounds and total flavonoid content in hydroalcoholic extracts of Romanian and Lebanon wild thyme

Analysed plant	Total phenolic content (TPC) mg GAE/g DW	Total flavonoid content (TFC) mg CE/g DW
Lebanon wild thyme	13.78 ± 0.95	8.54 ± 0.84
Summer savory	12.14 ± 0.97	7.49 ± 0.79

Values are the average of duplicates ± standard deviation.

The concentration of phenols in plants is dependent on climate and geographical position (Liu et al., 2018). *Satureja hortensis* L. (summer savory) from Muscel County, Romania and *Thymus serpyllum* (wild thyme) growing in Lebanon contain important concentrations of phenols and flavonoids. These findings are similar with other results regarding *Satureja hortensis* L. and *Thymus serpyllum* phenol and flavonoid contents (Ballester−Costa et al., 2017; Plánder et al., 2012; Kulisic et al., 2005). Lebanon wild thyme extract have shown that contains a high content of phenolic 13.78 ± 0.95 mg GAE/g DW and flavonoid 8.54 ± 0.84 mg CE/g DW

compounds when compare to Romanian thyme which had 12.14 ± 0.97 mg GAE/g DW and 7.49 ± 0.79 mg CE/g DW (Table 1). It was calculated that for both types of thyme, about 60% of the phenols were flavonoids. It was also calculated that the Lebanese wild thyme had 14% more polyphenols than the Romanian one. Also, the concentration of flavonoids was lower for the Romanian thyme compared to the Lebanese one with 14%. Statistically significant difference and positive correlation between the concentration of phenol and flavonoid contents of Romanian and Lebanese thyme ($p < 0.05$, $R^2 = 0.9999$).

DPPH radical scavenging activity. DPPH (2,2 - diphenyl - 1 - picrylhydrazyl) free radical scavenging activity was used to investigate the antioxidant activity of two thyme extract by comparation with gallic acid. The effect of antioxidants on DPPH radical scavenging is due to their ability to donate hydrogen. DPPH▪ is a stable free radical which accepts a hydrogen radical from an antioxidant molecule (AH) to become a stable diamagnetic molecule, in accordance with the equation below:

$$DPPH▪ + AH \rightarrow DPPH▪▪H + A▪$$

The concentration of antioxidant needed to decrease the initial DPPH concentration by 50% (IC_{50}) is a parameter widely used to measure the antioxidant activity (Brighente et al., 2007).

The free radical scavenging activity is higher when IC_{50} value is lower. The amount of extract needed to decrease the initial radical DPPH concentration by 50% is used for the free radical scavenging activity and is established as IC_{50}. Results of the DPPH radical scavenging activity test are shown in Table 2. IC_{50} of Lebanon thyme extract (85.25 ± 7.31 µg/ml) was significantly ($p < 0.05$) higher than that found for Romanian thyme extract (102.94 ± 8.14 µg/ml) radical scavenging activity when compared to BHA.

Some researchers reported that the phenolic compounds with hydroxyl groups attached to their structures present in aromatic plants are responsible for the important antioxidant effect (Shahidi et al., 1992). When comparing the sample results with the standard gallic acid (4 ± 0.35 µg/ml), the extracts in the current study had a much lower free radical scavenging activity. A significantly higher correlation was established between total flavonoid content and DPPH radical scavenging activity ($p<0.05$). The results indicated that both Romanian and Lebanon thyme extract have effective DPPH radical scavenging activities. It was observed that Lebanon wild thyme extract had the most effective DPPH radical scavenging activity.

Table 2. DPPH radical scavenging activity of hydroalcoholic extracts of Romanian and Lebanon wild thyme

Sample	IC_{50} value of DPPH (µg/ml)
Lebanon wild thyme extract	15.25 ± 1.31
Summer savory extract	17.94 ± 2.14
Gallic acid 10 µg/100 ml	4.51 ± 0.35
BHA 10 µg/100 ml	14.84 ± 1.57

Values are the average of triplicates ± standard deviation.

The extract of Lebanon thyme, with significant DPPH • scavenging activity, also had a higher quantity of total phenolics. This extract, which have a high antioxidant activity, also had a great quantity of flavonoids, as summarized in Table 1.

The ferric reducing antioxidant power (FRAP)

Prior et al. (2005) found out that FRAP mechanism is based on electron transfer rather than hydrogen atom transfer. The basis of FRAP assay is the ability of antioxidant compounds to reduce Fe^{3+} to Fe^{2+}.

The FRAP reaction is taking place in acidic medium (pH value equal to 3.6) in order to maintain iron solubility. The reaction mechanism is based on decreasing of ionization potential at low pH that drives hydrogen atom transfer and increases the redox potential. In the presence of 2,4,6-trypyridyl-s-triazine the reduction of Fe^{3+} to Fe^{2+} occurs. The reduction reaction is accompanied by the formation of a blue complex with Fe^{2+} with a maximum absorption at 593 nm. The reducing power appears to be related to the degree of hydroxylation and extent of conjugation in antioxidant compound (Huang et al., 2005). The results of evaluation of Ferric reducing antioxidant power (FRAP) are shown in Table 3.

Antioxidative activity of examined plant extracts measured with FRAP method bring more information regarding benefic effects of them. Results of the evaluations presented as

µmols Fe^{2+}/g DW, showed increasing activity with phenol extract's concentration. Highest FRAP values were found for ascorbic acid (54.14 ± 5.61 µmols Fe^{2+}/g DW) and in sample of Lebanon thyme extract (42.71 ± 4.24 µmols Fe^{2+}/g DW) and Romanian thyme extract (39.55 ± 4.21 µmols Fe^{2+}/g DW). Also, high activity showed BHA (34.12 ± 3.42 µmols Fe^{2+}/g DW). Similar results were observed by Gedikoğlu et al. (2019) and Birasuren et al. (2013). As the result of the FRAP analysis herbs extracts and standards were ranked as follows: BHA < Romanian thyme extract < Lebanon wild thyme extract < ascorbic acid.

Statistical analysis of relationships between ferric reducing antioxidant power of ethanol herbs extracts and total polyphenol content showed high correlations (R^2 = 0.9999, $p < 0.05$). Also, a significantly higher correlation was established between FRAP and DPPH radical scavenging activity ($p < 0.05$).

Table 3. Ferric reducing antioxidant power (FRAP) value of hydroalcoholic extracts of Romanian and Lebanon thyme

Sample	FRAP (µmols Fe^{2+}/g DW)
Lebanon wild thyme extract	42.71 ± 4.24
Summer savory extract	39.55 ± 4.21
Ascorbic acid (10 µg/100 ml)	54.14 ± 5.61
BHA (10 µg/100 ml)	34.12 ± 3.42

Values are the average of duplicates ± standard deviation.

Khosh-Khui et al. (2012) find out that water deficiency might increase antioxidants levels depending on plant genotypes and this can explain why Lebanon wild thyme extract showed higher antioxidant activity.

Antimicrobial activity. Two Gram-positive bacteria (*Staphylococcus aureus* ATCC 9144, *Staphylococcus epidermidis* ATCC 12228) and three Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, and *Salmonella typhimurium* ATCC 14028) were used to test antimicrobial activity of Romanian thyme extract and Lebanon thyme extract. Table 4 present the antimicrobial activities of thyme extracts and BHA against various organisms. The two extracts possessed antimicrobial activity against all tested bacteria, but the highest activity was showed by Lebanon wild thyme extract. BHA showed the lowest antimicrobial activity. This result regarding the antimicrobial activity of synthetic antioxidant were similar with other researches (Gavarić et al., 2015).

Thyme extracts are known to possess some antimicrobial activities and are used in various food preparations as flavour enhancers (Nzeako et al., 2006). Even the antimicrobial pathways are not fully known and understood, it looks that its action is due to the compounds present in the thyme extract (phenols and flavonoids among the others) (Gavarić et al., 2015). The synergistic effects of different compounds present into extracts can contribute to the antimicrobial activity through different mechanisms. Different extract compounds can interfere with bacterial membrane and thereby increase the cell leakage or act indirectly antimicrobial by facilitating the influx of antimicrobial phenolic compounds (Burt, 2004). For this reason, it is recommended to use plant extracts than pure compounds (Burt, 2004).

Table 4. Antimicrobial activity of hydroalcoholic extracts of BHA and Romanian and Lebanon wild thyme

Sample / Microorganism	Lebanon wild thyme extract	Summer savory extract	BHA (10 µg/100 ml)
	Zone of inhibition in mm		
Staphylococcus aureus	27.5 ± 3.1	28.1 ± 2.1	13.6 ±0.9
Staphylococcus epidermidis	24.1 ± 1.5	23.3 ± 1.9	14.1±1.0
Escherichia coli	25.2 ± 1.4	25.6 ± 2.1	11.2 ±0.9
Salmonella enteritidis	18.4 ± 1.1	16.4 ± 1.5	14.2 ±1.2
Salmonella typhimurium	13.2±1.1	11.0 ± 1.1	6.±0.7

Values are the average of triplicates ± standard deviation.

CONCLUSIONS

Both hydroalcoholic extracts were found to contain a noticeable amount of phenolics and flavonoids. Phenolics and flavonoids, may be the compounds responsible their antioxidant and antimicrobial activities in these plants extracts. The thyme extracts contain compounds with antioxidant activity that act as hydrogen/electron donors. Romanian thyme extract and Lebanon wild thyme extracts showed strong DPPH radical scavenging activity and strong ferric reducing antioxidant power when compared with standard. The results of this study show that the extract possessed antimicrobial activity against the tested bacteria and can be used as an easily accessible source of natural antibiotic. In

addition, the extracts of these plants can be regarded as plant-derived antioxidant and antimicrobial mixture in different fields (foods, cosmetics, pharmaceuticals).

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CLINICAL SCIENCES

COMPARATIVE STUDY OF THE INFLUENCE OF HAWTHORN (CRATAEGUS MONOGYNA) BERRIES ETHANOLIC EXTRACT AND BUTYLATED HYDROXYLANISOLE (BHA) ON LIPID PEROXIDATION, MYOGLOBIN OXIDATION, CONSISTENCY AND FIRMNESS OF MINCED PORK DURING REFRIGERATION

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ABSTRACT

BACKGROUND: Following public concern on the use of synthetic food antioxidants, there is an increasing demand for the application of mixed or purified natural antioxidants to maintain meat produces quality during storage.

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The aim of this research was to investigate the effect of hawthorn berries' ethanolic extract compared to butylated hydroxylanisole (BHA), on lipid peroxidation, myoglobin oxidation, protein electrophoresis pattern, consistency and firmness of minced pork during refrigeration at 4 ^{0}C and to identify the relationship between chemical modifications and consistency variation.

RESULTS. After 6 days of refrigeration it was found that: thiobarbituric acid reactive substances value of minced pork containing 200 mg GAE kg^{-1} total phenolics in minced meat (200 HP) was significant lower (0.1543±0.006) mg compared to BHA treated meat. The ratio of oxymioglobin / metmyoglobin in treated minced pork was respectively 0.845 for 200 HP and 0.473 for BHA treated minced meat; Concentrations of 100 HP or 300 HP will generate statistically higher firmness than the BHA in minced pork.

CONCLUSION:

The hawthorn berries' ethanolic extract was more effective than BHA in reducing lipid oxidation and protein degradation, for maintaining firmness and consistency of minced pork during six days of refrigeration at 4 ^{0}C.

INTRODUCTION

During refrigeration, meat and meat produces are subject to oxidative process that are responsible for the alteration of products colour and odour (oxidation of lipids, proteins and vitamins) and the modifications of their textural properties and nutritional value.

Meat lipid peroxidation and myoglobin oxidation are coupled processes. Aldehydes resulted from lipid peroxidation alter the stability of myoglobin oxidation and promote the formation of metmyoglobin and reactive species resulted from

myoglobin oxidation and metmyoglobin itself induce lipid peroxidation[1]. Antioxidants inhibit myoglobin oxidation by two mechanisms: (1) decrease unsaturated aldehydes levels, resulted from fatty acids oxidation, by decreasing lipid peroxidation, (2) scavenge hydrogen peroxide which can react with Fe^{2+} from myoglobin in Fenton reaction[1]. Moreover it was demonstrated that meat protein oxidation during cold storage lead to changes in amino acid structures which generate carbonyl formation and decrease sulfhydryl content.[2] Meat protein oxidation is also responsible for changes in water holding capacity and sensory tenderness.[3]

Synthetic antioxidants such as BHA (butylated hydroxylanisole), BHT (butylated hydroxytoluene) and PG (propyl gallate) are delaying the oxidation of meat produces during processing and storage.

Several reports suggest that synthetic antioxidants have toxic and carcinogenic effects,[4-6] thus there is an increased consumers' demand for the replacement of synthetic antioxidants with natural ones.[7]

An alternative to synthetic antioxidants are plant phenolic compounds. Plant phenolics are bioactive substances widely distributed in plants, with antioxidant properties and beneficial to human health[8] due to their demonstrated effect for improving heart function[9,10], protecting against atherosclerosis and the removal of LDL (low density lipoproteins) from the bloodstream[11]. Plant phenolics are also considered as a class of antitumor agents[12]. Among the beneficial effects of plant phenolics are the inhibition of carcinogenesis at the initiation, promotion and progression stages. [9,12]

Alongside their pharmaceutical value, due to their antioxidant capacity, the plant phenolic extracts are valuable additives for the food industry and especially for the meat industry[7,8,13,14].

Plant phenolics can slow lipid peroxidation by several mechanisms: (1) scavenging reactive species that initiate peroxidation, (2) chelating transitional metal ions, such as Fe^{2+} and Cu^{+}, preventing them from participating in the Fenton reaction, (3) breaking the free chain reaction by donating H• to the free radical formed during lipid oxidation, (4) acting as electrons donors.

A large number of studies reported many plant species as good candidates for obtaining phenolic extracts with antioxidant properties.[15-21] A large number of investigations were carried out on many plant families such as Asteraceae (daisy family), Polygonaceae (sorrel family), Rosaceae (dog-rose family), and Lamiaceae (mint family) with a main focus on the phenolics content of the extracts obtained from the aerial plant organs. The fruits, basal or other leaves, midribs, young stems, shoots, and inflorescences, were studies for species growing in many countries[9,14,16,19,22].

Among the plants fruits studied for their antioxidant capacity and richness in phenols are the berries belonging to the genus Crataegus which comprises about 265 species, such as *C. monogyna*, generally known as hawthorns [9,16,19,22]

Simirgiotis[16] reported that *C. monogyna* berries contain mainly flavonoid glycosides, phenolic acids, anthocyanins and flavonoid aglycons, have an antioxidant capacity of 3.61 ± 0.01 μg mL^{-1} in the DPPH radical assay and have the total phenolics content of 28.30 ± 0.02 mg GAE g^{-1} dry material (GAE- gallic acid equivalent) . Moreover he also reported that *C. monogyna* berries have the total flavonoids content of 8.77 ± 0.00 mg QE g^{-1} dry material (QE-quercetin equivalents).

Plant phenolic extracts can decrease lipid peroxidation,[23] myoglobin oxidation [8,19,24] and reduce metmyoglobin to oxymioglobin in the presence of thiols.[25]

Turgut et al [3] measured carbonyl and thiol (sulfhydryl) to evaluate the oxidative reactions of muscle proteins and demonstrated that pomegranate peel extract (containing high polyphenols concentration) influenced the oxidative reactions of muscle proteins during meatball refrigerated storage (4^0C). Plant phenolics extracts added to meat, can also influence protein conformation and stimulate the protein cross-linking process which modifies the rheological properties of minced meat. Apple powder containing transglutaminase (TG) and polyphenol oxidase (PPO) added to pork meat homogenate increased the value of storage modulus (G_0)[26] which is an evidence of improved consistency and firmness. Moreover, they found that the apple powder improved gel hardness of unheated meat homogenate at 4^0C.

Estevez et al.[27] found that pork liver pâté treated with rosemary extracts and sage extracts, showed a significant decrease of firmness after 30 days of refrigerated storage but had a very significant increase of firmness after 90 days of cold storage.

Maqsood and Benjakul[28] showed that SDS-PAGE (Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis- developed by Laemmli[29]) method revealed that protein patterns was modified for fresh ground beef and ground beef treated with sterilized distilled water and with tannic acid, stored in air and under modified atmosphere packaging for 15 days at 4C. They found that all samples contained tropomyosin, troponin-T and troponin-C, MHC (myosin heavy chain) and actins were degraded. They reported that under modified atmosphere packaging with high oxygen concentration, band intensity of MHC of the sample treated with sterilized distilled water decreased by 29.76%, whereas for the sample treated with tannic acid the band intensity of MHC decreased by 25.93% .

Modifications of protein pattern under phenolics treatment may also influence textural properties of minced meat such as consistency and firmness.

The rheological behaviour of food materials such as minced meat, liver pâté, and processed chesses is characterised with the help of back extrusion tests or rheometry. Back extrusion tests can measure textural properties such as consistency and firmness, which are important quality factors and attributes highly appreciated by consumers. During extrusion the food material is forced to flow in the gap between an inner and outer cylinder and Bingham law under different wall boundary conditions (stick, slip with friction and perfect slip) may well describe the food material flow behaviour.

Back extrusion, compression and puncture tests can provide important information on the structural modifications, which are taking place in meat due to added plants phenolics extracts. Presently little information is available on textural properties of minced meat containing different types of plant antioxidants (extracts).

Phenolics extracts added as antioxidants to meat and meat products will improve their quality and nutritional value and will be beneficial for human health as well. Plant phenolics are known to protect the biologically important cellular components, such as DNA, proteins, and membrane lipids, from reactive oxygen species (ROS).[30]

Hawthorn *(Crataegus monogyna)* is commonly found as a shrub or a small tree of 5 to 10 m tall and it is kown as common hawthorn**.** *Crataegus monogyna* is native to Europe, Asia and Northwest Africa however it may be an invasive specie which now can be found on territories from America's temperate regions too. Its small dark red fruits possess antioxidant activity due to the presence of different bioactive compounds such as flavonoids as hydroxycinnamic acids.[31-33] These compounds are reported to have neuroprotective, cardioprotective, hepatoprotective and nefroprotective effects. Several studies have shown that hawthorn berriescould reduce cardiovascular risk factors such as hypertension and hypercholesterolemia [34-36].

Although phenols from many plant species extracts, including *C. monogyna,* were largely studied and characterised [9,13,31-36], information on the influence of total phenolics from *C. monogyna* berries ethanolic extract on pork minced meat during refrigeration over time was not yet studied. The main aim of present research is to demonstrate that hawthorn fruits' ethanolic extracts are very useful additives for the food industry, taking into consideration that the detailed chemical characterization of the ethanolic extracts is beyond the scope of the present investigation. The main objectives of the present research was to investigate the effect of hawthorn's berries ethanolic extract compared to synthetic antioxidant BHA, on lipid peroxidation, myoglobin oxidation, protein electrophoretic pattern and textural parameters (consistency and firmness) of minced meat during refrigeration for 6 days at 4 ^{0}C.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents were of analytical grade and were purchased from Redox Life Tech, Bucharest, Romania Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of hawthorn berries ethanolic extract

Hawthorn berries harvesting, drying, grinding and sieving

Hawthorn berries were manually harvested in October from shrubs, aged about 10 to 15 years, from a South-East Carpathian hill forest. The berries were dried in an oven at 60°C for 10 hours. The dried berries were grinded and homogenise using the Grindomix GM 200 knife mill and manually sieved using a 500µm mesh size sieve. The

resulting fraction was used for the preparation of the hawthorn berries ethanolic extract (HBE).

Total phenolics extraction

The 500µm fraction of dried and grinded hawthorn berries was used for phenols extraction with ethanol 60% (v/v) in 1:10 ratio (w/v). The mixture was stirred for 12 hours at room temperature and then heated into a water bath at 60°C for 3 hours. After filtration, the mixture was centrifuged at 10000 x g for 15 min at room temperature using a Sigma centrifuge Sigma (2 – 16K). The ethanol was evaporated at 80°C using a Heidolph (Laborota 4000) rotary evaporator. The pH of the extract was adjusted to neutral with 2M NaOH.

Preparation of phenolics solutions for meat treatments

As liquid addition to the minced meat will influence its chemical and rheological properties, we have prepared three different solutions of 100 ml each using the neutralised extract. The solutions were prepare by using the neutralise extract and sterilised ultrapure water at 0^0C . The concentration of phenolics in each solution was calculated in order so that the three minced meat treatments would contain hawthorn berries total phenolics (HP) of 100 mg GAE kg^{-1} of minced meat (S1), 200 mg GAE kg^{-1} of minced meat (S2) and 300 mg GAE kg^{-1} of minced meat (S3) respectively (HP – denotes the total phenolics in the minced meat, expressed as mg GAE kg^{-1}minced meat) (Table 1).

Preparation of the BHA solution for meat treatments

The forth treatment (S4) consisted of minced meat treated with 100 ml of solution containing BHA. The solution was prepared so that the meat treated with the synthetic antioxidant will contain BHA as much as of 100 mg kg^{-1} of minced meat. (Table 1).

Meat samples preparation

Meat preparation

About 1.2 kg of fresh pork leg muscle, from 4 different carcasses was purchased from a local slaughter house located in Bucharest, Romania. The meat was packed in a polyethylene bag and kept in a cooler during transportation. Same day, the pork meat was washed with sterilized distilled water (4°C) and dried using filter paper.

The meat was cut into 1cm x 1cm x 1 cm pieces. The convective tissues and fat were manually removed. The resulting 4 kg of meat was minced together and homogenized. The pork meat was minced using a commercially available electric grinder for home use containing a grinder plate of 5 mm holes. The grinder was chose to mimic the majority of the home grinders and professional grinding systems.

The resulting minced meat was divided into 4 aliquots of 1000g each. Each aliquot was divided into 3 portions, resulting 12 portions of 330g.

Treatment of minced pork

The three prepared solutions containing phenolics and the BHA solution were mixed with minced meat to obtain four meat treatment (S1, S2, S3 and S4) as described in table 1.

For each treatment 3 portions of 330 g of minced pork were distinctively treated with the phenolics solutions (Table 1) resulting 3 replicates per treatment. To each treatment was assigned a code as described in Table 1.

After the treatment, each of the 330g portions was divided in half (165g each) resulting 24 half-portions, thus chemical and rheological measurements used the same number of treatments and treatment replicates, 12 of the half- portions were used for the chemical analyses and 12 for the rheological measurements.

Chemical and rheological analyses were made in 3 replicates for each of the treatments' replicates.

All the treated minced meat was packed in polyethylene bags and stored at 4°C.

The chemical and rheological measurements were made simultaneously, every 2 days during the 6 days of refrigeration (day 0, day 2, day 4 and day 6). The SDS PAGE and GC (Gas chromatography) analyses were made for the all treatments at day 0 and day 6 The chemical and rheological measurements for "day 0" were made 5 hours after the treatments preparation.

Chemical analysis

Determination of total phenolic content (TPC) of the hawthorn berries ethanolic extract

The total phenolics content (TPC) of the hawthorn berries ethanolic extract was spectrophotometrically determined using Folin-Ciocalteu reagent[37] as following: 0.5 mL of the diluted sample and 7.0 mL distilled water reacted with 0.5 mL of Folin-Ciocalteu reagent for 3 minutes. Then 2 mL saturated sodium carbonate solution (about 20%) were added to the reaction mixture. The absorbance readings were taken at 765 nm after incubation at room temperature for 2 hours. The concentration of total phenolics in the

ethanolic extract was derived from a standard curve of gallic acid (GA). The results were expressed as *mg gallic acid equivalent/100 mL* (mg GAE/100 mL). All analyses were performed in triplicate.

Determination of conjugated diene (CD) and conjugated triene (CT) hydroperoxides

Total lipids extraction[38] was performed as following: the sample was homogenised with 2:1 (v/v) chloroform-methanol mixture to a final dilution of 20-fold sample volume. After filtration and washing, the extract was used for evaluation of conjugated dienes (CD) and conjugated trienes (CT). 20 μL lipid extract was mixed with 2 mL isooctane and absorbance was measured at 233 nm wave-length for CD and at 268 nm wave-length for CT, using isooctane as blank and a Jasco V670 spectrophotometer [39]. The results are in absorbance units.

Determination of thiobarbituric acid reactive substances (TBARS)

For TBARS analysis 0.5 g of minced meat was treated with 0.5 mL of synthetic antioxidant solution (BHA), and then was manually homogenized in 10 mL extracting solution represented by trichloroacetic reagent and distilled water 1:1 (v/v).

Trichloroacetic reagent contains 20% (w/v) tricloroacetic acid and 1.6% (w/v) *o*-phosphoric acid. The slurry was filtered into a 10 mL centrifuge tube. The volume was filled up to 10 mL with trichloroacetic acid/water (1:1, v/v). Subsequently, a 5 mL aliquot of this solution was transferred into another tube and was mixed with 5 mL of thiobarbituric acid solution (0.02 M). The mixture was then heated at 100°C in boiling water for 35 min until a pink colour developed. The mixture was cooled and the absorbance was measured at 532 nm using a UV-VIS spectrophotometer Jasko V670.

TBARS values were expressed as mg of malondialdehyde (MDA) per kg meat (mg MDA kg^{-1} meat), calculated using 1,1,3,3-tetramethoxypropane (TMP) as standard.[39]

Chromatographic profile of fatty acids

Lipid extraction and fatty acid methyl esters

Fatty acids profile of lipids from minced meat was determined as fatty acids methyl esters (FAME). Lipid extractions were made according to Bligh and Dyer method and FAME were prepared by transmethylation using 2M KOH in methanol and normal heptane according to the method described by Oz. [41]

Gas-chromatographic conditions

FAME were quantified by gas-chromatography (GC) using a Perkin-Elmer-Clarus 500 system with a flame ionisation detector (FID), capillary column injection system and a silica capillary column SGE (BPX70, 60 m; 0.25 mm inner diameter, 0.25 μm film, Agilent). The oven starting temperature was 180^0C and was increased by 5^0C/min to the final temperature of 220^0C, while the injector and the detector temperature were set at 220^0C and 260^0C, respectively. The split ratio used was 1:100 and split flow 50mL/min. The sample size was 1 μL. The carrier gas was hydrogen (35 cm/s, 180^0C) and the burning gas was the air oxygen (420 mL/min). FAME were quantified taking into consideration macromolecular chain length, unsaturation and double bonds geometry. Fatty acids are identified by comparing the retention times of FAME with the ones of FAME standard components mixture. The GC analyses were made in three replicates and the results were expressed as percentage (% of the total fatty acid methyl esters).

Determination of oxymyoglobin and metmyoglobin

Oxymyoglobin (OxyMb) and metmyoglobin (MetMb) concentrations in samples were determined according to the method described by Maqsood and Benjakul[42]: 2 g of minced meat were transferred into a centrifuge tube (50 mL) and 20 mL of cold 40 mM phosphate buffer (pH 6.8) were added. The mixture was homogenized and the homogenate was centrifuged at 3000 x g at 4°C for 30 min in a Sigma (2-16K) centrifuge. The supernatant was filtered with Whatman no. 1 filter paper and the filtrate was diluted to 25 mL with the same tampon buffer. The clear supernatant was scanned between 500 and 700 nm using a UV-VIS spectrophotometer (Jasco V670). OxyMb and MetMb contents were calculated[43]:

$$\% \text{ OxyMb} = (0.882R_1 - 1.267R_2 + 0.809R_3 - 0.361) \times 100$$

$$\% \text{ MetMb} = (-2.514R_1 + 0.777R_2 + 0.800R_3 + 1.098) \times 100$$

Where, R_1 is A_{572}/A_{525}, R_2 is A_{565}/A_{525}, and R_3 is A_{545}/A_{525}.

Proteins pattern by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE[28,29] was made according to Laemmli[29] method. The samples (15 µg protein) were loaded in polyacrylamide gel and subject to electrophoresis at a constant current, 15 mA per gel, using a mini Protein II unit (Biorad Laboratories Inc., Richmond, CA, USA).The molecular weight of proteins was estimated with wide range molecular marker. The protein band intensity was measured using GS-700 Imaging Densitometer (Bio-Rad laboratories, Hercules,Ca,USA) and the 1.4Molecular Analyst Software.

Rheological analysis

The rheological analysis was made using a Texture Analyser TA-*Plus* (Lloyds

company) using the NEXIGEN software. The back extrusion cell had a 30 mm diameter and the extrusion plunger (stainless steel) diameter was 11 mm with a flat head. Each measurement was made in three replicates for each treatment replicate. The weight of each sample used for the extrusion tests was 3.0000g ± 3%. Trigger force: 0.001 N; Test Speed: 1.0 mm/s; Post-Test Speed: 1.0 mm/s; Compression Distance: 8mm; Samples temperature: 6^0C

When a trigger force of 0.001N has been attained at the sample surface, the plunger compresses the sample over the specified distance of 8 mm. Once the specified distance has been reached, the plunger begins to withdraw from the sample.

The maximum force recorded is a measure of sample firmness over the specified distance. The sample consistency is represented by the work done (energy) by the plunger during the compression over 8 mm depth into the sample. The work (energy) is identified as the area below the force-displacement curve.

Textural analyses were performed every 2 days the same days as the chemical analyses.

Statistical analysis

Data statistical analysis was conducted using the JMP5.0 software (SAS Company) and IBM SPSS software (version 22). Analysis of variance was performed on all the variables measured using the General Linear Model (GLM) procedure and ANOVA. The tests used for testing that the variances are equal are: O'Brien, Brown-Forsythe, and Levene and for means comparison is Tukey-Kamer HSD.

ANOVA analyses are used to evaluate the influence of treatments and refrigeration time on the textural parameters. One way ANOVA was used to identify significant differences in fatty acids chromatographic profile for day 0 and 6 and conjugated dienes, conjugated trienes, TBARS, oxymioglobin percentages,

metmyoglobin concentration, All pairs Tukey- Kramer ($\alpha = 0.05$) and Duncan's New Multiple Range test was used to identify significant differences between means. The influence of the treatment was considered significant for $p<0.05$ and the means significantly different for $p<0.05$.

RESULTS AND DISCUSSIONS

Total phenolic content (TPC) of the Romanian hawthorn berries ethanolic extract. The TPC of the ethanolic extract obtained from Crataegus monogyna berries harvested in the SE Carpatian hill forest of Romania was of 1933 mg GAE / 100 g dry fruits. The TPC of the ethanolic extract we have obtained in our study is superior to the TPC reported by Baladica et al[9] obtained from the distilled water infusion of Romanian *C. monogyna* dry leaves (206,6 mg GAE/100 leaves powder). Using a methanolic extract of *C. monogyna* dried berries from Chile, Simirgiotis[16] found a TPC value of 28,30 mg GAE/100g dried fruits after extraction with 0.1%HCl in MeOH. Higher TPC (7480 mg GAE/100 g dry fruits) were also found in methanolic extracts of dried berries of cornelian cherry [44] and in acetone extracts of *Crataegus monogyna* Jaqc. (1282.0 ± 50.0.8) mg GAE/100 g dry fruit[22]. The difference between the TPC of different extracts was highlighted also by the study of Ganhão et al[45] who found that for the dry berries of *Crataegus monogyna* the TPC was 450 GAE/100 g dry fruit for the water extract, 600 GAE/100 g dry fruit for the methanol extract and 2068 GAE/100 g dry fruit for the ethanol extract. Our study is confirming that ethanol extracts from *Crataegus monogyna* dried fruits have high TPC. Our findings are in good accordance with the other reports[22, 45] however these results show that usually ethanol, methanol and acetone howthorn's fruits extracts are higher in TPC than extracts obtained by other extraction methods[9,16] .

The phenolics compounds in berries have been largely studied [9,16,45] including experiments for the identification of the composition of different types of extracts such as water , ethanolic , methanolic and acetone extracts. According to the extracting methods different concentrations of hydroxybenzoic acids, hidroxicinnamic acids, anthocianins, flavonols, flavan-3-ols, proanthocyanidins, ellagic acids and ellagitannins were found[9,16,45].

Simirgiotis[16] found that for the *Crataegus monogyna* berries methanolic extract on the basis of freeze-dried starting material, the total phenolics content (TPC) was of 28.30 mg GAE/g dry material, the total flavonoid content(TFC) was of 8.77mgQE/g (QE-quercetine equivalent), the ferric reducing antioxidant power (FRAP) was of 85.65μmolTE/g, the scavenging capacity DPPH of 3.61(IC_{50} μg/mL) and the extraction yield (w/w) of 12,36%

The phenolic compounds have different structures and number of hydroxyl groups having different molecular properties mode of action and stability.

The effect of hawthorn berries total phenolics on minced pork lipid peroxidation

Over the last three decades, a lot research work was developed to identify the main factors influencing lipid peroxidation in meat and meat produces. There is a general agreement that lipid peroxidation is influenced by animal specie, muscle type, anatomical location and type of processing[46,47]. However there is no general agreement yet on which of the two factors, content of unsaturated fatty acids[46] and total pigment and myoglobin concentrations[47], may be considered as a major factor for lipid peroxidation in meat. This controversy is supported by conflicting conclusions from different studies. Cheng [46] considered that due to the high content of unsaturated fatty acids the rate of lipid peroxidation is higher in pork compared to beef . However Min

and Ahn[47] citing Rhee and Ziprin[48] showed that lipid peroxidation in raw beef was more susceptible to lipid peroxidation than pork and chicken, suggesting that total pigment and myoglobin concentrations are responsible for the differences in lipid peroxidation. Moreover processing such as chopping and mincing, liberates the membrane-bound phospholipids, which will oxidise easier[46,49]. The disruption of muscle tissue and the incorporation of air during processing will facilitate several phenomenons such as: unsaturated fatty acids reaction with the oxygen from the air incorporated during processing; increased contact with enzymes and free ionic iron released from heme pigments, as major catalyst for lipid peroxidation in raw meat.

Lipid peroxidation of unsaturated fatty acids in meat may occur by non-enzymatic or enzymatic reactions. The most important pathway of lipid oxidation in meat is the non-enzymatic oxidation, which is a free radical chain reaction. Reactive species (RS), such as hydroxyl radical (from Fenton reaction), singlet oxygen and hypervalent myoglobin are the major initiators of the non-enzymatic oxidation in the meat system. Enzymatic oxidation represents the dioxygenation of fatty acids containing cis, cis-1,4-pentadiene structure catalysed by lipoxygenases'. The hydroperoxides resulted from these reactions are considered the primary products of lipid peroxidation.

Polyunsaturated fatty acids (PUFA) from meat are more susceptible to oxidation than monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs), and form hydroperoxides with conjugated double bonds (conjugated diene hydroperoxides and conjugated triene hydroperoxides).

Unsaturated fatty acids with two double bonds generate conjugated dienes hydroperoxide (CD) while fatty acids with three double bonds generate conjugated trienes hydroperoxide (CT).

Hydroperoxides are relatively stable in moderate conditions (low temperature, absence of transitional metal ions) however, in meat, due to the large presence of Fe^{2+} ions, they become susceptible to further reactions, such as isomerization and decomposition. The resulted products, including pentanal, hexanal, 4-hydroxynonenal, and malondialdehyde, known as thiobarbituric acid reactive substances (TBARS), are the secondary products of lipid peroxidation.[50, 51]

Modiffications of conjugated dienes (CD) and conjugated trienes (CT) values

There is scarce information on the influence of natural/synthetic antioxidants on CD and CT. Maqsood and Benjakul[49] reported the effect of phenolic compounds (100mg/L) on the formation of lipid oxidation products in menhaden oil-in-water emulsion stored at 30^0C for 168 hours. The influence of phenolics on the CD formation in the emulsion was tested separately for: catechin, caffeic acid, ferulic acid and tannic acid. Among the tested phenolics the most efficient in preventing CD formation was found to be the tannic acid. Maqsood si Benjakul[49] showed that for minced mackerel treated with catechin, caffeic acid, ferulic acid and tannic acid, the CD absorbance increased constantly during ice storage for 15 days. The lowest increase rate of CD absorbance was found for the samples treated with tannic acid. The rate of formation of conjugated diene will reach a maximum, increasing constantly during the process of oxidation, and will decrease when the rate of the decomposition of hydroperoxydes exceed the rate of their formation [47].

Our research shows the influence of total hawthorn berries' phenolics and BHA treatments on the formation of CD and CT in minced pork (figure 1 and figure 2).In figure 1 the absorbance of CD after 2 days of refrigeration is smaller than the absorbance measured immediately after the HBE addition to minced pork (day 0). This

reaction could not be observed for the minced mackerel samples treated separately with catechin, caffeic acid, ferulic acid and tannic acid after 15 days of ice storage[49].

In our experiment during the first two days of refrigeration the significant decrease of CD and CT (table 2 and 3) in all minced pork samples, suggests important oxidative processes on PUFAs, during samples preparation (Day 0) and important decomposition of peroxides after 2 days of refrigeration. Maqsood and Benjakul[49] suggested that the mechanism of CD formation implies an initiation stage followed by oxidation propagated by hydrogen subtraction near double bonds generating the formation of isomeric hydroperoxides that carry CD groups. They also reported that all samples of fish oil emulsions containing added phenolics showed a lower CD formation than the control.

In our experiment, after 6 days of refrigeration the S4 treatment containing BHA, was the least oxidized sample (lowest CD absorbance) being significantly different from S1 and S2 treatments while S1 treatment (100 HP) and S2 treatment (200 HP) are not significantly different from each other (table 2). The S1 and S2 treatments showed the highest significant oxidation compared to S3 and S4 treatments (figure 1)

After 6 days of refrigeration, the minced pork containing 100 HP (S1) and 200 HP (S2) showed the highest oxidation of fatty acids with three double bonds (Fig. 2). Moreover the mean CT absorbance for meat sample containing 300 HP (S3), was significantly lower ($p<0.01$), followed by the values obtained for S1 and S2 treatments.

The influence of treatments over the six days of refrigeration on minced pork conjugated trienes was highly significant ($p<0.0001$). After six days of refrigeration, there was a significant difference between CT absorbance of S4 treatment compared to the S1 and S2 treatments (Figure 2). After 2 days of refrigeration the CT absorbance of S3 and S4 treatments were not significantly different. Moreover no significant

differences were observed for CT absorbance corresponding to S2, S3 and S4 treatments after 4 days of refrigeration (figure2).

The antioxidantive efficacy of the treatments used in our research was also influenced by the delivery of the hawthorn berries ethanolic extract solutions in the meat system. All measured parameters had large standard deviations. The large standard deviations observed suggests that insufficient sample homogeneity may be suspected or that the chemical processes are not taking place at the same rate and simultaneously in all the samples' volume. Our results also confirm the well recognised fact that the main practical difficulty in minced meat antioxidant treatments is to make a uniform dispersion of the antioxidant in the meat system[46]. We also observed a colour gradient, from meat samples edges towards their bulk centre. We suspect that the colour variation in the bulk samples may be due to oxidation as a result of samples contact with air and air diffusion into the samples. Although the colour was obviously degraded at the margins of the minced meat samples after 6 days of refrigeration, it remained unaltered in the bulk centre. We suspect that inhomogeneous samples oxidation and dispersion of the antioxidant in the meat system might have generated large standard deviations of the analysed parameters.

Changes in thiobarbituric acid reactive substances (TBARS)

TBARS values variation during refrigeration was specific for each type of treatment, suggesting the existence of possible secondary reactions of condensation of TBARS with amino acids and peptides to form Schiff's base or decomposition of TBARS with the fomation of volatile low molecular weight compounds (table 4, figure 3).

At the beginning (Day 0) TBARS concentration was significantly higher for the S2 treatment compared to all treatments (figure 3). Our results are consistent with the results obtained by Atcan et al [22] who showed that the raw pork patties treated with 200 ppm and 800 ppm phenolics from hawthorn 100% acetone extract were not significantly different. In their study, both treatments kept TBARS values below 0.5mg MDA/kg patty. In our experiment, for Day0, the lowest TBARS value (below 0.30 mg MDA / kg minced pork), was obtained for the S3 treatment containing 300 HP. Our results suggest that the treatments with phenolics from hawthorn berries' ethanolic extract is more efficient in controlling peroxidation than the acetone extract.

After 2 days of refrigeration (figure 3, table 4), TBARS values decreased for all minced meat treatments. The lowest TBARS value was found in the S1 treatment, containing 100 HP, which was significantly different from S2, S3, and S4 treatments.

Figure 3 shows an alternative decrease and increase of TBARS during refrigeration, which is consistent with other findings reporting the alternative increase and decrease of TBARS values during refrigeration[42,52] and shows that HBE solutions could decrease lipid oxidation in minced pork subject to refrigeration.

Maqsood and Benjakul[49] studied the retardation of heme-mediated lipid oxidation by tannic acid in fish muscle (Asian see bass) during ice storage and reported the increase followed by a decrease of TBARS value and the lipid oxidation retardation in the presence of phenolic compounds. The decreased TBARS value was probably due to the loss in oxidation products formed, particularly the volatile ones. Malondialdehyde and other short chain carbon products of lipid oxidation are not stable and decompose to organic alcohols and acids, which are not measured by the TBARS test.[53,54] Supplementary, the decrease in TBARS was also probably due to the interaction of thiobarbituric acid reactive substances with proteins[53]

Changes of chromatographic profile of fatty acids

The percentages of fatty acids in minced pork treated with hawthorn ethanolic extract and in control samples in the day 0 and after 6 days of refrigeration are shown in table 5 and 6.

Studies carried out on different meat types showed that lipid oxidation decreases meat quality and leads to low consumers'acceptability following colour deterioration and development of off-odours and off-flavours [55,56] The susceptibility of PUFA to rapid oxidation decrease nutritional value of meat due to destruction of essential fatty acids and generates organoleptic modifications. Following oxidation a complex mixture of volatile secondary products with objectionable off-flavours are produces.[57,58] For Day 0 the concentration of C16:1 fatty acid in BHA treated minced pork was significantly different from the concentration of this fatty acid in the HP treated minced meat (Table 5).

The PUFA concentration decreased significantly with refrigeration time (Table 6), reflecting the lipid oxidation and potentially PUFA oxidation. The minced meat containing hawthorn etanolic extract, 200 HP and 300 HP had significantly increased PUFA concentrations ($P<0.05$) compared to S4 (100 BHA). For S4 (BHA treated minced pork) the LC-PUFA n-6 partial sums was significantly lower than in the minced pork containing 200 HP and 300 HP. Inhibitory action of total polyphenolic extract on minced pork's PUFA oxidation was observed for several individual PUFA such as: 18:2*n–6*, 20:2*n–6*, 20:3*n–6*, 20:3*n –3*, 20:4*n–6* and for LC-PUFA *n–6* and *n–3* partial sums.

The following classification applies for minced pork treated with HBE and BHA:

a) For PUFA partial sum:

S1 (100 HP)< S4 (100 BHA) < S2 (200 HP) < S3 (300 HP)

b) For LC-PUFA *n-3* partial sum:

S1 (100 HP)< S2 (200 HP) = S3 (300 HP) < S4 (100 BHA)

c) For LC-PUFA *n-6*, partial sum:

S4 (100 BHA) < S1 (100 HP) < S2 (200 HP) = S3 (300 HP)

Ganhao et al[59] also reported the inhibitory activity of some Mediterranean berries (including *Cartaegus monogyna*) ethanolic extract on lipid peroxidation in porcine burger patties subject to cooking and chilled storage.

Similar results were reported by other researchers for extracts from other plats/seeds but on raw, not minced, beef meat. Rojas and Brever[60] reported that grape seed extract reduces lipid oxidation in raw meat. McBride et al[61] demonstrated that rosemary polyphenols were more effective than BHA/BHT in preserving oxidative stability of beef. Brannan and Mah[62] found that for the minced meat, grape seed extract was more effective than gallic acid.

In conclusion, HBE treatments have a similar or better antioxidant effect than the BHA (synthetic) antioxidant. The HBE can be successfully used instead of synthetic antioxidants for enhancing the shelf life of minced pork by controlling the lipid peroxidation.

Variation of oxymyoglobin (OxyMb) and metmyoglobin (MetMb) concentrations

Oxidation of myoglobin to metmyoglobin is an undesirable process in meat because the bright red colour is associated with freshness.[24]

The effect of phenolics from hawthorn berries ethanolic extract on meat colour was investigated by evaluating the oxymyoglobin's and metmyoglobin's concentration variationFor all minced pork treatments, OxyMb concentration (Fig. 4) decreased

significantly ($p<0.05$) during the first four days of refrigeration. After six days of refrigeration OxyMb concentration increased in the minced pork treated with hawthorn etanolic extract (S1, S2, and S3) and BHA (S4), the mean concentrations complying with the following order:

$$S1 > S2 = S3 = S4$$

After 6 days of refrigeration, the highest increase of OxyMb concentration was found for the S1 treatment, which is by 27.42% higher compared to the concentration of S1 for day 4. The same was observed for S2 and S4 treatments, which, compared to day 4, have higher OxyMb concentrations by 21.31% and by 20.29% respectively compared to day 4.

Variation of metmyoglobin (MetMb) concentrations

Changes in metmyoglobin concentration in minced pork treated with HBE and BHA and stored for 6 days at 4^0C are shown in Figure 5 . MetMb concentration increased significantly ($p<0.0001$) during refrigeration in all minced pork treatments. The increase of metmyoglobin concentration was coincidental with the decrease in oxymioglobin concentration.

After six days of refrigeration the MetMb concentration of the S1treatment had the lowest concentration of all treatments, being significantly different compared to the other treatments (Fig. 5).

After six days of refrigeration the concentrations, compared to day 4, the MetMb concentrations decreased significantly in all treatments (Fig. 5). The mean concentrations can be classified as following:

S1 (100 HP) = S2 (200 HP) < S3 (300 HP) < S4 (100 BHA)

Our results for the first four days of the experiment are consistent with the experiment of Maqsood and Benjakul [28] who studied the combined effect of tannic acid and modified atmospheric packaging on oxymyoglobin and metmyoglobin concentration in refrigerated minced beef reporting also the decrease of oxymyoglobin concentration and the increase of metmyoglobin concentration during refrigerate storage time.

The reducing activity of hawthorn berries ethanolic extract (HBE)

Oxymyoglobin/metmyoglobin ratio values suggest that HBE is able to reduce metmyoglobin to myoglobin (Fig. 6 and Table 7) which was confirmed by samples' colour change from brownish red (day 4) to light red (day 6) . This phenomenon was strongly evident for the minced pork containing 200 HP (Fig. 6,) showing that HBE as well as BHA may reduce metmyoglobin ($MbFe^{3+}$) to myoglobin ($MbFe^{2+}$).

A similar phenomenon was identifies by Inai et al.[25] who studied the effect of 20 polyphenols on metmyoglobin. They found that different polyphenols can have a distinct reducing character being strong or weak reducers. Thus, kaempferol, quercetin and myricetin showed reducing activity even in very low concentrations and may be considered as strong reducers, while synaptic acid, catechin, taxifolin, morin and feluric acid were identified as weak reducers. However, in the present study it is still unclear why, after six days of refrigeration, the S1 and S3 treatments did not show an increase of the ratio of OxyMb to MetMb (Fig 6).

A possible explanation could be that inside each treatment sample the chemical processes are not evolving in an identical environment and are not occurring simultaneously in every point of the sample volume. Therefore samples were naturally isotropic. This aspect was observed macroscopically too, as the color of the minced

meat was different on the samples edges or the samples surfaces which were in contact with the plastic bag (used during refrigeration), compared to the samples core.

This fact suggest that special refrigeration boxes have to be constructed which can support sampling the treated minced meat from inside the boxes in order to obtain aliquots kept in identical storage condition. (i.e., aliquots from the core of the refrigeration box, from the corners, or which were in contact with one of the box walls).

Changes of protein patterns

Proteins patterns of minced pork treated with hawthorn berries etahnolic extract and BHA, on day 0 and after six day of refrigeration, are shown in Figure 7. All treatments contained the same major and minor proteins.

The major proteins identified were Myosin heavy chain (MHC - 200 kDa), Actin (44 kDa) and Tropomyosin β (38 kDa) (Fig. 7).

After 6 days of refrigeration, MHC band intensity, which correlates with protein concentration, decreased by 5.29% (S1), 4.95% (S2), 5.33% (S3), and 7.14% (S4) compared to Day 0.

Actin band intensity decreased by 4.29% (S1), 5.1% (S2), 6.82% (S3) and 6.93% (S4) after 6 days of refrigeration.

Muroya et al.[63,64] reported that a band corresponding to ~ 30 kDa resulted from the degradation of Troponin T in beef muscle.

The present results show that both HBE and the synthetic antioxidant BHA, can prevent protein degradation in minced pork during refrigeration which is consistent with the results found by Maqsood and Benjaku[28] on minced beef treated with tannic acid. Moreover hawthorn berries etahnolic extract did not induce cross-links in the soluble proteins (to form protein aggregates) and therefore there is no risk of reducing their ability to retain water.

The influence of hawthorn berries ethanolic extract and BHA treatments on minced pork consistency and firmness

Mince pork consistency The consistency was significantly influenced by treatments with hawthorn berries ethanolic extract and BHA. An interesting result is that after 2 and 4 days of refrigeration no significant differences were observed between treatments containing hawthorn berries etahnolic extract and (Table 9)

Although after 4 days of refrigeration the consistency of S4 treatment containing BHA was significantly higher compared to minced pork containing phenolics (Table 9) its consistency decreased significantly after 6 days of refrigeration compared to treatments containing HBE.

After six days of refrigeration the consistency of S2 and S3 treatments were not significantly different and the S1 treatment had the highest significant consistency while the BHA treatment (S4) had the lowest significant consistency (Table 9) which demonstrates an excellent antioxidant capacity of the hawthorn ethanolic extract.

The consistency of minced pork also showed large standard deviations confirming that minced meat oxidation over time is an anisotropic process and that antioxidant homogenisation may be one of the factors influencing the consistency's variance.

Mince pork firmness (maximum back extrusion force)Minced pork firmness was also influenced by treatments. During the first 2 days of refrigeration the samples treated with hawthorn berries ethanolic extract showed statistically similar firmness (table 10). After six days of refrigeration the highest firmnesses were observed for the S1 treatment containing 100 HP and S3 treatment containing 300 HP (Table 10). Therefore, concentrations of 100 HP or 300 HP in minced pork will generate a statistically higher

firmness than the BHA treatment, and may successfully replace the synthetic antioxidants treatments.

There are scarce information on consistency and firmness of raw minced meat treated with natural or synthetic antioxidants. Most of the reported data are referring to cooked patties' textural properties resulting from sensory analysis or instrumental measurements [59 Ganhão]. Instrumental data on pork cooked patties containing an antioxidant extract [59 Ganhão], refer mostly to texture profile analysis parameters such as hardness, chewiness and cohesiveness which may be correlated to sensory data for cooked patties. However these data cannot be related to the raw minced meat consistency and firmness during refrigeration due to several issues. One important issue is the fact that textural attributes as developed by Szczesniak et al[65] are not considered in most of the reported textural sensory analysis on meat patties. Researchers usually consider texture as a single factor characterizing the product and use a 9 point hedonic scale (1, dislike extremely; 2, dislike very much; 3, dislike moderately; 4, dislike slightly; 5, neither like nor dislike; 6, like slightly; 7, like moderately; 8, like very much; 9, like extremely) to describe it [66]. This approach is a useful indicator for consumers acceptability however it does not offer the adequate quantitative description of textural attributes such as hardness or chewiness which could be correlated to the corresponding instrumental measurements before and after cooking.

The relationship between TBARS concentrations and the minced pork consistency

We have suspected that over the six days of refrigeration the consistency of the minced pork is influenced by the concentrations of TBARS in the treated samples. The coefficient of determination (Table 11) between treatments' TBARS concentrations and the treatments' consistency showed that between 80% to over 90% of the consistency

variance may be explained by the variance of the TBARS concentrations in the minced meat.

Our findings suggest that the minced pork consistency during storage at 4^0C is influenced by the concentration as well as the type of antioxidant used. For the treatments containing 200 HP, 300HP and 100 BHA there is a negative dependency between consistency and TBARS concentrations (negative slope). For minced meat containing 100 HP (Table 11) the consistency will increase with increasing TBARS concentrations (positive slope).

All samples containing over 200 HP showed an increase of consistency (day 6) for low values of TBARS concentration (Table 4, Table 9 and Table 11).

Moreover we found that also peroxide concentrations (μmol peroxide / Kg sample) variance are negatively related to the variance in TBARS for samples containing over 200 HP, hence consistency may also be influenced by the peroxide concentrations in minced pork containing over 200 HP.

CONCLUSIONS

The addition of hawthorn berries ethanolic extract to minced pork was highly effective in reducing lipid oxidation and protein degradation over six days of refrigeration at 4 ^{0}C.

Hawthorn berries ethanolic extract stabilized the colour of minced pork during refrigeration by: 1) delaying myoglobin oxidation and 2) reducing metmyoglobin to myoglobin.

The addition of 100 HP and 200 HP to minced pork may enhance colour and support lipid oxidation stability.

Metmyoglobin was reduced to oxyhemoglobin and samples colour was improved and turned to light red after six days of refrigeration in the minced pork containing 100 HP. It was found that hawthorn berries ethanolic extract reduce metmyoglobin to oxymioglobin however the ability of aldehydes resulting from lipid peroxidation may destabilize oxymyoglobin and promote its oxidation to metmyoglobin.

The concentrations of 100 HP and 200 HP are the most efficient in reducing metmyoglobin.

Oxymioglobin/metmyoglobin ratio ($R_{OxyMb / MetMb}$) variation in treated minced pork refrigerated for 6 days is highly time dependent, however the mathematical expression of the dependency is distinct for each treatment.

Conjugated dienes' (CD) and conjugated trienes' (CT) absorbance was significantly higher after 6 days of refrigeration ($t=4^0C$) for the minced pork containing 100 HP compared to the BHA treated minced meat.

The minced pork containing 200 HP had the best colour and the highest ratio of oxymioglobin to metmyoglobin after six days of refrigeration.

Minced pork consistency and firmness during refrigeration was significantly influenced by treatments with hawthorn berries ethanolic extract.

After six days of refrigeration the consistencies of the minced pork, containing 100 HP and respectively 300 HP were not significantly different however, they were significantly higher than the consistency of the treatments containing BHA and 200 HP.

After six days of refrigeration, the firmness of minced pork was not significantly different for the samples containing 200 HP and BHA indicating that BHA treatments may be successfully replaced by hawthorn total polyphenolic extract treatments.

The samples treated with BHA showed a significant decrease of consistency after 2 and 6 days with a minimum after 6 days of refrigeration at 4^0C.

As much as 83% to 97% of the consistency variance for the samples treated with hawthorn berries ethanolic extract can be explained by the TBARS concentration variance. However only 63.5% of the consistency variance could be explained by TBARS concentration variance for the BHA treated minced pork. As peroxide concentrations variance are negatively related to the variance of TBARS concentrations in minced pork containing over 200 HP, consistency may also be influenced by the peroxide concentrations

To increase minced meat consistency the recommended content of hawthorn berries ethanolic extract in minced pork is 100 HP.

After 6 days of refrigeration minced pork treated with BHA showed significant lower oxymioglobin concentrations, conjugated dienes (CD) and conjugated trienes (CT) absorbance, consistency and firmness compared to the minced pork treated with hawthorn berries ethanolic extract.

Hawthorn berries ethanolic extract can successfully replace BHA for the antioxidant treatment of minced pork.

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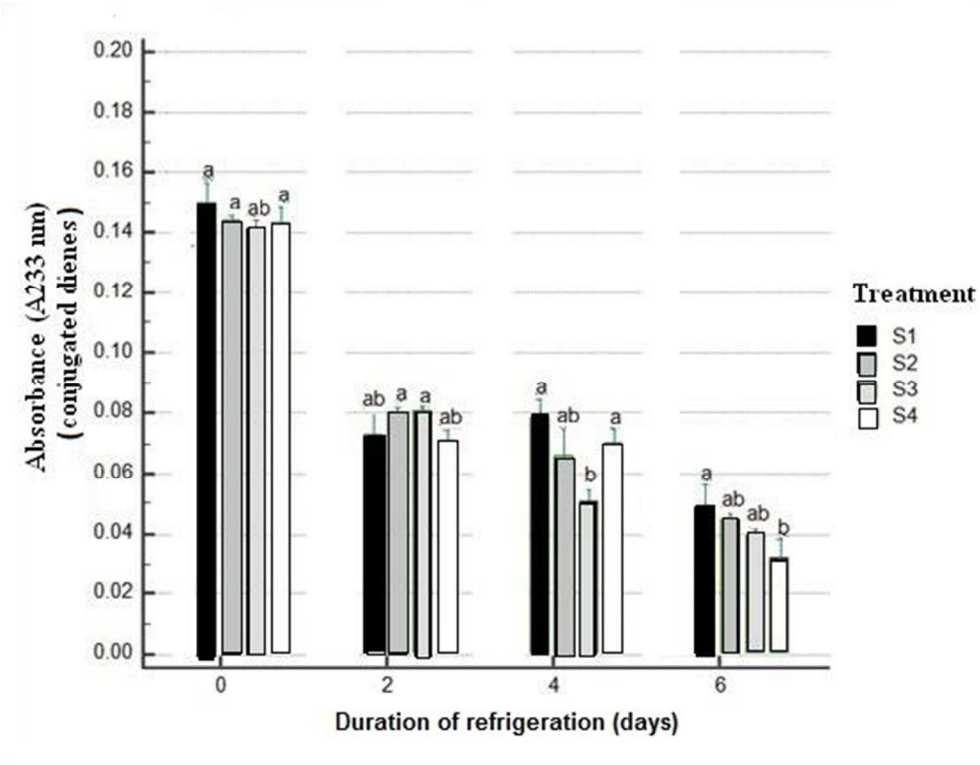

Figure 1. Conjugated dienes (CD) absorbance values of minced pork treated with HBE and BHA. Values are presented over six days of refrigeration (mean + standard deviation, $p < 0.05$, Duncan's multiple range test). No comparison between measurements made in different days can be made. Levels that are not connected by same letter are significantly different

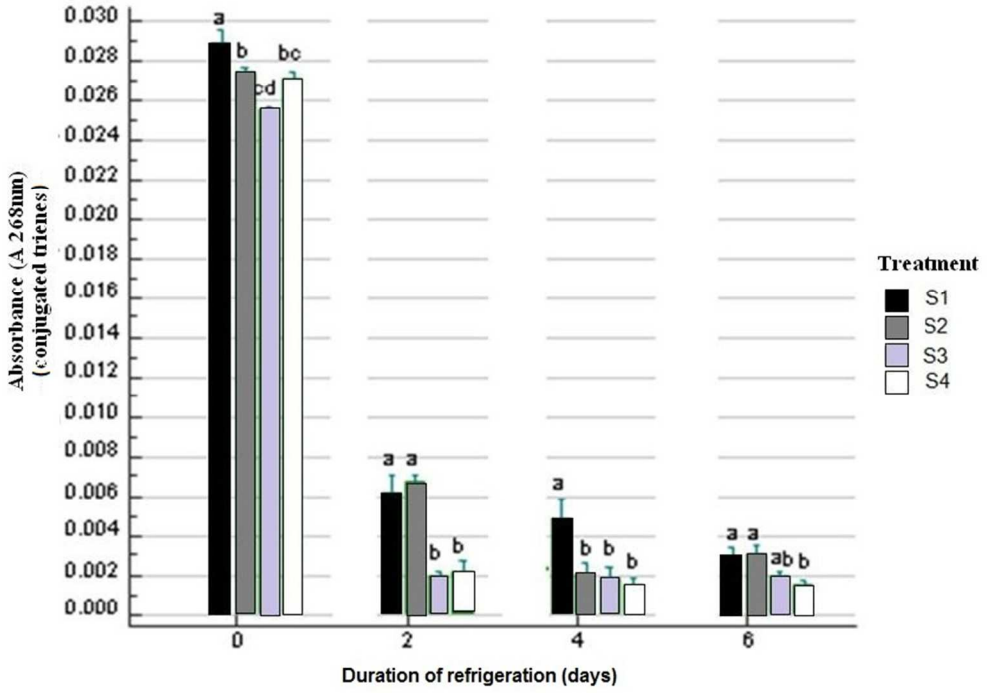

Figure 2. Conjugated trienes (CT) values for minced pork treated with HBE and BHA, during six days of refrigeration (mean + standard deviation). The statistical significance ($p < 0.05$, Duncan's multiple range test) is shown for each group. No comparison between measurements made in different days can be made. Levels that are not connected by same letter are significantly different.

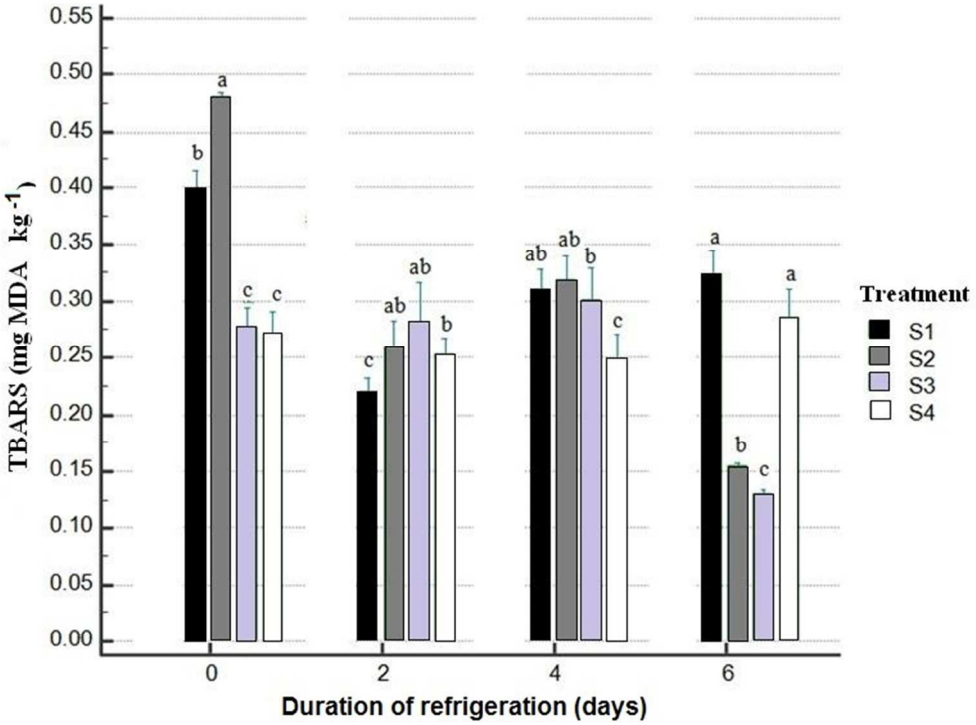

Figure 3. TBARS values in minced pork treated with HBE and BHA over six days of refrigeration (mean+standard deviation). Levels in a group, which are not connected by same letter are significantly different ($p < 0.05$, Duncan's multiple range test).

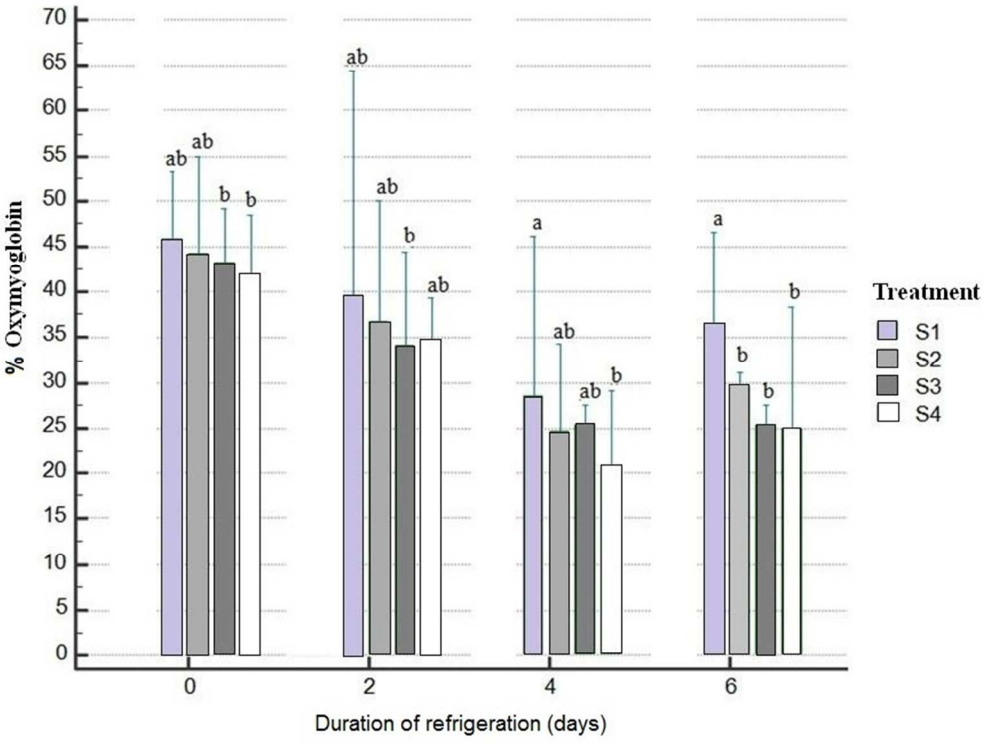

Figure 4. Changes in oxymyoglobin concentrations in minced pork treated with hawthorn berries ethanolic extract and BHA over six days of refrigeration. Levels in a group, which are not connected by the same letter, are significantly different. No comparison can be made between values corresponding to different refrigeration time (days).

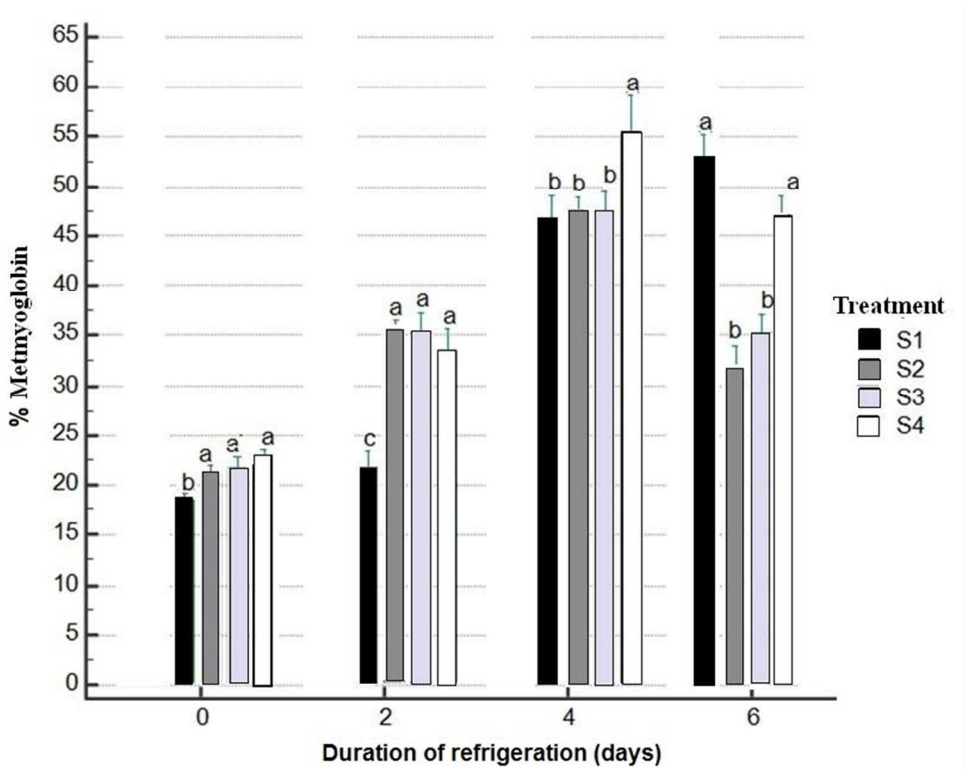

Figure 5.Variation of metmyoglobin concentrations during six days of refrigeration for minced pork treated with hawthorn berries ethanolic extract and BHA. Levels in a group, which are not connected by the same letter are significantly different. No comparison can be made between values corresponding to different refrigeration time (days).

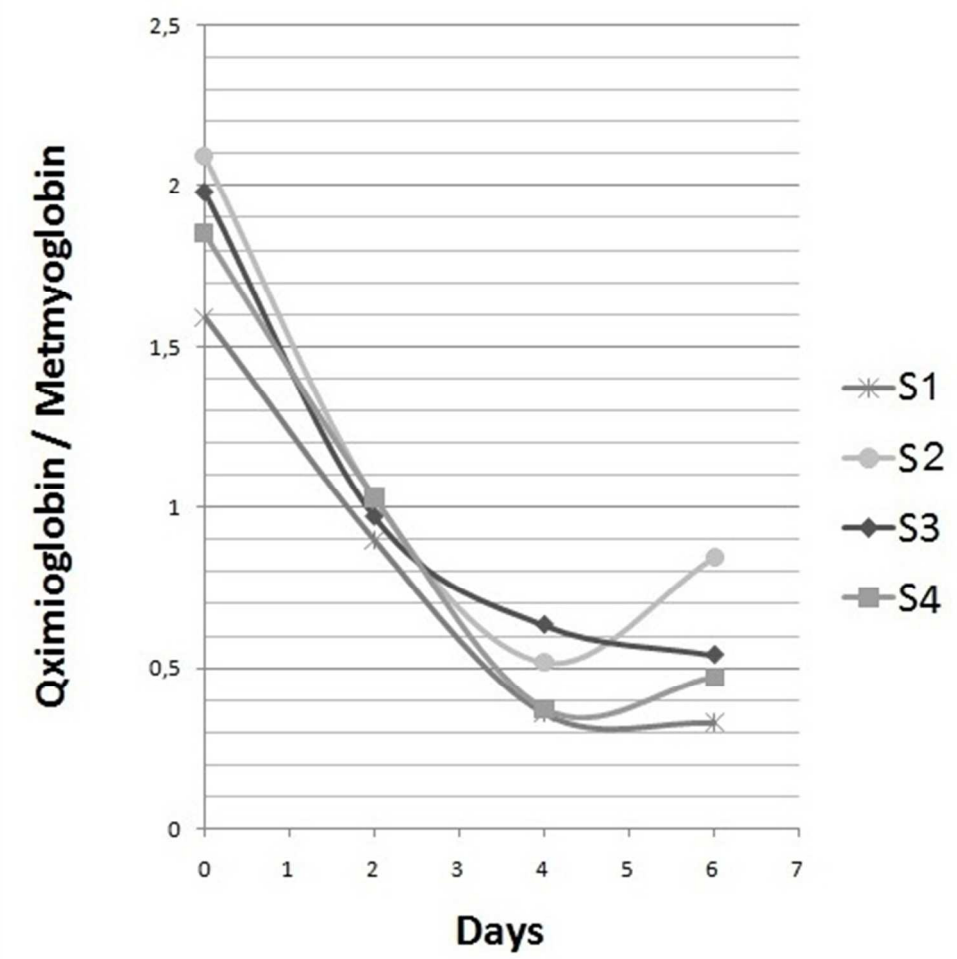

Figure 6. The ratio of oxymioglobin to metmyoglobin in treated minced pork as a function of refrigeration time. Refrigeration temperature: + 4 0C.

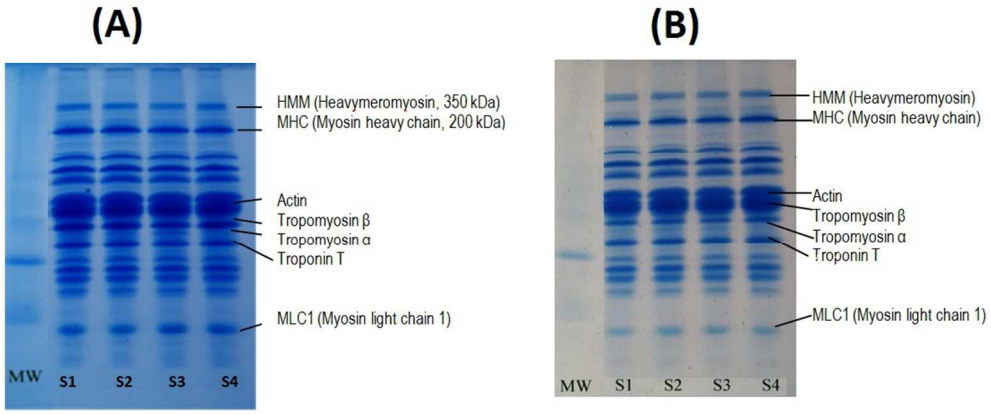

Figure 7. SDS-PAGE patterns of muscle proteins from minced pork treated with hawthorn berries ethanolic extract and BHA: (A) for day 0; (B) after 6 day of refrigeration. MW-molecular weight marker

Table 1. Minced pork treatments

Sample code	Grinded meat weight used for the treatment (g)	Treatment solution	Concentration of antioxidant in minced meat (mg kg^{-1})	Total volume of solution added to 1000 g of minced pork (mL)
S1	1000	Neutralized hawthorn ethanolic extract	100	100
S2	1000	Neutralized hawthorn ethanolic extract	200	100
S3	1000	Neutralized hawthorn ethanolic extract	300	100
S4	1000	BHA	100	100

Table 2. Comparison of conjugated dienes (CD) mean absorbances

Treatment and number of days of refrigeration		Mean	Treatment and number of days of refrigeration		Mean
S1 - day0	a	0.1498	S4 - day2	defg	0.0706
S2 - day0	ab	0.1439	S4 - day4	defg	0.0703
S4 - day0	ab	0.1431	S2 - day4	defgh	0.0659
S3 - day0	ab	0.1410	S3 - day4	defgh	0.0505
S3 - day2	cd	0.0804	S1 - day6	defgh	0.0491
S2 - day2	cd	0.0802	S2 - day6	defgh	0.0449
S1 - day4	cd	0.07851	S3 - day6	fgh	0.0398
S1 - day2	def	0.0732	S4 - day6	h	0.0319

Levels not connected by same letter are significantly different

Table 3. Comparison of conjugated trienes (CT) mean absorbances

Treatments and number of days of refrigeration		Mean	Treatments and number of days of refrigeration		Mean
S1-day 0	a	0.0288	S1-day 6	defg	0.0029
S2-day 0	ab	0.0274	S4-day 2	efg	0.0021
S4-day 0	ab	0.0270	S2-day 4	fg	0.0020
S3-day 0	b	0.0255	S3-day 2	fg	0.0019
S2-day 2	c	0.0067	S3-day 6	g	0.0019
S1-day 2	C	0.0062	S3-day 4	g	0.0018
S1-day 4	cdef	0.0048	S4-day 4	g	0.0014
S2-day 6	defg	0.0030	S4-day 6	g	0.0014

Levels not connected by same letter are significantly different

Table 4. Thiobarbituric acid reactive substances (TBARS) (mg MDA kg-1) in treated minced pork*

Days after antioxidant addition	Treatments*			
	S1	S2	S3	S4
	TBARS concentrations (mg MDA Kg^{-1})			
Day 0	0.3988 ± 0.028^{b}	0.4810 ± 0.006^{a}	0.2779 ± 0.0283^{d}	0.2709 ± 0.034^{d}
Day 2	0.2203 ± 0.021^{c}	0.2596 ± 0.041^{bc}	0.2822 ± 0.059^{bc}	0.2526 ± 0.024^{c}
Day 4	0.3101 ± 0.031^{bc}	0.3198 ± 0.034^{bc}	0.3010 ± 0.051^{c}	0.2483 ± 0.039^{c}
Day 6	0.3242 ± 0.036^{a}	0.1543 ± 0.006^{b}	0.1304 ± 0.006^{b}	0.2864 ± 0.041^{a}

* Levels in a row, which are not connected by same letter are significantly different ($p < 0.05$, Duncan's multiple range test. No comparisons between rows can be made

Table 5. Fatty acids chromatographic profile in treated minced pork – day 0

Fatty Acid (%)	Treatments*			
	S1	S2	S3	S4
C10:0	0.10±0.01	0.09±0.01	0.10±0.02	0.10±0.02
C12:0	0.12±0.02	0.12±0.01	0.13±0.04	0.13±0.01
C14:0	2.33±0.05	2.38±0.14	2.33±0.10	2.40±0.17
C15:0	0.42±0.01	0.43±0.02	0.42±0.04	0.42±0.03
C16:0	26.33±0.03	26.25±0.05	26.19±0.04	26.17±0.04
C17:0	0.72±0.05	0.60±0.10	0.70±0.26	0.70±0.10
C18:0	12.44±0.09	12.44±0.07	12.50±0.08	12.46±0.03
C21:0	0.26±0.03	0.26±0.02	0.28±0.03	0.26±0.04
C23:0	0.16±0.02	0.15±0.03	0.14±0.03	0.15±0.04
C14:1	0.64±0.04	0.66±0.03	0.62±0.04	0.67±0.03
C15:1	0.57^{ab}±0.02	0.71^{a}±0.01	0.68^{ab}±0.03	0.66^{bc}±0.03
C16:1	2.67^{b}±0.06	2.68^{b}±0.07	2.62^{b}±0.06	2.85^{a}±0.04
C17:1	0.52±0.07	0.52±0.07	0.50±0.05	0.49±0.05
C18:1n-9	38.97±0.34	38.98±0.28	39.00±0.31	38.92±0.33
C18:2n-6	9.19±0.20	9.20±0.22	9.21±0.21	9.21±0.20
C18:3n-6	0.71±0.11	0.71±0.09	0.70±0.13	0.70±0.15
C20:2n-6	0.19±0.04	0.20±0.05	0.19±0.04	0.21±0.05
C20:3n-6	0.27±0.05	0.27±0.06	0.28±0.06	0.27±0.05
C20:3n-3	0.44±0.06	0.44±0.09	0.45±0.08	0.46±0.06
C20:4n-6	1.80±0.11	1.79±0.09	1.79±0.08	1.81±0.18
SFA(1)	42.88±0.88	42.72±1.54	42.79±1.11	42.79±1.14
MUFA(2)	43.47±1.26	43.55±1.48	43.42±1.26	43.59±1.40
PUFA(3)	12.60±0.82	12.61±1.09	12.62±1.17	12.66±1.15
LC-PUFA n-3(4)	0.44±0.06	0.44±0.09	0.45±0.08	0.46±0.06
LC-PUFA n-6(5)	2.26±0.10	2.26±0.32	2.26±0.50	2.29±0.36
n-6/n-3(6)	5.13±0.53	5.13±0.79	5.02±0.66	4.97±0.54
Other Fatty Acid	1.05±0.26	1.12±0.32	1.17±0.32	0.96±0.24

*Values are presented as mean ± standard deviation, n=3. Levels in a row, which are not connected by same letter, are significantly different ($p < 0.05$, Duncan's multiple range test.) (1)SFA: Saturated Fatty Acid. (2)MUFA: Monounsaturated Fatty Acid. (3)PUFA: Polyunsaturated Fatty Acid. (4)PUFA n-3: Polyunsaturated Fatty Acid series n-3. (5)PUFA n-6: Polyunsaturated Fatty Acid series n-6. (6)n-6/ n-3: PUFA n-6/PUFA n-3, ratio.

Table 6. Fatty acids chromatographic profile in treated minced pork after 6 days of refrigeration

Fatty Acid (%)	Treatments *			
	S1	S2	S3	S4
C10:0	0.10±0.01	0.09±0.01	0.10±0.02	0.10±0.02
C12:0	0.12±0.02	0.12±0.01	0.13±0.04	0.13±0.01
C14:0	2.33±0.05	2.38±0.14	2.33±0.10	2.40±0.17
C15:0	0.42^{b}±0.01	0.43^{ab}±0.02	0.42^{b}±0.04	0.42^{b}±0.03
C16:0	25.59±0.50	25.59±0.55	25.50±1.17	25.53±1.17
C17:0	0.72±0.05	0.60±0.10	0.70±0.26	0.70±0.10
C18:0	13.59±1.68	13.49±1.75	13.42±1.85	13.47±1.74
C21:0	0.26±0.03	0.26±0.02	0.24±0.06	0.23±0.07
C23:0	0.16±0.02	0.15±0.03	0.14±0.03	0.15±0.03
C14:1	0.61±0.19	0.60±0.14	0.52±0.13	0.54±0.17
C15:1	0.67^{ab}±0.02	0.71^{a}±0.01	0.60^{ab}±0.16	0.65^{c}±0.04
C16:1	2.63^{b}±0.08	2.63^{b}±0.09	2.62^{b}±0.06	2.85^{a}±0.04
C17:1	0.52±0.07	0.50±0.04	0.50±0.05	0.44±0.09
C18:1n-9	38.98±2.34	38.93±2.21	39.02±1.98	38.90±1.67
C18:2n-6	9.16±1.10	9.18±1.18	9.21±1.19	9.12±1.13
C18:3n-6	0.71±0.11	0.71±0.09	0.70±0.13	0.70±0.15
C20:2n-6	0.15^{a}±0.04	0.15^{a}±0.04	0.15^{a}±0.06	0.21^{a}±0.05
C20:3n-6	0.24±0.08	0.26±0.07	0.28±0.06	0.27±0.05
C20:3n-3	0.38±0.11	0.42±0.08	0.42±0.08	0.43±0.05
C20:4n-6	1.71±0.20	1.77±0.13	1.75±0.15	1.60±0.24
SFA[1]	43.29±2.13	43.11±2.49	43.08±2.05	43.13±2.60
MUFA[2]	43.41±1.17	43.37±1.44	43.16±1.87	43.38±1.41
PUFA[3]	12.21 ± 1.16^{d}	12.44 ± 0.94^{b}	12.56 ± 1.08^{a}	12.33 ± 1.10^{c}
LC-PUFA n-3[4]	0.38 ± 0.11^{c}	0.42 ± 0.09^{b}	0.42 ± 0.08^{b}	0.43 ± 0.05^{a}
LC-PUFA n-6[5]	2.10 ± 0.35^{b}	2.18 ± 0.44^{a}	2.18 ± 0.59^{a}	2.08 ± 0.73^{c}
n-6/n-3[6]	5.52±0.80	5.19±0.71	5.19±0.70	4.83±0.68
Other Fatty Acid	1.09±0.33	1.08±0.37	1.10±0.20	1.12±0.04

*Values are presented as mean ± standard deviation, n=3. Levels in a row, which are not connected by same letter, are significantly different ($p < 0.05$, Duncan's multiple range test). [1]SFA: Saturated Fatty Acid. [2]MUFA: Monounsaturated Fatty Acid. [3]PUFA: Polyunsaturated Fatty Acid. [4]PUFA n-3: Polyunsaturated Fatty Acid series n-3. [5]PUFA n-6: Polyunsaturated Fatty Acid series n-6. [6] n-6/ n-3: PUFA n-6/PUFA n-3, ratio.

Table 7. Comparison between metmyoglobin concentrations in treated minced meat over the refrigeration period

Treatments and number of days of refrigeration		Mean	Treatments and number of days of refrigeration		Mean
S4 - day 6	a	57,7319	S3 - day 2	bc	35,1704
S2 - day 4	ab	47,4774	S1 - day 6	bc	31,7632
S3 - day 4	ab	47,3954	S4 - day 0	bc	31,1521
S3 - day 6	ab	46,9494	S4 - day 2	bc	31,0155
S1- day 4	ab	46,5248	S3 - day 0	c	21,8573
S4 - day 4	ab	44,5026	S1- day 2	c	21,5562
S2 - day 2	bc	35,8196	S2 - day 0	c	21,2021
S2 - day 6	bc	35,2579	S1 - day 0	c	18,4331

Levels not connected by same letter are significantly different

Table 8. Oxymioglobin/metmyoglobin ratio (R OxyMb / MetMb) variation with time in treated minced pork as a function of the number of days (D) of refrigeration at 4C

Treatment	Antioxidant content	Time (days) dependency of $R_{OxyMb / MetMb}$	Coefficient of determination
S1	100 HP	$R_{OxyMb / MetMb} = 0,086\ D^2 - 0,733\ D + 2,105$	0,997
S2	200HP	$R_{CxyMb / MetMb} = 0,041D^2 - 0,464\ D + 1,609$	0,994
S3	300 HP	$R_{OxyMb / MetMb} = 0,057D^2 - 0,576D + 1,959$	0,992
S4	100 BHA	$R_{OxyMb / MetMb} = 0,057D^2 - 0,583D + 1,879$	0,987

Table 9. Back extrusion consistency (mean value (J) ± standard deviation (J)) of treated minced pork

Treatments	Consistency (J)* (mean value ± standard deviation)			
	DAY 0	DAY 2	DAY 4	DAY 6
S1	(0,0053±0.0005)^{Aabc}	(0,0044±0.0003) ^{Ab}	(0,0052±0.0011) ^{Aa}	(0,0060±0.0006)^{Aab}
S2	(0,0043±0.0007) ^{Abc}	(0,0042±0.0006) ^{Ab}	(0,0041±0.0007) ^{Aa}	(0,0048±0.0003) ^{Aab}
S3	(0,0049±0.0010) ^{Abc}	(0,0044±0.0005) ^{Ab}	(0,0045±0.0013) ^{Aa}	(0,0056±0.0013)^{Aab}
S4	(0,0064±0.0007) ^{Aab}	(0,0042±0.0006) ^{Bb}	(0,0064±0.0004) ^{Aab}	(0,0033±0.0007) ^{Bb}

*Two-way ANOVA-levels not connected by the same letter are significantly different.

Table 10. Firmness (N) mean values (maximum back extrusion force) and standard deviations (N) of treated minced pork

Treatments	DAY 0	DAY 2	DAY 4	DAY 6
	Mean value of the firmness* (N)			
S1	(1.35±0.08) ^{aB}	(1.18±0.19) ^{aB}	(1.58±0.19) ^{aA}	(1.58±0.30) ^{aC}
S2	(1.29±0.17) ^{bB}	(1.21±0.20) ^{bB}	(1.15±0.09) ^{bA}	(1.36±0.18) ^{bB}
S3	(1.53±0.17) ^{cB}	(1.34±0.31) ^{cB}	(1.28±0.49) ^{cA}	(1.56±0.35) ^{cC}
S4	(1.66 ±0.28) ^{dC}	(1.29±0.09) ^{dB}	(1.75±0.35) ^{dA}	(1.11±0.21) ^{dB}

*Two-way ANOVA. Levels not connected by the same letter are significantly different.

Table 11. Coefficients of determination between concentrations of TBARS (mg Kg-1) and consistencies (J) of treated minced pork samples.

	S1- TBARS	S2- TBARS	S3- TBARS	S4- TBARS
S1- Consistency	R^2=0,83 (positive slope)	-	-	-
S2- Consistency	-	R^2=0,96 (negative slope)	-	-
S3- Consistency	-	-	R^2=0,97 (negative slope)	-
S4- Consistency	-	-	-	R^2=0,635 (negative slope)

Comprehensive REVIEWS in Food Science and Food Safety

Plant Polyphenols as Antioxidant and Antibacterial Agents for Shelf-Life Extension of Meat and Meat Products: Classification, Structures, Sources, and Action Mechanisms

Camelia Papuc, Gheorghe V. Goran, Corina N. Predescu, Valentin Nicorescu, and Georgeta Stefan

Abstract: Oxidative processes and meat spoilage bacteria are major contributors to decreasing the shelf-life of meat and meat products. Oxidative processes occur during processing, storage, and light exposure, lowering the nutritional and sensory value and acceptability of meat and generating toxic compounds for humans. Polyphenols inhibit oxidative processes in 3 ways: as reactive species scavengers, lipoxygenase inhibitors, and reducing agents for metmyoglobin. Thus, polyphenols are candidate antioxidants for meat and meat products. The cross-contamination of meat with spoilage and pathogenic microorganisms can occur in production lines and result in economic losses. The ability of polyphenols to interact with bacterial cell wall components and the bacterial cell membrane can prevent and control biofilm formation, as well as inhibit microbial enzymes, interfere in protein regulation, and deprive bacterial cell enzymes of substrates and metal ions. Thus, polyphenols are candidate antimicrobial agents for use with meat and meat products. Commercially available polyphenols can decrease primary and secondary lipid peroxidation levels, inhibit lipoxygenase activity, improve meat color stability, minimize the degradation of salt-soluble myofibrillar protein and sulfhydryl groups, and retard bacterial growth. Further studies are now needed to clarify the synergistic/antagonistic action of various polyphenols, and to identify the best polyphenol classes, concentrations, and conditions of use.

Keywords: antioxidant, antimicrobial, meat/meat products, polyphenols, shelf-life

Introduction

Polyphenols are organic compounds with one or more hydroxyl groups attached to a phenyl ring. Polyphenols are not involved in the normal growth and development of plants but do have important roles in plant defense mechanisms against viruses, bacteria, fungi, and herbivores. Polyphenols are biosynthesized from phenylalanine or tyrosine. The antioxidant and antimicrobial activities of these molecules make them suited for use as natural preservatives for the meat industry (Güllüce and others 2003; Cui and others 2012). In the meat industry, sensory quality and appearance (color, texture, and flavor) are important traits that affect consumers' acceptance of meat and meat products (Min and Ahn 2005). Oxidation of lipids and myoglobin and meat spoilage bacteria are major contributors to a reduced shelf-life for meat/meat products. Lipid oxidation decreases the nutritional value of meat through the deterioration of essential fatty acids, causing unacceptable flavor for consumers, generating potentially toxic products, and promoting the oxidation of other important molecules, such as myoglobin. Important bacteria associated with meat spoilage are *Brochothrix thermosphacta*, *Carnobacterium* spp., *Enterobacteriaceae*, *Lactobacillus* spp., *Leuconostoc* spp., *Pseudomonas* spp., and *Shewanella putrefaciens* (Borch and others 1996). Spoilage by bacteria in meat causes off-odor, off-flavor, discoloration, gas production, slime production, and reduced pH, leading to significant economic losses. Improper slaughtering, manipulation, and storage can lead to meat contamination with pathogens, including *Salmonella* spp. and *Listeria monocytogenes*, which cause foodborne illnesses. Many suspected toxins are used to extend the shelf-life of meat/meat products, such as sodium/potassium nitrite and synthetic antioxidants (Kahl and Kappus 1993; Knekt and others 1999). However, in recent years, consumers have demanded that such chemicals be replaced with natural compounds with beneficial effects on human health. Because of their antioxidant and antimicrobial properties, commercially available polyphenols and plant extracts rich in polyphenols might be useful for preserving meat/meat products from oxidative deterioration, bacterial spoilage, and the growth of pathogens. In the last decade, it has been shown that plant polyphenols have a positive impact on human health, reduce oxidative processes, inhibit growth of many pathogens [such as bacteria (Nakayama and others 1993; Song and others 2005), viruses (Shin and Chung 2007; Kohda and others 2008; Nantz and others 2013), and fungi (Park and others 2006)], stimulate the

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growth of commensal and beneficial bacteria (Cardona and others 2013; Duda-Chodak and others 2015; Mills and others 2015), and prevent chronic diseases (Yang and Hong 2013), including cancer (Casado and others 2016). For these reasons, polyphenols are increasingly being used in the meat industry as an alternative to chemical antioxidants and antimicrobials.

Polyphenol Classifications by Chemical Structure and Sources

Polyphenols can be subdivided into 3 main classes, flavonoids, stilbenoids, and phenolic acids (Table 1). Flavonoids are the most prevalent of these. Chemically, the backbones of flavonoids are composed of two phenyl rings (A and B) and an oxygenated heterocyclic ring (C), that gives a general structure with a 15-carbon skeleton (C6–C3–C6 backbone). Flavones, flavanones, flavonols, flavanonols, flavan-3-ols, isoflavones, neoflavonoids, chalcones, and anthocyanidins are the most widely known flavonoids (Table 1). Flavones and flavonols are the most common flavonoids, ubiquitous in the plant kingdom, with the exception of algae and fungi (Bravo 1998). Monomeric flavan-3-ols (catechins) and their derivatives (epicatechin, gallocatechin) are the major flavonoids found in tea leaves and cocoa beans. Condensed tannins or proanthocyanidins are common constituents of woody plants, but are often found in fruits and beverages, such as beer and wine. These polymeric compounds can precipitate proteins and have antimicrobial activities. Isoflavonoids possess an isoflavan (3-phenylchroman) backbone but are structurally diverse, despite having a low distribution in the plant kingdom. Genistein and daidzein, which are found in soy, are important isoflavonoids with antioxidant and estrogenic activities. Neoflavonoids have a 4-phenyl coumarin backbone and are rarely found in food plants. Dalbergin is the most widely distributed neoflavonoid in the plant kingdom (Tsao 2010). Chalcones have an aromatic ketone 1,3-diphenyl-1-propen-3-one backbone. Chalcones are found in fruits and vegetables in the form of monomers, dimers, oligomers, Diels-Alder adducts, and as various conjugates. Plant pigments, whose color changes from yellow to orange in some plants, such as *Coreopsis* and *Asteraceae taxa* species, contain chalcones, dihydrochalcones, and aurones (Aksöz and Ertan 2012). Anthocyanidins are the principal components of red, blue, and purple pigments distributed in flower petals, fruits, and vegetables. The glycosidic forms of anthocyanidins found in plants are known as anthocyanins (Bravo 1998). More than 500 anthocyanins have been identified, which differ by the pattern of hydroxylation or methoxylation of the B-ring, and glycosylation with different sugar units (Tsao 2010). The color of anthocyanins is pH-dependent (orange or red at a pH of 3.0 or lower, bluish red at pH values between 6 and 7, and blue in basic conditions) (Brouillard 1988). *Stilbenoids* are 1,2-diphenylethene polyphenols (derivatives of stilbene) and have a 14-carbon skeleton (C6–C2–C6 backbone) (Table 1). Stilbenoids may have monomeric, oligomeric, or polymeric structures. Due to their various biological activities, such as antioxidant, anticancer, estrogenic, and antibacterial actions, *trans*-resveratrol has attracted the attention of many researchers (Gorham and others 1999; Zhang and others 2012). 3, 4′, 5-Trihydroxystilbene (resveratrol) is found in various plants (grapes, berries, peanuts, cocoa), as well as in red wine. Phenolic acids are derived from benzoic acid (C1–C6 backbone) or cinnamic acid (C3–C6 backbone) (Table 1). In fruits and vegetables, hydroxybenzoic acids are found in a very low concentration, especially in the free-form. In grains and seeds, hydroxybenzoic acids are typically in the bound form. Black radishes, onions, and tea leaves are important sources of free hydroxybenzoic acids (Rice-Evans and others 1996; Manach and others 2004). The phenolic acids found in the bound form may be hydrolyzed in alkaline media or in enzymatic reactions. Hydroxycinnamic acids are phenylpropanoid derivatives (C6–C3) found in all parts of fruits and vegetables. They are present in bound forms (glycosylated derivatives or esters of quinic acid, shikimic acid, or tartaric acid) in fruits and, rarely, in the free form (only in processed foods subjected to fermentation, sterilization, or freezing) (Manach and others 2004).

Polyphenols as Antioxidants: Structure–Activity Relationship

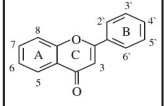
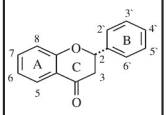
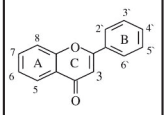
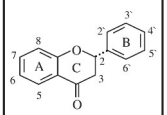
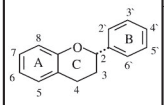
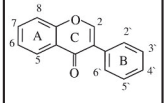
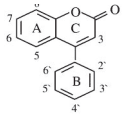
Polyphenols as antioxidants via the reactive species scavengers' pathway

The effectiveness of phenolic compounds in the inhibition of oxidative processes in foods is related to their reactive species (RS) scavenging activity. RS are formed in meat during processing, storage, and light exposure, thereby damaging lipids, proteins, and vitamins, and thus lowering the nutritional value and sensory acceptability of foods, and even generating toxic compounds for humans, such as carbonyls (Campo and others 2006; Dietze and others 2007; Papuc and others 2017), alcohols (Min and Ahn 2005), furans (Ruiz and others 1999), cross-linked proteins (Kim E and others 2013), volatile sulfur compounds (Ahn 2002), and oxidized amino acids (Dunlop and others 2013). Generally, RS are free radicals (HO·, $O_2\cdot^-$, ROO·, RO·, NO·, RS·, PUFA·), non-radicals (H_2O_2, HOCl, $^1O_2$, O_3), or transition metal ions (Fe^{2+}, Cu^+) that promote oxidation (Papuc and others 2017).

Polyphenols as free radical scavengers. Due to their structure [hydroxyl group(s) on a benzene ring(s)], polyphenols scavenge free radical by H-atom transfer from the active OH group(s) of the polyphenolic to the free radical (Ar-OH + R· → ArO· + RH) (Di Meo and others 2013). Di Meo and others (2013) proposed four mechanisms for this reaction, which are shown in Figure 1.

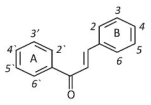
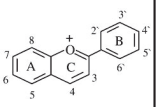
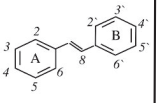
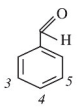
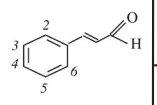
Several researchers have reported strong antioxidant activity for flavonoids. It was reported that flavonoids are free radical scavengers during food oxidation, including the most aggressive free radical, hydroxyl radical (HO·). By chemiluminescence, Chen and others (2002) studied the relationship between the structure of 12 natural flavonoids and HO· scavenging activity and found that quercetin, heliosin, hyperoside, kaempferol, baicalin, corylifolin, lysionotin, matteucynol, corylifolinin, and genistein could scavenge HO·. They suggested that the phenolic hydroxyl is the main active group in the HO· scavenging activity, and the positions of the hydroxyl groups in flavonoids are more important than the total number of phenolic hydroxyl groups. The comparative studies undertaken by Chen and others (2002) on flavonoids with an *otho*-dihydroxyl group on the A-ring (on 5, 6 positions) (baicalin) and flavonoids with *meta*-dihydroxyl group on the A-ring (on 5, 7 positions) (lysionotin and matteucinol) have shown that the HO· scavenging ability of flavonoids with an *ortho*-dihidroxyl group is much stronger than those with *meta*-dihydroxyl groups. Also, they showed that flavonoids with a *meta*-hydroxyl group on the A-ring and *ortho*-hydroxyl group on the B-ring (on 3′, 4′ positions), such as quercetin, heliosin, and hyperoside, can effectively scavenge HO·. They also found that the HO· scavenging activity of the flavonoids mentioned above is higher than that of flavonoids with only a *meta*-dihydroxyl group on the A-ring. Amic and others (2003) suggested that the flavonoids with an *ortho*-dihydroxy group on the B-ring (on 3′, 4′ position) or with a hydroxyl group

Table 1–Polyphenols: classes, subclasses and sources

Class name, subclasse name and the backbone structure	Examples	Position of hydroxyl groups / substituted hydroxyl groups / other substituents												Sources	References
1. Flavonoids															
		2	**3**	**4**	**5**	**6**	**7**	**8**	**2`**	**3`**	**4`**	**5`**	**6`**		
Flavones 	Luteolin	–	–	–	OH	–	OH	–	–	OH	OH	–	–	Curly endive (*Cichorium endivia),* Alfalfa (*Medicago sativa*), Indian trumpet flower (*Oroxylum indicum*), Baikal skullcap	Kawaii and others (1999); Chen and others (2003); Tsao and McCallum (2009); Barros and others (2012); El-Shafey and AbdElgawad (2012); Mishima and others (2015); Kim and others (2016)
	Apigenin	–	–	–	OH	–	OH	–	–	–	OH	–	–		
	Chrysin	–	–	–	OH	–	OH	–	–	–	–	–	–		
	Baicalein	–	–	–	OH	OH	OH	–	–	–	–	–	–		
Flavanones 	Hesperetin	–	–	–	OH	–	OH	–	–	OH	OCH_3	–	–	Citrus and grapefruit peels	Lien and others (2008); Mullen and others (2008); Tomás-Navarro and others (2014)
	Naringenin	–	–	–	OH	–	OH	–	–	–	OH	–	–		
Flavonols 	Quercetin	–	OH	–	OH	–	OH	–	–	OH	OH	–	–	Propolis, honey, chamomile and linden, *Ginkgo biloba,* Blue gum eucalyptus.	Chan and others (2007); Bertelli and others (2012); Kurtagić and others (2013); Şanlıa and Lunte (2014); Dezsi and others (2015)
	Kaempferol	–	OH	–	OH	–	OH	–	–	–	OH	–	–		
	Galangin	–	OH	–	OH	–	OH	–	–	–	–	–	–		
	Fisetin	–	OH	–	–	–	OH	–	–	OH	OH	–	–		
	Myricetin	–	OH	–	OH	–	OH	–	–	OH	OH	OH	–		
	Morin	–	OH	–	OH	–	OH	–	OH	–	OH	–	–		
	Hyperoside	–	OGal	–	OH	–	OH	–	–	OH	OH	–	–		
	Heliosin	–	OdiGal	–	OH	–	OH	–	–	OH	OH	–	–		
Flavanonols 	Taxifolin	–	OH	–	OH	–	OH	–	–	OH	OH	–	–	Dahurian larch (*Larix gmelinii*), Chinese lacquer tree	Kim J and others (2010); Liu and others (2014)
	Fustin	–	OH	–	–	–	OH	–	–	OH	OH	–	–		
Flavan-3-ols 	***Monomers***													Green tea, black tea, cocoa	Zaveri (2005); Subhashini and others (2010); Gadkari and Balaraman (2015)
	(+)-Catechin	–	βOH	–	OH	–	OH	–	–	OH	OH	–	–		
	(-)-Epicatechin	–	αOH	–	OH	–	OH	–	–	OH	OH	–	–		
	(-)-Epigallocatechin	–	αOH	–	OH	–	OH	–	–	OH	OH	OH	–		
	(-)-Epicatechin-3-gallate	–	αOGallate	–	OH	–	OH	–	–	OH	OH	–	–		
	(-)-Epigallogatechin-3-gallate	–	αOGallate	–	OH	–	OH	–	–	OH	OH	OH	–		
	Polymers Proanthocyanidins or condensed tannins	Oxidative condensation of the monomeric units (a flavan-3-ols) between C4 and C6 or C8												Woody plants, fruits and beverages such as beer and wine	Porter (1989); Bravo (1998)
Isoflavones 	Genistein	–	–		OH	–	OH	–	–	–	OH		–	Leguminous family plants (soybean), red clover	Wang and Murphy (1994); Mazur and others (1998); Dixon and Ferreira (2002); Tsao and others (2003); Kim and others (2012); Kuligowski and others (2017)
	Genistin	–	–	–	OH	–	OGl	–	–	–	OH	–	–		
	Daidzein	–	–	–	–	–	OH	–	–	–	OH	–	–		
	Daidzin	–	–	–	–	–	OGl	–	–	–	OH	–	–		
	Biochanin A	–	–	–	OH	–	OH	–	–	–	OCH_3	–	–		
	Formononetin	–	–	–	–	–	OH	–	–	–	OCH_3	–	–		
Neoflavonoids (4-phenyl coumarine) 	Dalbergin		–	–	–	OH	OCH_3	–	–	–	–	–	–	Widely distributed in plant kingdom	Garazd and others (2003); Laure and others (2008)
	Calophyllolide	Complex structure													
	Inophyllums B, P, G, F	Complex structure													

(*Continued*)

Table 1–Continued.

Class name, sub-classe name and the backbone structure	Examples	Position of hydroxyl groups / substituted hydroxyl groups / other substituents												Sources	References
		2	3	4			7	8	2`	3`	4`	5`	6`		
Chalcones 	Isoliquiritigenin	–	–	OH	–	–	–	–	OH	–	OH	–	–	Apples, flowers, hop, beer	Tsao and others (2003); Tsao and McCallum (2009); Aksöz and Ertan (2011); Suwito and others(2014); Phang and others(2016)
	Flavokawain A	–	–	OCH_3	–	–	–	–	OH	–	OCH_3	–	OCH_3		
	Flavokawain B	–	–	–	–	–	–	–	OH	–	OCH_3	–	OCH_3		
	Flavokawain C	–	–	OH	–	–	–	–	OH	–	OCH_3	–	OCH_3		
	Gymnogrammene	–	–	OCH_3	–	–	–	–	OH	–	OCH_3	–	OH		
Anthocyanidins 	Cyanidin	–	OH	–	OH	–	OH	–	–	OH	OH	–	–	Red and blue flowers petals, fruits and vegetables	Mazza (1995); Bravo (1998); Pietta (2000)
	Pelargonidin	–	OH	–	OH	–	OH	–	–	–	OH	–	–		
	Peonidin	–	OH	–	OH	–	OH	–	–	OCH_3	OH	–	–		
	Delphinidin	–	OH	–	OH	–	OH	–	–	OH	OH	OH	–		
	Petunidin	–	OH	–	OH	–	OH	–	–	OH	OH	OCH_3	–		
	Malvidin	–	OH	–	OH	–	OH	–	–	OCH_3	OH	OCH_3	–		
	Anthocyanins														
	Cyanidin-3-glucoside	–	OGl	–	OH	–	OH	–	–	OH	OH	–	–		
	Cyanidin-3-rutinoside	–	ORu	–	OH	–	OH	–	–	OH	OH	–	–		
	Cyanin	–	OGl	–	OGl	–	OH	–	–	OH	OH	–	–		
	Pelargonidin-3-glucoside	–	OGl	–	OH	–	OH	–	–	–	OH	–	–		
2.Stilbenoids															
Stilbenoids 	*trans*-Resveratrol	–	OH	–	OH	–	–	–	–	–	OH	–	–	Red grapes, wine, blueberries, peanuts, dark chocolate, *Cajanus cajan*, sorghum	Siemann and Creasy(1992); Sanders and other (2000); Burns and others (2002); Lyons and others (2003); Counet and others(2006); Bröhan and others (2011); Zhang and others(2012)
	trans-Piceatannol	–	OH	OH	–	–	–	–	–	OH	–	OH	–		
	trans-Piceid	–	OGl	–	OH	–	–	–	–	–	OH	–	–		
	trans-Pterostilbene	–	OCH_3	–	OCH_3	–	–	–	–	–	OH	–	–		
	Cajanotone	C_5H_9	OCH_3	–	OH	–	–	OH	–	–	–	–	–		
	Cajanamide	C_5H_9	OCH_3	–	OH	CO		NH	–	–	–	–	–		
3.Phenolic acids															
Benzoic acids 	***Monomers***													Fruits and vegetables	Rice-Evans and others (1996); Manach and others (2004)
	p-Hydroxybenzoic acid	–	–	OH	–	–	–	–	–	–	–	–	–		
	Gallic acid	–	OH	OH	OH	–	–	–	–	–	–	–	–		
	Protocatechuic acid(3,4)	–	OH	OH	–	–	–	–	–	–	–	–	–		
C6 – C1	*Ellagic acid*	*Dilactone of the gallic acid*													
	Polymers *Hydrolyzable tannins (gallotannins and ellagitannins)*	*Derivatives of gallic acid or ellagic acid*												Chinese rhubarb	Jourdes and others (2013)
Hydroxycinnamic acids 	Caffeic acid	–	OH	OH	–	–	–	–	–	–	–	–	–	Fruits and vegetables, coffee	Guzman (2014)
	p-Coumaric acid	–	–	OH	–	–	–	–	–	–	–	–	–		
	Ferulic acid	–	OCH_3	OH	–	–	–	–	–	–	–	–	–		
	Sinapic acid	–	OCH_3	OH	OCH_3	–	–	–	–	–	–	–	–		
C6 – C3	Curcumin (diferuloylmethane)	Condensation of two ferulic acid by a methylen group												Turmeric (*Curcuma longa*)	Kloesch and others (2013)
	Chlorogenic acids Chlorogenic acid 5-Caffeoylquinic acid *Caffeoylferuloylquinic acids*	Esters of hydroxycinnamic acids with quinic acid 3-O-Ester of caffeic acid with (-)-quinic acid 5-O- Ester of caffeic acid with (-)-quinic acid Esters of caffeic and ferulic acids with quinic acid												Fruits, coffee, potatoes, roselle (*Hibiscus sabdariffa*)	Liang and Kitts (2015)

in the C-ring at the position 3, or both, are the most effective radical scavengers. They also supposed that the antiradical potential of the flavonoids with an *ortho*-dihydroxy group on the B-ring is due to the stability conferred to the flavonoid phenoxyl radicals by the *ortho*-dihydroxy group structure, which participates in electron delocalization. There has been much discussion in the literature about the reaction mechanisms of the antioxidant activity of flavonoids. Flavonoids can scavenge free radicals both by hydrogen atom donating reaction (Fl-OH → Fl-O· + H·) and by electrochemical oxidation (Fl-OH → Fl-O· + e^- +

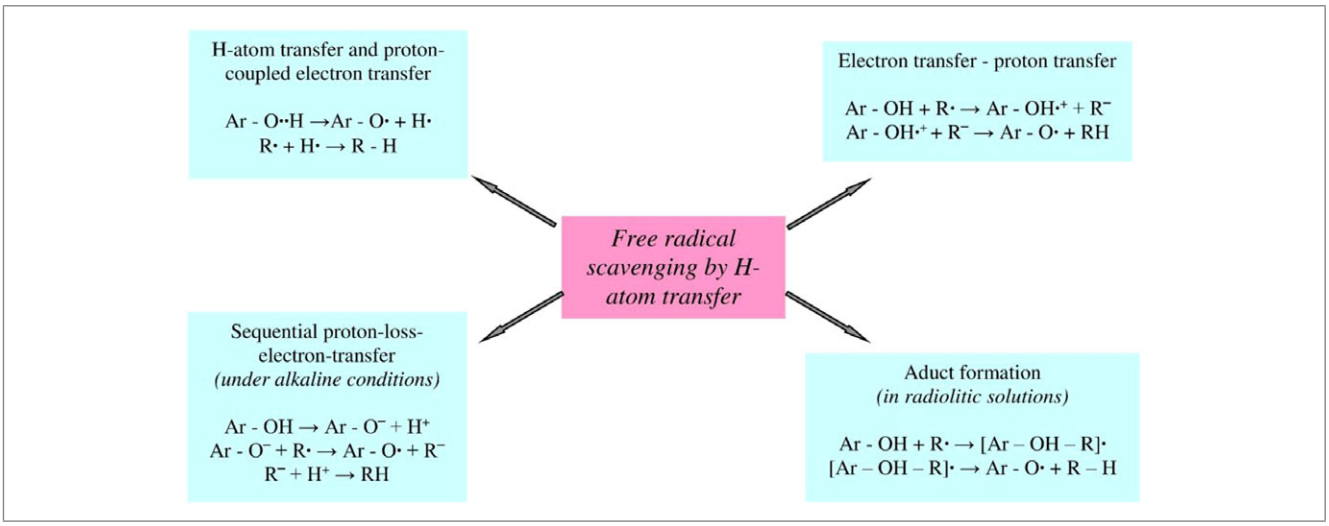


Figure 1–Polyphenols free radical scavenging by hydrogen-transfer mechanisms. R·, free radical; Ar-OH, phenolic compound (data for reaction mechanisms collected from Di Meo and others 2013).

H^+). Jovanovic and others (1994) suggested that flavonoids are thermodynamically able to reduce free radicals with redox potential in the range of 0.13 to 1.0 V, by hydrogen atom donation. The resulted phenoxyl radicals could donate also another hydrogen atom forming quinones, thus terminating the chain reaction. The data obtained by Meng and others (2014) provide evidence that radical scavenging activity depends on the conjugation between the B- and C-rings, which is affected by the structure, the number, and positions of hydroxyl groups, and the structural groups. Some researchers have suggested that flavonoids act as free radical scavengers by forming less reactive phenoxyl radicals when donating a hydrogen atom from their hydroxyl group (Arora and others 1998; Amic and others 2003). By redistributing the unpaired electron on the aromatic core, flavonoid phenoxyl radicals form a resonance structure with a much lower activity, compared to free radical and phenoxyl radicals. Brett and Ghica (2003) studied the mechanism of electrochemical oxidation of 3,5,7,3′,4′-pentahydroxyflavone (quercetin) and found that the oxidation of the 3′,4′-dihydroxyl group occurs at very low positive potentials, compared to the others hydroxyl groups, and is a 2-electron 2-proton reversible reaction. The electrochemical oxidation of quercetin, undertaken by Brett and Ghica (2003), demonstrated by electrochemical techniques that the quinone, the final product of quercetin oxidation, is not electroactive, and that is blocks the electrode surface. These authors suggested that quercetin oxidation reactions act in a cascade, connected with all hydroxyl groups, but that the catechol groups (3′, 4′dihydroxyl) are more active where a two-electron and two-proton reaction occurs. For this reason, in food systems, quercetin may be the terminator in the chain reaction because it could easily scavenge a free radical (R·) by hydrogen atom donation from its 3′-hydroxyl and then from its 4′-hydroxyl group, or by electron transfer—proton transfer, forming a quinone with low activity. Figure 2 shows the hypothetical mechanisms for free radical scavenging activity of quercetin by hydrogen atom donation (A) and by electron transfer—proton transfer (B).

Simic and others (2007) investigated the electrochemical oxidation of the flavonoids quercetin and rutin using cyclic voltammetry and the inhibition of lipid peroxidation, and they found that the introduction of a second hydroxyl group in the benzene ring decreased first oxidation potential and increased the percent inhibition of lipid peroxidation. Pietta (2000) suggested that the glycosylation of 3-hydroxyl group, as in rutin, reduces the free radical scavenging activity of flavonoids. The presence of a 3′,4′-dihydroxy group on the B-ring of flavonoids is not necessary for a high free radical scavenging activity (Amic and others 2003), but the presence of a hydroxyl group in position 3 flavonoids is important for free radical scavenging activity (Lupea and others 2008).

The antioxidant activity of flavan-3-ol was demonstrated in the same way. The electrochemical oxidation of flavan-3-ol (+)-catechin revealed that the catechol B-ring (3′,4′-dihydroxyl group) is more easily oxidizable than the resorcinol A-ring (5,7-dihydroxyl group), and so the oxidation occurs at the 3′,4′-dihydroxyl group, by a similar mechanism as quercetin oxidation, so that the final oxidation product is not electroactive and blocks the electrode surface (Janeiro and Brett 2004). Huang and Frankel (1997) studied the antioxidant activity of tea catechins in different lipid systems and suggested that these biomolecules have antioxidant or prooxidant functions depending on their different reduction potentials, stability, and relative partitions between phases in different lipid systems. Thus, they observed that the oxidation of corn oil triglycerides at 50 °C was inhibited by epigallocatechin (EGC), epigallocatechin gallate (EGCG), and epicatechin gallate (ECG), better than epicatechin (EC) and catechin. In contrast, the oxidation of soy lecithin liposomes at 50 °C was inhibited best by EGCG, followed by EC, EGC, ECG, and catechin. Oxidation of liposomes at 37 °C induced by 10 μM cupric acetate was better inhibited by catechin and EC than ECG, and promoted by EGCG and EGC. Ruiz-Larrea and others (1997) investigated the $ABTS^+$ total antioxidant activity of some phytoestrogenic isoflavones, and they found the following relation for the scavenging activity of radical in an aqueous phase: genistein > daidzein = genistin = biochanin A > formononetin ≈ ononin, the last one exhibiting no antioxidant activity. Studies on the oxidation inhibition of low-density lipoproteins, both in aqueous and lipophilic phases, support the observation that genistein is the most potent antioxidant among the investigated phytoestrogenic isoflavones. The authors suggested that the antioxidant activity of isoflavones is due to its single 4′-hydroxyl group and to the 5,7-dihydroxy group. Calliste and others (2001) studied the antioxidant activity of some substituted chalcones with different numbers and at different positions of hydroxyl groups, and they suggested that the alpha-beta double bond and 6′-hydroxy group are

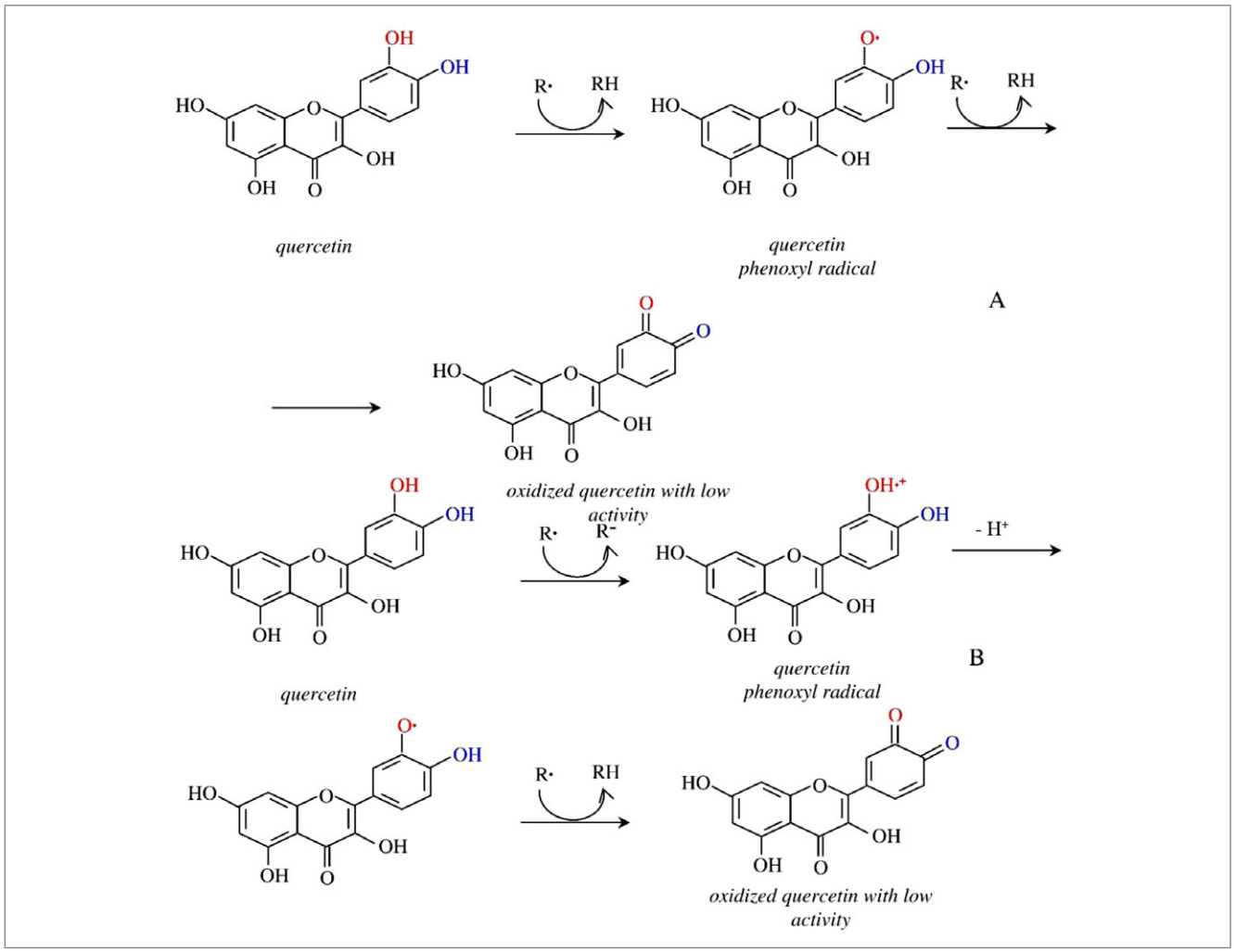


Figure 2–Hypothetical mechanisms for free radical scavenging activity of quercetin. (A) Hydrogen atom donation. (B) Electron transfer—proton transfer.

important for the antioxidant activity. Vasil'ev and others (2010) studied the peroxyl radical (ROO·) scavenging activity of six chalcones by a chemiluminescence method, and suggested that the abstraction of hydrogen atom from chalcones by the ROO· is the main reaction path of these phenolics. However, they also found a contribution by a secondary reaction of chalcones that resulted in a phenoxyl radical (ArO·). Sivakumar and others (2011) studied the superoxide anion scavenging activity, hydrogen peroxide scavenging activity, reducing power, transition metal chelating ability, and DPPH· scavenging activity of 25 synthesized chalcones, and they concluded that the antioxidant activity of chalcones is due to (i) hydrogen or electron donation capacity, (ii) their ability to stabilize and delocalize the unpaired electron, and (iii) their potential to chelate transition metal ions. Kähkönen and Heinonen (2003) investigated the DPPH· scavenging activity and free radical scavenging activity of anthocyanidins pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, and their glycosidic forms (anthocyanins) in lipid-containing models, and they reported strong antioxidant activity, comparable with α-tocopherol, Trolox, catechin, and quercetin in human low-density lipoproteins (LDL), and a weak or even prooxidant activity in bulk methyl linoleate. Compared to anthocyanins, the antioxidant activity of anthocyanidins did not differ remarkably in methyl linoleate emulsion, but was higher in LDL and lower in bulk oil.

Many researchers have reported on the antioxidant activity for the most representative stilbenoid, *trans*-resveratrol (Bastianetto and others 2000; Stojanovic and others 2001; Sinha and others 2002; Lorenz and others 2003; Queiroz and others 2009; Gülçin 2010). It was demonstrated that hydrogen donation (or atom hydrogen abstraction) is one of the mechanisms that explain the antioxidant activity of *trans*-resveratrol (Cao and others 2003). There are two hydrogen abstraction possibilities: (i) from a *para*-OH group (position 4') and (ii) from a *meta*-OH (position 3 or 5). So, for the first abstraction case, a π conjugation between an unpaired electron of the phenoxyl radical from the 4' position and π electrons of the diphenylethene skeleton occurs, generating stable *trans*-resveratrol semiquinone radicals with electron delocalization over the entire molecule. An electron-delocalized system between the B- and A-rings explains why *trans*-resveratrol is a strong scavenger of free radicals. In the second case, electron delocalization over the entire molecule did not occur. Therefore, hydrogen abstraction from the *para*-OH group is more favorable in the antioxidant mechanism. Stojanovic and others (2001) studied the antioxidant activity of *trans*-resveratrol compared to its analogs (*trans*-4-hydroxystilbene and *trans*-3,5-dihydroxystilbene) in the radical liposome oxidation and concluded that the similarity between the spectral and kinetic properties of *trans*-resveratrol and *trans*-hydroxystilbene scavenging free radicals demonstrates that the *para*-OH group of

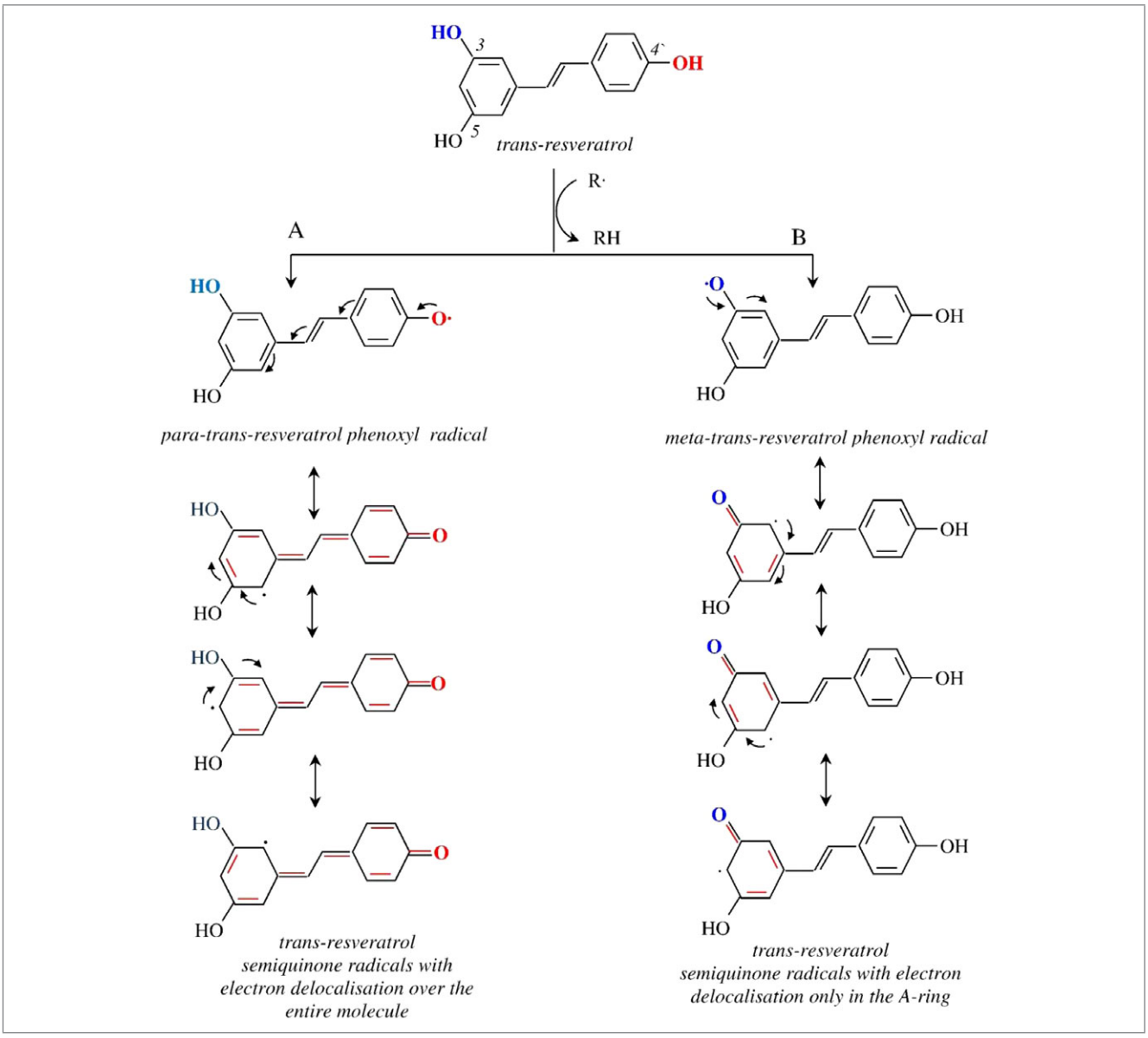


Figure 3–Possible mechanism for free radicals scavenging activity of *trans*-resveratrol. (A) Hydrogen atom abstraction from *para*-OH group. (B) Hydrogen atom abstraction from a *meta*-OH group.

trans-resveratrol is more active than the *meta*-OH groups. A possible mechanism for free radical scavenging activity of *trans*-resveratrol is presented in Figure 3.

Queiroz and others (2009) determined the pharmacophore responsible for the antioxidant activity of *trans*-resveratrol and concluded that their semiquinone free radicals have several resonance structures in which unpaired electrons are mainly distributed on the 4-hydroxystilbene. Gülçin (2010) found that resveratrol inhibits lipid peroxidation in linoleic acid emulsion more efficiently than BHA, BHT, α-tocopherol, and Trolox, at the same concentration, and it was effective at scavenging DPPH·, ABTS·$^{+}$, DMPD·$^{+}$, $O_2{\cdot}^{-}$, H_2O_2, as well as having good reducing power and Fe^{2+}-chelating activity.

The electron-donating ability of polyphenols reflects the reducing power of these biomolecules and is also associated with their antioxidant activity. Polyphenols can break the free radical chain by donating electrons and, for this reason, the transition metal reducing power of polyphenols is correlated with their antioxidant activity. Mira and others (2002) investigated the ability of some flavonoids to reduce copper and iron and concluded that they depend on both the standard redox potential of the metals (E^0) and flavonoid structure. Thus, all studied flavonoids showed higher reducing power for Cu^{2+} ($E°_{Cu2+/Cu+} = +0.15$ V) than for Fe^{3+}($E°_{Fe3+/Fe2+} = +0.77$ V), except the flavones myricetin (3,5,7,3',4',5'-OH) and quercetin (3,5,7,3',4'-OH), which showed high Fe^{3+}-reducing activity. Mira and others (2002) have proposed that the concomitant presence of the catechol group (3',4'-OH in B-ring) and 3-OH group (C-ring) is an important structural feature for reducing the power of flavonoids. The copper-reducing activity of the investigated flavonoid decreased in the order: myricetin (3,5,7,3',4',5'-OH) > quercetin (3,5,7,3',4'-OH), taxifolin (3,5,7,2′,3′-OH) and catechin (3,5,7,3',4'-OH) > luteolin (5,7,3',4'-OH) and kaempferol (3,5,7,4′) > apigenin (5,7,4′-OH), which emphasized the importance of the number of hydroxyl groups, catechol structure in the B-ring, and the presence of the 3-OH group for the high reducing activity of flavonoids.

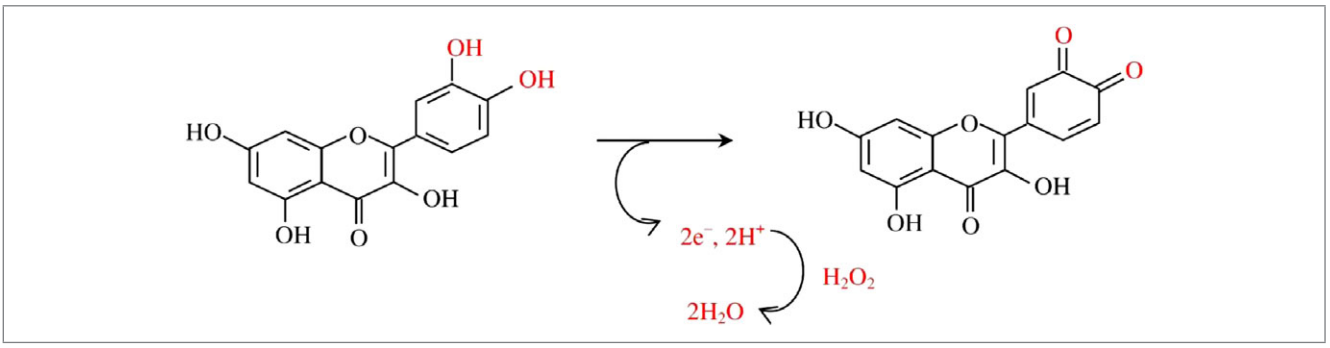


Figure 4—Quercetin possible mechanism of hydrogen peroxide scavenging.

Polyphenols as nonradical reactive species scavengers. Hydrogen peroxide is a nonfree radical reactive species with strong oxidizing properties in meat systems. This is because, in the presence of Fe^{2+}, it can generate HO·. Hydrogen peroxide can be formed in cells by many oxidized enzymes (for example, superoxide dismutase). Since phenolic compounds are good electron and protons donors, hydrogen peroxide can be converted into water by these compounds (Figure 4).

Many researchers have studied the hydrogen-scavenging activity of commercially available polyphenols in order to establish a relationship between structure and antioxidant activity. Sroka and Cisowski (2003) studied the hydrogen peroxide scavenging activity of some natural hydroxybenzoic acids and found strong antioxidant activity. They reported that phenolics with three hydroxyl groups bonded to the aromatic ring exhibited a stronger antioxidant activity than those with two hydroxyl groups and that phenolics with *ortho*-substitution of hydroxyl groups had stronger antioxidant activity than others. For this reason, they suggested that the *ortho*-substitution is the most favorable structure for hydrogen peroxide-scavenging activity. Comparative studies were undertaken on benzoic acids (gallic, protocatechuic, p-hydroxybenzoic, m-hydroxybenzoic and vanillic acids) and hydroxycinnamic acids (caffeic, *p*-coumaric, ferulic, hydrocaffeic acids) by a chemiluminescence method, which reported the following trend for the hydrogen-scavenging activity: protocatechuic acid < *p*-hydroxybenzoic acid < *m*-hydroxybenzoic acid < gallic acid < vanillic acid, respectively *m*-coumaric acid < *p*-coumaric acid < ferulic acid < caffeic acid < hydrocaffeic acid. Between these 2 groups, benzoic acids had a higher antioxidant activity (Mansouri and others 2005). By a chemiluminescence method, Gülçin and others (2010) found that tannic acid is a more efficient hydrogen scavenger than the synthetic antioxidants BHA, BHT, and α-tocopherol, and they suggested that tannic acid works as an electron donor and proton donor. Singlet oxygen ($^1O_2$) is another nonradical and highly reactive molecule appearing in meat systems, from triplet oxygen in the presence of a photosensitizer (such as, porphyrins) and light (Min and Boff 2002). Singlet oxygen in meat produces oxidative processes correlated with meat spoilage. It was demonstrated that polyphenols can minimize singlet-oxygen oxidation processes. Using a near-IR $^1O_2$ luminescence method, Tournaire and others (1993) studied the ability of 13 flavonoids (flavonol, flavone, flavanone, and flavane) to quench singlet oxygen, with the aim of establishing a structure-activity relationship. They suggested that the presence of a catechol moiety on the B-ring controls the quenching, whereas the structure of the C-ring (particularly in the presence of an OH activating the double bond) is the main factor determining $^1O_2$ scavenging activity.

Polyphenols as chelators of transition metal ions. Almost all of the transition metal ions have the ability to function in various oxidation states. In the active redox state, these ions can act as a catalyst in the autoxidation of many biomolecules (Miller and others 1990). In most cases, the oxidation of biomolecules can be initiated by the hydroxyl radical (HO·) generated in the reaction between the redox-active transition metal ions state with hydrogen peroxide, known as Fenton and Fenton-like reactions (Bokare and Choi 2014). In biological systems, these ions can be involved in cyclic redox reactions, generating ROS other than HO· (Stohs and Bagchi 1995). Transition metal ion chelators can sequester these ions and control their prooxidant activity. The ability of transition metal ion chelators to inhibit a biomolecule oxidation, especially lipid oxidation, has been investigated (Shahidi and others 1986; Gülçin 2010; Torres-Fuentes and others 2014). Iron, the most abundant ion in meat, released from heme pigments and ferritin, may be an important catalyst in the oxidation of lipids and proteins (Min and Ahn 2005). It was demonstrated that transition metal ion chelators significantly decrease the oxidative processes in biological systems (Ahn and others 1993; Chen and Ahn 1998). Chen and Ahn (1998) studied the Fe^{2+}-chelating ability of 6 natural phenolics and the inhibitory effect on lipid peroxidation induced by this ion in oil systems. They found that the investigated phenolics can control lipid peroxidation and form phenolics-metal complexes with the following stoichiometry: quercetin, rutin, catechin, and caffeic acid to Fe^{2+} were 3:1, 2:1, 1:1, and 1:1, respectively. Studies undertaken in recent years have demonstrated that the polyphenols can be excellent chelators for iron and copper.

Many researchers have reported on the ability of flavonoids to chelate transition metal ions (Morel and others 1998; Mira and others 2002; Riha and others 2014; Liu and Guo 2015). Flavonoids containing hydroxyl groups at the 3, 5, 3', and 4' positions, and carbonyl at the 4 position, possess 3 sites where metal complexes can form. These sites are between the 3'-OH and 4-carbonyl groups, the 5-OH and 4-carbonyl groups, and the 3′-OH and 4'-OH groups (Morel and others 1998). Mira and others (2002) studied the interaction of flavones, isoflavones, flavanones, and flavan-3-ol catechin with iron and copper ions by ultraviolet spectroscopy and electrospray ionization mass spectrometry, and they suggested that only flavones and flavan-3-ol catechin interact with metal ions. In their study, they supposed that the chelating activity of flavonoids is flavonoid structure-, pH-, and metal ion-dependent. Thus, at pH 7.4 and pH 5.5,all studied flavones chelated Cu^{2+} at the 5-OH-4-carbonyl site; at pH 7.4, myricetin and quercetin additionally chelated at the 3′-OH-4'-OH (*ortho*-catechol) site, which was also the chelating site for catechin at pH 7.4. Also, they found that, at pH 5.5, Fe^{3+} interacts strongly with myricetin and quercetin, probably at the 5-OH-4-carbonyl site.

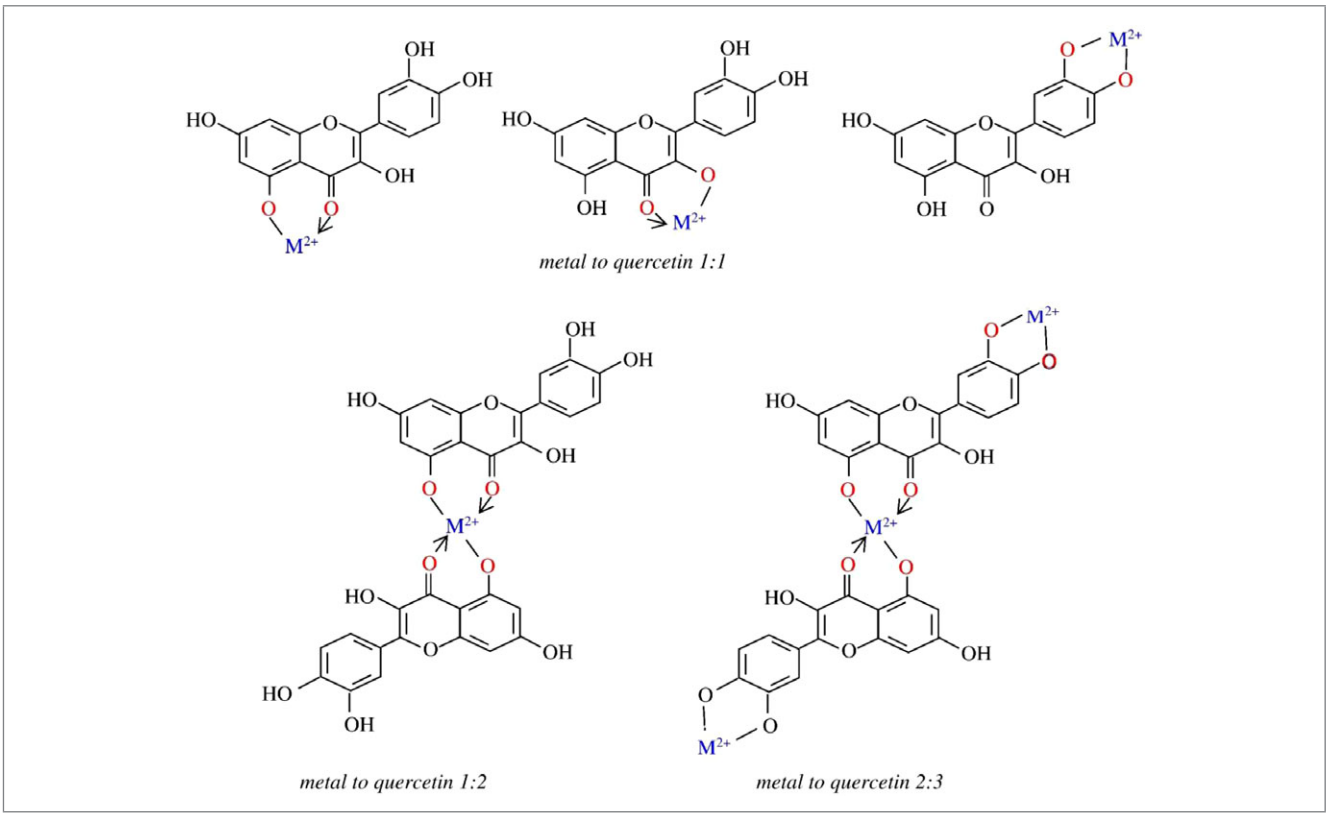


Figure 5–Structure of divalent metal-quercetin complexes stoichiometry 1:1, 1:2, and 2:3. M^{2+}—divalent metallic ion

Riha and others (2014) studied the interaction of Cu^{2+} and Cu^{+} with 26 flavonoids at four pH conditions (4.5, 5.5, 6.8, and 7.5) by spectrophotometric methods, and found that the most active sites were the 3-OH-4-carbonyl in flavonols and 5,6,7-tri OH groups in flavones. Thus, it seems that the presence of 2,3double bonds in flavones and flavonols is essential in forming copper chelates (Riha and others 2014) due to the increases of the backbone planarity (Mira and others 2002). Liu and Guo (2015) investigated the transition of metal-quercetin complexes using electrospray ionization tandem mass spectrometry. By full-scan mass spectra, they found that the stoichiometry metal to quercetin was 1:2 and 2:3 for Fe^{2+}, 1:1 and 1:2 for Cu^{2+}, and 1:1, 1:2, and 2:3 for Co^{2+}, Ni^{2+}, and Zn^{2+}. Also, they suggested that the reduction of the metal ionic radius decreases the number of chelating molecules, and the most important quercetin site in the forming of the metal complexes is 5-OH-4-carbonyl. For the investigated transition metal ions, they found an increasing stability of metal-complexes as follows: $Zn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+}$. Figure 5 shows the possible structure of these metal-quercetin complexes, realized between a divalent transition metal (M^{2+}) and quercetin for stoichiometry metal to flavonoid 1:1, 1:2, and 2:3.

Riha and others (2014) claimed that the stoichiometry of the metal complexes depends on the number and type of the functional groups and that the stability is pH-dependent. Thus, the stoichiometry of flavonoid to copper at pH 6.8 was found to be 2:1 for the flavonoids with 3-OH-4-carbonyl sites, and flavonoids with 5-OH-4-carbonyl sites; 1:2 for the flavonoids containing both (3-OH)-carbonyl and 5-OH-4-carbonyl sites and a catechol B-ring; and 1:1 for the flavonoids with 5,6,7-OH sites. Decreasing the pH to 5.5 resulted in a drop in the chelating activity of the flavonoids with a catechol B-ring, 5-OH-4-carbonyl, and even 5,6-OH or 5,6,7-OH chelating sites. The reactive species scavenger pathway of polyphenol antioxidants is summarized in Table 2.

Polyphenols as antioxidants via the pathway of lipoxygenase inhibition. Lipoxygenases (LOXs) are a family of nonheme iron-containing enzymes that catalyze the dioxygenation of polyunsaturated fatty acids with a *1-cis*,*4-cis* pentadiene structural unit to give a polyunsaturated fatty acid hydroperoxide with a *cis-trans* conjugated diene unit. They are distributed in bacteria, plants, fungi, invertebrates, and mammals (Ivanov and others 2010). LOXs are classified according to the specific position at which they produce dioxygenation of linoleic acid in plants and arachidonic acid in mammals. In plants, 9- and 13-LOXs have been identified. In mammals, 5-, 8-, 12-, and 15-LOXs have been identified. Also, the stereoconfiguration of LOXs may be specified; letter R (rectus—the priorities decrease in a clockwise manner) or letter S (sinister—the priorities decrease in a counterclockwise manner) is used in the LOXs designation (for example, 12S-LOX and 12R-LOX). Based on the structural features of the proteins, plant LOXs are divided into type 1 and type 2 (Baysal and Demirdoven 2007; Hayward and others 2017). Based on the phylogenetic relatedness, mammalian LOXs were divided into 4 main subtypes: platelet-type 12S-LOX, 15S/12S-LOX, epidermis-type 12S-LOX, and 5S-LOX (Kühn and Thiele 1999). The soybean lipoxygenase (sLOX) (isoenzyme LOX-1) is the most extensively studied LOX. The dioxygenation of a polyunsaturated fatty acid consists of 4 major steps: (1) abstraction of a labile hydrogen from a *bis*-allylic methylene group; (2) delocalization of a double bond and formation of a *cis*, *trans* conjugated diene lipid radical; (3) dioxygenation of the lipid radical and formation of the peroxy radical; and (4) peroxy radical reduction and the protonation of the peroxy anion (Ivanov and others 2010; Papuc and others 2017). The active form of LOXs contains Fe^{3+} that is reduced to Fe^{2+} when the abstraction of the

Table 2–Polyphenols as antioxidants: structure – activity relationship

Example of polyphenols and investigated activity	Relation structure-antioxidant activity	Analysis method	Sources
Polyphenols as antioxidants via reactive species scavenger's pathway			
Quercetin, heliosin, hyperoside, kaempferol, baicalin, corylifolin, lysionotin, matteucinol, corylifolinin, and genistein – HO· scavenging activity	The positions of hydroxyl groups in the structure of flavonoids is more important than the numbers of phenolic hydroxyl groups; Flavonoids with an *otho*-diOH on the A-ring (on 5,6 positions) shown a HO· scavenging activity much stronger than flavonoids with *meta*-diOH on the A-ring (on 5,7 positions); Flavonoids with *meta*-diOH on the A-ring and *ortho*-OH in the B-ring (on 3′, 4′ positions) are more reactive than flavonoids only with *meta*-diOH, and can effectively scavenge HO·.	Chemiluminescence	Chen and others (2002)
Morin, taxifolin, kaempferol, fustin, galangin, rutin, quercetin, luteolin 7-gl, quercetin 3,7-digl, larycitrin, larycitrin 3′-gl, robinetin, fisetin, myricetin, kaempferol 3,7-digl, 3-hydroxyflavone, apigenin 7-gl, hesperetin, vitexin, 3,5,7,3′,4′,5′-hexamethoxyflavone, naringenin, naringin, 7-hydroxyflavone, flavanone, flavone, chrysin, apigenin, 8-methoxyflavone, 5-hydroxyflavone - DPPH· scavenging activity	Flavonoids with *ortho*-diOH on the B-ring (on 3′,4′ position) and/or with a OH on the C-ring at the position 3, are the most effective radical scavengers, due to the degree of stability conferred on the flavonoid phenoxyl radicals by *ortho*-diOH structure, participants in electron delocalization; The presence of 3′,4′-diOH on the B-ring of flavonoid structure is not a necessary condition for the high free radical scavenging activity expression.	Colorimetric	Amic and others (2003)
3,5,7,3′,4′-Pentahydroxyflavone (quercetin) – antioxidant activity	Oxidation of 3′,4′-diOH occurs at very low positive potentials, comparatively with the others hydroxyl groups, and is a 2 electron 2 proton reversible reaction.	Cyclic, differential pulse and square-wave voltammetry at different pH	Brett and Ghica (2003)
Quercetin and rutin – lipid peroxidation inhibition	Introduction of a second OH in the benzene ring conducted to the decrease of the first oxidation potential and to increase of the percent inhibition of lipid peroxidation.	Cyclic voltammetry	Simić and others (2007)
(+)-Catechin and resorcinol – oxidation potential	Catechol B-ring (3′,4′-diOH) is more easily oxidizable than resorcinol A-ring (5,7-diOH).Oxidation occurs at 3′,4′-dihydroxyl group, by a similar mechanism as quercetin oxidation.	Cyclic voltammetry	Janeiro and Brett (2004)
Genistein, daidzein, genistin, biochanin A, formononetin – LDL oxidation inhibition	Activity of isoflavones is due to single 4′-hydroxyl group and to 5,7-dihydroxy group.	Colorimetric	Ruiz-Larrea and other (1997)
2′-Hydroxychalcone, 4′-hydroxychalcone, 4-hydroxychalcone, 2′,4-dihydroxychalcone, isoliquiritigenin, 2′,4′-dihydroxychalcone, phloretin and naringenin chalcone – reactive species scavenging activity	Alpha-beta double bond and 6′ OH group are very important for the antioxidant activity of chalcones.	Spectrofotometric	Calliste and others (2001)
6 Chalcones – peroxyl radical (ROO·) scavenging activity	Presence of a secondary reaction of chalcones and resulted phenoxyl radical (ArO·).	Chemiluminescence	Vasil'ev and others (2010)
Pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, and their glycosidic forms (anthocyanins) – antioxidant and DPPH· scavenging activity	Glycosilation decreases antioxidant activity.	Spectrophotometric	Kähkönen and Heinonen (2003)
trans-Resveratrol and *trans*-hydroxystilbene – reactive species scavenging activity	*para*-OH group of *trans*-resveratrol is more active than *meta*-OH groups in reactive species scavenging activity.	Spectrophotometric	Stojanovic and others (2001)
Phenolic acids – hydrogen peroxide scavenging activity	Antioxidant activity increase with the number of OH groups; *ortho*-hydroxyl substitution for OH groups is the most favorable structure.	Spectrophotometric	Sroka and Cisowski (2003)
Benzoic acids and hydroxycynnamic acids – hydrogen peroxyde scavenging activity	Benzoic acids exhibit higher antioxidant activity.	Chemiluminescence	Mansouri and others (2005)
Tannic acid – hydrogen peroxide scavenging activity	Tannic acid acts as electron and proton donor.	Spectrophotometric	Gülçin and others (2010)
13 Flavonoids (flavonol, flavone, flavanone and flavane)	Presence of a catechol moiety on B-ring controls the quencing; the C-ring structure is the main factor determining $^1O_2$ scavenging activity.	Near-IR luminescence	Tournaire and others (1993)

(Continued)

Table 2–Continued.

Example of polyphenols and investigated activity	Relation structure-antioxidant activity	Analysis method	Sources
Flavones, isoflavones, flavanones and flavan-3-ol catechin – Cu^{2+} and Fe^{3+} reducing power	The concomitant presence of catechol group in B-ring and 3-OH group (C-ring) is very important in reducing power;Reducing power increases with the number of OH groups.	Colorimetric	Mira and others (2002)
Flavones, isoflavones, flavanones and flavan-3-ol catechin – Cu^{2+} and Fe^{3+} chelating ability	Only flavones and flavan-3-ol catechin interact with metal ions in reactions dependent of structure, metal ion and pH. The active sites are:-5-OH-4-carbonyl (interaction of all flavones with Cu^{2+} at pH 5.5);-5-OH-4-carbonyl and 3′-OH-4′-OH (myricetin and quercetin interaction with Cu^{2+} at pH 7.4),- 3′-OH-4′-OH (interaction of catechin with Cu^{2+} at pH 7.4);-5-OH-4-carbonyl (myricetin and quercetin interaction with Fe^{3+} at pH 5.5).	UV spectroscopy and electrospray ionisation mass spectrometry	Mira and others (2002)
26 Flavonoids – Cu^{2+} and Cu^{+} chelating ability	The most active sites were 3-OH-4-carbonyl in flavonols and 5,6,7-tri OH groups in flavones;2,3-Double bond in flavones and flavonols is essential in forming cooper chelates.	Spectrophotometric	Říha and others (2014)
Quercetin – transition metals chelatation ability	The stoichiometry metal to quercetin was:-1:2 and 2:3 for Fe^{2+},-1:1 and 1:2 for Cu^{2+},-1:1, 1:2, and 2:3 for Co^{2+}, Ni^{2+}, and Zn^{2+}.The most important site is 5-OH-4-carbonyl.	Electrospray ionisation tandem mass spectrometry	Liu and Guo (2015)
Polyphenols as antioxidants via pathway of lipoxygenase inhibition			
3,4 Dihydroxybenzoic and 4′,5,7-trihydroxyflavone acids – sLOX inhibitory activity	Inhibitory activity is due to the interactions of phenolics with amino acid residues from folding polypeptide chain and nonheme iron from the catalitic site of sLOX.	Molecular docking study	Sudha and Srinivasan (2014)
Curcumin – LOX1 inhibitory activity	Curcumin bound to phosphatidylcholine micelles is a competitive inhibitor for soybean LOX1.	Lineweaver-Burk plot analysis and spectroscopic analysis	Began and others (1998)
18 Flavonoids – inhibitory activity on rabbit reticulocyte 15-LOO-1	(1) The presence of a OH in flavonoid structure is not an essential condition for inhibitory activity; (2) a catechol arrangement fortify the inhibitory activity;(3) In the presence of a catechol arrangement the inhibitory activity is reversed correlated to the number of OH groups; (4) the presence of a 2,3-double bond in the heterocyclic C-ring increases the inhibitory activity.	Lineweaver-Burk plot analysis and spectroscopic analysis	Sadik and other (1995)
Catechin, epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate – the inhibitory activity on the mackrel *(Scomber scombrus)* muscle lipoxygenase	Mixed noncompetitive type inhibition.	Lineweaver-Burk plot analysis and spectroscopic analysis	Banerjee (2005)
Polyphenols as reducing agents for metmyoglobin			
Kaempferol, myricetin and quercetin, sinapic acid, catechin, nordihydroguaiaretic acid, taxifolin, morin and ferulic acid – reducing activity on MetMb	The rapid convertibility of the quinine form to the phenol form of flavanols.	Spectrophotometric	Inai and others (2014)
Caffeic acid, didydrocaffeic acid and hydroxtyrosol – reducing activity	Highly reducing agents in the presence of equimolar amounts of cysteine.	Spectrophotometric	Miura and others (2014)
Cysteinyl polyphenols – reducing activity on MetMb	The number of cysteinyl sulfur substitution in polyphenols influences both MetMb reducing activity and MbO_2 maintenance.	Spectrophotometric	Honda and others (2016)

hydrogen from *bis*-allylic methylene group and formation of diene lipid radical occur (Funk and others 1990). Reoxidation of Fe^{2+} occurs in the fourth step when the peroxy lipid radical is reduced to form a peroxy lipid anion. Many researchers have studied the 3D structure of LOXs from different sources (Boyington and others 1993; Skrzypczak-Jankun and others 1997; Choi and others 2008; Neau and others 2009; Rapp and others 2009; Gilbert and others 2011). They concluded that most LOXs consist of a single polypeptide chain folding into 2 domains: a smaller N-terminal β-barrel domain and a larger C-terminal mostly α-helix domain, which contains the substrate-binding pocket and the redox-active nonheme iron, essential for the catalyzed reaction. Protein X-ray crystallography studies have shown that, for all LOXs, the catalytic nonheme iron is octahedrically coordinated by 5 amino acid residues from the polypeptide chain and a hydroxide ligand (Ivanov and others 2010; Hayward and others 2017). In the case of soybean LOX1 and coral 8R-LOX, the amino acid residue ligands are three His, one Asn and the C-terminal amino acid residue, Ile

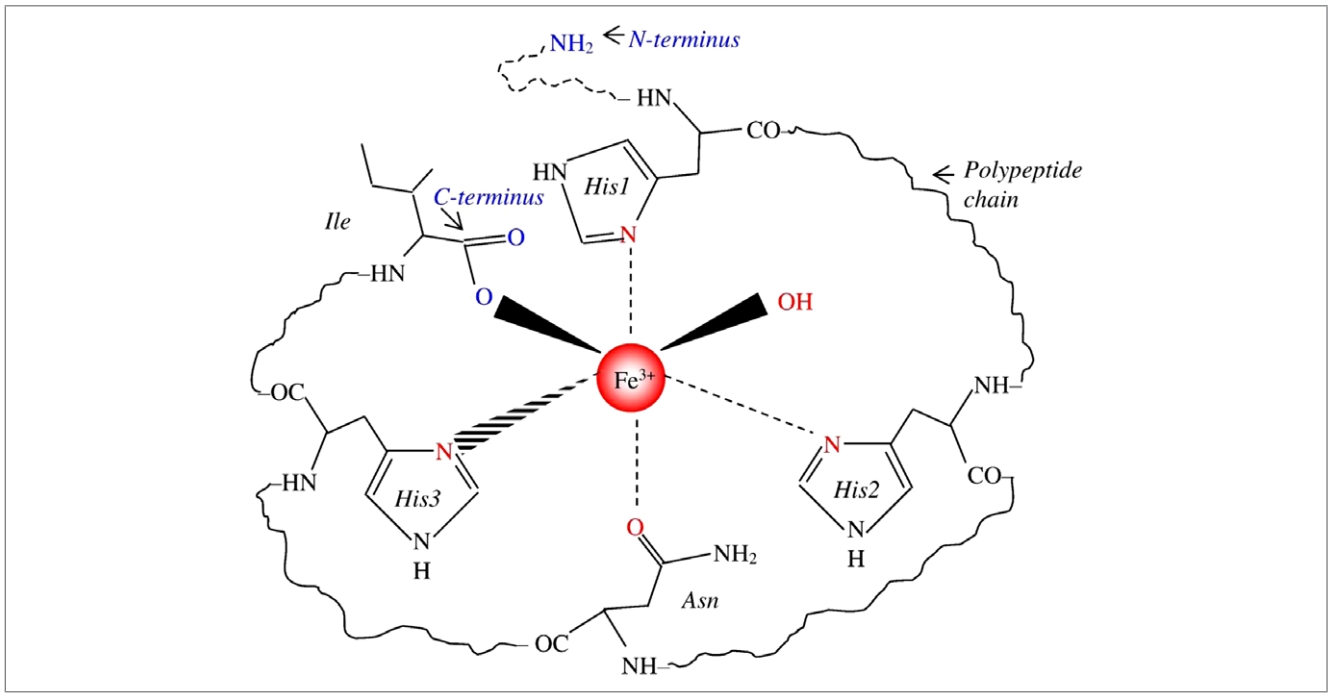


Figure 6–Octaedrically coordination of nonheme iron in the active site of LOXs (adapted from Hayward and others 2017).

(Figure 6). Because the primary structures of LOXs are different, the position of histidine ligands in the polypeptide chain may be different. For example, *His 1* in the rabbit reticulocyte 15-LOX is His-361, but in the soybean, the LOX1 equivalent is His 499; *His 2* is His 366, equivalent His 504; *His 3* is His 541 equivalent His 690, respectively (Brash 1999).

In the existing cleft between the 2 domains, the soybean LOX1 and the rabbit 15-LOX contain 2 major cavities, one for substrate and one for molecular oxygen, which intersect in the proximity of catalytic nonheme iron (Ivanov and others 2010). The ligand-free coral 8R-LOX contains only an internal cavity, forming a U-shaped channel accessible from opposite directions for substrate and molecular oxygen (Ivanov and others 2010). Hsieh and Kinsella (1989) reported specific volatile flavor carbonyl compounds (1-octen-3-ol, 2-octenal, 2-nonenal, 2-nonadienal, 1,5-octadien-3ol, and 2,5-octadien-1-ol) in fish tissue as a result of arachidonic and eicosapentaenoic acid oxidation initiated by lipoxygenase. In recent years, many researchers have addressed the inhibition of LOX-catalyzed reactions (Maccarroni and others 1995; Began and others 1998; Clemens and others 2001; Skrzypczak-Jankun and others 2003), because an understanding of the inhibition mechanism might help identify solutions that could reduce the oxidative processes induced by LOXs. Thus, many molecules, including polyphenols, have been shown to inhibit LOX activity. Polyphenols can inhibit LOX activity by 3 mechanisms: (1) binding to the hydrophobic active site, (2) lipid radical scavenging, and (3) interaction with the hydrophobic fatty acid substrate (Schurink 2007; Maqsood and Benjakul 2010a). Sudha and Srinivasan (2014) studied the inhibitory activity of 3,4 dihydroxybenzoic acid and 4′,5,7-trihydroxyflavone, isolated from the chloroform fraction of *Anisomeles malabarica*, and they found a significant soybean LOX inhibitory activity for 3,4 dihydroxybenzoic acid (74.04%) and 4′,5,7-trihydroxyflavone (34.68%). They suggested that the inhibitory activity of the investigated phenolics is due to the interactions of these compounds with amino acid residues from the folded polypeptide chain and nonheme iron from the catalytic site of sLOX. In the molecular docking studies by Sudha and Srinivasan (2014), the interaction between sLOX and the identified phenolics were compared with the known LOX inhibitor nordihydroguairetic acid (NDGA). This approach demonstrated that all 3 compounds were docked into the active site of sLOX, but that their interactions are different. NDGA is connected by hydrogen interactions (with His 513, His 518, and Ile 857), π-π stacking interaction with 3 amino acid residues (Phe 576, His 518, His 523 and Ile 857), and iron chelation by the hydroxyl groups; 4′,5,7-trihydroxyflavone by a hydrogen bond (between His 518 and a carbonyl oxygen of pyranone moiety) and π-π stacking interactions with 2 amino acid residues (His 518 and Phe 576); and 3,5 dihydroxybenzoic acid does not interact with any hydrogen bond, but through a π-π stacking interaction with His 518 and chelation with iron. They also suggested that the inhibitory activity of 3,5 dihydroxybenzoic acid is due to its ability to block the entry of the substrate in the active site through covalent binding of redox-active iron. Because the tertiary structure and catalytic mechanism of soybean LOX1 were characterized in detail, many researchers have studied LOXs' inhibition using this enzyme in different experimental model systems. Maccarrone and others (1995) investigated the inhibitory activity of chain-breaking antioxidants such as ascorbic acid, 6-palmitoylascorbic acid, and Trolox on the sLOX1 activity. To do this, they monitored the enzymatic formation of oxodienes (by of Lineweaver-Burk double-reciprocal plots and Yoshino's graphical method). They reported that these antioxidants are competitive inhibitors for LOX1 (Ki of 27 μM for ascorbic acid, 3 μM for 6-palmitoylascorbic acid and 18 μM for Trolox). They also suggested that the inhibition is competitive, complete, and reversible. Began and others (1998) studied the inhibition of soybean LOX1 by curcumin, in aqueous solution and bound to phosphatidylcholine micelles, in the dioxygenation reaction of linoleic and arachidonic acids. They reported no inhibition for the curcumin in aqueous solution and 50% inhibition of

linoleic acid dioxygenation in the case of curcumin bound to phosphatidylcholine micelles. Based on Lineweaver-Burk plot analysis and spectroscopic analysis, they suggested that curcumin was a competitive inhibitor for soybean LOX1 (Kiof 1.7 μM for linoleic and 4.3 μM for arachidonic acids) and that the inhibition was due to the binding of the active center iron, after its binding to phosphatidylcholine micelles. After dilution of the enzyme/inhibitor complex with the buffer, LOX1 recovered its catalytic activity. Based on this finding, Began and others (1998) concluded that the inhibition of LOX1 by curcumin bound to phosphatidylcholine is reversible. The inhibitory effect of 18 flavonoids on rabbit reticulocyte 15-LOO-1, using linoleic acid as substrate, was studied by Sadik and others (2003) in order to elucidate the relationship between the flavonoid structure and its inhibitory effect. They suggested that: (1) the presence of a hydroxyl group in the flavonoid structure is not an essential condition for inhibitory activity; (2) a catechol arrangement fortifies the inhibitory activity; (3) in the presence of a catechol arrangement, the inhibitory activity is reversely correlated with the number of hydroxyl groups; and (4) the presence of a 2,3-double bond in the heterocyclic ring (C) increases the inhibitory activity. Lipoxygenases activity is found in various vertebrate and invertebrate tissues, and their post-mortem release may be an important factor in the generation of lipid peroxides, which can then initiate the rancidity of muscle tissue. In muscle tissues, a variety of lipid molecules, such as free fatty acids and phospholipids, can be used as substrates for LOXs to initiate lipid peroxidation. Thus, the inhibition of LOXs activity in muscle tissue treated with polyphenols during storage can be a solution to increase the oxidative stability of muscle tissues. The inhibitory activity of polyphenols on LOXs in muscle tissue was investigated using commercially available compounds or plant extracts rich in polyphenols. Banerjee (2006) used catechin (CT), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) to study their inhibition activity on mackerel (*Scomber scombrus*) muscle lipoxygenase, compared to synthetic antioxidants, and they found that EGCG was the strongest inhibitor tested (IC50 value 0.13 nM). From Lineweaver-Burk plots, for all investigated catechins, Banerjee (2006) found a mixed noncompetitive-type inhibition. In Table 2, the lipoxygenase inhibition pathway of polyphenol antioxidant activities is presented.

Polyphenols as reducing agents for metmyoglobin. Myoglobin (Mb) is a heme Fe(II)-containing protein that gives color to the meat. Oxidation of Fe(II) from Mb to Fe(III), resulting in metmyoglobin (MetMb), is accompanied by a color change in meat, from red to brownish-red, which is associated with meat spoilage but does not always indicate that the meat is spoiled. Molecular and muscle model studies have demonstrated that commercially available polyphenols and extracts rich in polyphenols can inhibit myoglobin oxidation or reduce MetMb, or both. Inai and others (2014) studied the MetMb reduction to the MbO_2 activity of some plant polyphenols and observed a potent activity for flavonols (kaempferol, myricetin, and quercetin) in a concentration of 300 μmol/L against 60 μmol/L MetMb. They also found that the investigated polyphenols (sinapic acid, catechin, nordihydroguaiaretic acid, taxifolin, morin, and ferulic acid) reduced MetMb when at concentrations of 600 μmol/L. Miura and others (2014) reported that the studied polyphenols are myoglobin-reducing agents only in the presence of the cysteine. They found that, in the reduction reaction of MetMb to oxymyoglobin (MbO_2), caffeic acid, dihydrocaffeic acid, and hydroxytyrosol (600 μmol/L) each transformed from nonreducing agents into highly-reducing agents in the presence of equimolar amounts of cysteine (Miura and others 2014). Honda and others (2016) have shown that the reaction products from the peroxidase-catalyzed oxidation of polyphenols in the presence of cysteine have high activity for reducing MetMb to MbO_2. After purification of the cysteinyl polyphenols resulting from the enzymatic oxidation of some commercially available polyphenols (catechin, chlorogenic acid, dihydrocaffeic acid, hydroxytyrosol, nordihydroguaiaretic acid, and rosmarinic acid), they tested these compounds in the MetMb reduction reaction and found that the MetMb-reducing activity was concentration-dependent in the first hour and that the MbO_2 concentration decreased in some reactions after the first hour. Also, they found that the number of cysteinyl sulfur substitutions in polyphenols influences both the MetMb-reducing activity and MbO_2 maintenance. The hydroxytyrosol derivative with 2 cysteine residues had the highest MetMb-reducing activity and no effect on MbO_2 concentration. High phenolics, and other active ingredient extracts, obtained from different plants, provide a good source of antioxidants (Kumar and others 2015), and, for this reason, they were used to study the effects on myoglobin stability in different meat experimental model systems, some of which are presented below. Table 2 shows polyphenols as reducing agents for metmyoglobin.

Polyphenols as Antioxidants to Improve Stability to Oxidation and Color in Meat

Many recent studies have demonstrated that commercially available polyphenols and extracts rich in polyphenols (such as, from spices, berries, tea, roots, flowers, fruits, seeds, coffee residue, various by-products, bee pollen) inhibit lipid peroxidation in meat and meat products, and they also preserve meat color better than/or as well as, the synthetic antioxidants butylhydroxyanisole (BHA), butylhydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate (PG), which have been suspected to have negative effects on human health. The most common indicators for lipid peroxidation and protein oxidation in meat/meat products are peroxide (PV), conjugated dienes (CD), thiobarbituric acid-reactive substances (TBARS), fatty acid contents, volatiles, total carbonyl, and color. Maqsood and Benjakul (2010c) found that tannic acid (100 or 200 ppm) effectively decreased peroxide and TBARS values in striped catfish slices stored 15 d at 4 °C in air and under modified atmospheric packaging. Maqsood and Benjakul (2010b) studied the effect of 200 mg/kg tannic acid in refrigerated ground beef, packed in a modified atmosphere, and they reported low PV, TBARS, and nonheme iron content values in the meat treated with tannic acid. Also, they found less degradation of proteins and some color improvement in samples treated with tannic acid. Al-Hijazeen and others (2016) studied the effect of tannic acid on the oxidative processes in raw and cooked chicken breast meat during storage, compared to BHA, and they reported that tannic acid (5 ppm and 10 ppm) inhibited lipid and protein oxidation, maintained meat color, and lowered off-odor-related volatile formation more effectively than BHA, in both raw and cooked meat. Tannic acid (TA), catechin (CT), caffeic acid (CA), and gallic acid (GA), in concentrations of 200 ppm, were used to study the antioxidant activity in ground camel meat during 9 d of refrigeration by Maqsood and others (2015). They found that, in all samples treated with phenolic compounds, lipid peroxidation was retarded, but that the lowest levels for PV and TBARS value were found in meat treated with TA or CT. Also, phenolics inhibited the oxidative degradation of proteins, but the best results were reported for TA and CT. Liu and others (2015) studied the antioxidant activities of tea catechins and grape

seeds extracts, compared to vitamin E, carnosine, and BHA, in raw beef patties during 8 d of storage at 4 °C. They found that the antioxidant treatment significantly inhibited discoloration and lipid oxidation. The efficiency of the antioxidants in preserving the redness value decreased in the following order: carnosine > grape seed extract > vitamin E > tea catechin > BHA, while the inhibition rate of lipid oxidation, for the investigated antioxidant, decreased as follows: BHA > tea catechins > vitamin E > grape seed extract > carnosine. After 8 d of storage, the MetMb percent (MetMb%) in the patties treated with the mentioned antioxidants increased in the following order: carnosine < grape seed extract < vitamin E < tea catechin < BHA. All tested antioxidants reduced MetMb, but after 8 d of storage at 4 °C, the highest MetMb-reducing activity was reported in the patties treated with grape seed extract. Work by Maqsood and others (2016) suggested that pure phenolics (TA and CT) are more effective at retarding lipid oxidation in refrigerated camel meat sausages, compared to extracts rich in these phenolics (date seed and green tea extracts). Maqsood and Benjakul (2010a) studied the antioxidant activities of catechin, caffeic acid, ferulic acid, and tannic acid on lipids dioxygenation in fish mince, and they reported an increase of the inhibitory activity with increasing concentration for all phenolics tested, and the highest LOX inhibitory activity was shown for caffeic acid. Chicken meat treated with rosemary and clove ethanolic extracts rich in polyphenols (total phenolic content: 48.49, respectively 34.18 mg GAE/g) had a lower TBARS value and meat discoloration than either untreated meat or meat treated with BHT. A similar result was also reported by Zhang H and others (2016). Cho and others (2015) evaluated the effect of different concentrations of *Allium hookeri* root extract (AHE) on the lipid oxidation and color stability of raw sulfur-fed pork patties during 14 d of refrigeration, and they found that the CD and TBARS values decreased proportional to the increase of AHE level and inhibited meat discoloration. In that paper, Cho and others (2015) suggested that AHE polyphenols could be used to extend the shelf-life of food. Zhang Y and others (2016) prepared pork salami using an extract rich in flavonoids obtained from rape (*Brassica campestris* L.) bee pollen (RBP) as an antioxidant. They found that RBP extract decreased PV and TBARS values during salami processing. They also prepared salami using 0.05% RBP extract, 0.03% rutin, 0.03% quercetin, and 0.03% kaempferol as antioxidant and found that RBT and each of the investigated flavonoids delayed the decrease in the activities of the endogenous antioxidant glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) enzymes. Extracts rich in polyphenols obtained from coffee residue showed strong antioxidant activity in raw and cooked meat during 12 h of storage at 37 °C, and 5 d of storage at 4 °C (Kim and others 2016). Work by Sun and others (2016) demonstrated that young-apple polyphenols (YAP) retarded the decrease in of fatty acid contents (both unsaturated and saturated), and minimized the degradation of salt-soluble myofibrillar protein and sulfhydryl group in grass carp fillets. Because the YAP did not affect the taste of the fillets, Sun and others (2016) suggested that YAP could be used to extend the shelf-life of freshwater fish. Polyphenols extracted from leafy green vegetables consumed in East Asia were reported to improve the color and resistance to oxidation in ground beef meat patties refrigerated at 4 °C for 12 d (Kim SJ and others 2013). During cold storage of raw beef sausages treated with Ziziphus ethanolic extract (0.5% and 1.0%) and aqueous extract (1.0%), lipid peroxidation was inhibited in concentration- and solvent-dependent manner. After 14 d of storage at 2 to 4 °C the levels of secondary lipid peroxidation products increased in the following order: 1.0% ethanolic extract < 1.0% aqueous extract < 0.5% ethanolic extract (Abdulla and others 2016). Due to the higher antioxidant activity of Ziziphus extracts compared to synthetic antioxidant TBHQ, Abdulla and others (2016) suggested that these extracts might be useful as natural antioxidants in the meat industry. Polyphenols extracted from sea buckthorn fruits increased the resistance to oxidation of lipid and myoglobin from carp muscle subjected to storage by freezing (Papuc and others 2012). Hayes and others (2009) reported the decrease of lipid and oxymyoglobin oxidation in bovine and porcine muscle model systems treated with ellagic acid and olive leaf extract, stored 24 h at 4 °C. Table 3 summarizes the effects of polyphenols on lipid oxidation and meat color.

Antibacterial Activity of Polyphenols

After slaughtering, in the production lines, meat is exposed to environmental conditions, equipment, and tools that facilitate cross-contamination with spoilage and pathogenic microorganisms, which are potential vectors of meat/meat product spoilage and foodborne diseases. Recent studies carried out by Voloski and others (2016) revealed the importance of hygiene on the microbiological quality and shelf-life of meat stored under refrigeration. Vacuum-packaged buffalo meat, collected at the end of the deboning line, was stored under refrigeration for 2 mo and the impact of the microbiological contamination during cutting and vacuum-packaging on the meat shelf-life was evaluated. On the processing day, high counts of *Pseudomonas* spp and lactic acid bacteria (LAB) were observed on the meat cuts, whereas *Listeria grayi* and *Listeria innocua* were found only in some meat cuts during storage. For these reasons, Voloski and others (2016) concluded that cutting and deboning operations performed during meat production affect the microbiological quality and shelf-life of the refrigerated meat. Foodborne bacteria and spoilage bacteria are dangerous meat contaminants and pathogens for humans. *Brochothrix thermosphacta*, *Camobacterium* spp., *Enterobacteriaceae*, *Lactobacillus* spp., *Leuconostoc* spp., *Pseudomonas* spp., and *Shewanella putrefaciens* cause spoilage of refrigerated meat (Borch and others 1996). *Escherichia coli 0157:H7*, *Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, and *Campylobacter jejuni* cause foodborne diseases (Barbosa and others 2009). Meat spoilage and foodborne illnesses cause serious economic losses. Therefore, it is important to control the development of bacteria in meat and the meat processing line. Antimicrobial agents are the most common solution for this. Antimicrobials used in the meat industry can be natural, semisynthetic, or synthetic substances that kill or delay the growth of microorganisms in all meat matrixes. Natural antimicrobials may be of plant origin (essential oils, polyphenols, fermented juices rich in nitrites), animal origin (lactoferrin, defensins, chitosan), or microbiological origin (reuterin, pediocin, nisin) (Tiwari and others 2009; Sultanbawa 2011). Many researchers have demonstrated that commercially available polyphenols and plant extracts rich in polyphenols can be used as antioxidants and natural antimicrobials. *In vitro* studies have shown that polyphenols have antimicrobial activities against Gram-positive and Gram-negative bacteria. The mechanisms of antimicrobial activity of polyphenols have not yet been fully resolved. Due to the structural diversity of polyphenol classes, it is impossible to predict the structural features of each class responsible for the antimicrobial activity, and the interaction forces between bacteria and polyphenols. Cowan (1999) suggested that the antimicrobial activity of polyphenols was due to OH group/groups bonded to both the aromatic rings and the oxygen substituted ring. Studies on the aglycones and

Table 3–Polyphenols as antioxidants and antimicrobial for meat

Polyphenols / extracts rich in polyphenols	Meat model system	Effects on lipid oxidation	Effects on meat color	Antimicrobial activity	References
Tannic acid	Striped catfish slices stored for 15 d at 4 °C in air and under modified atmospheric packaging	Decreased PV and TBARS values	–	Retardation of total viable and psychrophilic bacteria grow	Maqsood and Benjakul (2010c)
Tannic acid	Ground beef stored for 15 d at 4 °C in air and under modified atmospheric packaging	Decreased PV and TBARS values	Color improvement	Inhibitory effect on the growth of psychrophilic bacteria	Maqsood and Benjakul (2010b)
Tannic acid	Raw and cooked chicken breast meat stored for 7 d at 4 °C	Decreased TBARS value	Maintained meat color	–	Al-Hijazeen and others (2016)
Tannic acid, catechin, caffeic acid and gallic acid	Ground camel meat stored for 9 d at 4 °C	Tannic acid and catechin decreased PV and TBARS values more effecient than caffeic acid and gallic acid	Tannic acid and catechin maintained the best the meat color	Retardation of psychrophilic and mesophilic bacteria grow	Maqsood and others (2015)
Tea catechins and grape seeds extracts	Raw beef patties stored for 8 d at 4 °C.	Tea catechins decreased TBARS value similar with BHA	Extracts preserves redness value	–	Liu and others (2015)
Tannic acid, date seed extract, catechin and green tea extract	Camel meat sausages stored for 12 d at 4 °C	Tannic acid and catechin were more efficent than the extracts used in lipid oxidation inhibition	Antioxidants did not affect the sensory color	Tannic acid and catechin were more efficient than the extracts used in retardation of bacterial growth	Maqsood and others (2016)
Catechin, caffeic acid, ferulic acid and tannic acid	Fish mince 15 d ice storage	All polyphenols decreased PV, CD, TBARS values and inhibited LOX activity	–	–	Maqsood and Benjakul (2010a)
Rosemary and clove ethanolic extractes rich in polyphenols	Chicken meat refrigerated for 15 d	Decreased TBARS leavel	Decreased meat discoloration	Total viable counts values decreased significantly with the addition of spice extracts	Zhang H and others (2016)
Allium hookeri root extract (AHE)	Raw sulfur-fed pork patties refrigerated for 14 d	CD and TBARS values decreased proportionally with the increase of AHE level	Inhibited meat discoloration	–	Cho and others (2015)
Rape bee pollen	Pork salami during processing	Decreased PV and TBARS values	–	–	Zhang Y and others (2016)
Coffee residue extracts	Raw chicken meat stored for 12 h storage at 37 °C; cooked chicken patties stored for 5 d at 4 °C	Ethanolic extracts decreased TBARS values as effective as BHA	–	–	Kim and others (2016)
Young apple extract	Grass carp fillets stored for 7 d at 4 °C	Retarded the decrease of fatty acids content	Minimize the degradation of salt-soluble myofibrillar protein and sulfhydryl group	Aerobic bacteria count values decreased, comparatively with the control samples	Sun and others (2016)
Polyphenols extracted from leafy green vegetables consumed in East Asia	Raw beef patties stored for 12 d at 4 °C	Decrease TBARS values	Improve the meat color stability	Decreased the number of microorganisms in beef patties	Kim SJ and others (2013)
Ziziphus ethanolic extracts	Raw beef sausages cold storage	Decreased TBARS values	Improve the sausages color stability	Inhibited the growth of all tested foodborne bacteria strains	Abdulla and others (2016)
Sea buckthorn extract	Fish muscle stored by freezing for 6 wk at −18 °C	Decreased PV, CD and TBARS values	Inhibited Mb oxidation	–	Papuc and others (2012)
Ellagic acid and olive leaf extract	Bovine and porcine muscle stored for 24 h at 4 °C	Deceased TBARS values	Decreased oxyMb oxidation		Hayes and others (2009)
Gallic and protocatechuic acids, gallic and caffeic acids, and quercetin and rutin, and three Argentinian red wine varieties	Fish meat stored at 4 and 20 °C	–	–	Decreased *L. Monocytogenes* and *E. coli* viability	Rodríguez-Vaquero and others (2013)
Quillay extract	Marinated chicken meat stored for 8 d at 6 °C	Decreased TBARS values	–	Reduced mesophilic aerobe count and total coliform count values	Fellenberg and others (2011)
Rumex tingitanus leaves extracts	Minced beef meat	–	–	Ethyl acetate fraction eradicates the *L. Monocytogenes* population in meat in a concentration and time-dependent manner	Mhalla and others (2017)

glycosylated flavonoids reported little antimicrobial activity for glycosylated flavonoids (Liu and others 2010; Wu and others 2013b). Thus, the antibacterial activity of flavonoids is strongly dependent on the backbone structure, number and positions of OH groups, the presence of glycosidic linkages, and alkylation of OH groups. Antimicrobial activity studies using extracts obtained from black, green, and white tea, and also commercially available tea polyphenols, have reported a positive correlation with catechins, total theaflavins, and total thearubigins concentrations (Koech and others 2013), and the bacterial killing effect was dose-dependent (Kao and others 2010).

How polyphenols kill or inhibit bacteria

How polyphenols kill or inhibit bacteria is unclear. The interaction of polyphenols with nonspecific forces such as hydrogen-bonding and hydrophobic effects, lipophilic forces, as well as by covalent bond formation, was related to microbial membranes, adhesins, enzymes, and cell envelope transport proteins (Cowan 1999; Kumar and Pandey 2013). The antibacterial activity of polyphenols may be also due to the capacity of these compounds to chelate iron, vital for the survival of almost all bacteria (Field and Lettinga 1992).

Interaction with the bacterial cell wall

The cell walls of Gram-negative and Gram-positive bacteria are different. Gram-negative bacteria cell walls consist of a thin layer of peptidoglycan and the outer membrane. The outer membrane (OM) is composed of a phospholipid bilayer and proteins and, on his outer leaflet contains lipopolysaccharides (LPS). In Gram-positive bacteria, cell walls lack the outer membrane and, thus, are composed of a thick layer of peptidoglycan and lipoteichoic acid (Brown and others 2015). In both Gram-positive and Gram-negative bacteria, the cell wall plays an important role in the osmotic protection of the cell. Thus, any damage to the cell wall will decrease the tolerance of the cell to high ionic strength and low osmotic pressure. The bacterial cell wall peptidoglycan layer is essential for bacteria viability, and it is the most important site for interactions with antibiotics. Many researchers have demonstrated the ability of polyphenols to interact with the bacterial cell wall, and found that the susceptibility to polyphenol action of Gram-positive bacteria is different from Gram-negative bacteria; the latter are more resistant to polyphenol actions. This is due to differences in cell wall composition; the outer hydrophilic membrane of Gram-negative bacteria is mainly composed of lipopolysaccharides (LPS) (Nohynek and others 2006) and hinders polyphenol connections to the peptidoglycan layers of these microorganisms (Cui and others 2012). Flavones, flavonoids, and flavonols are effective antimicrobial substances against a wide range of microorganisms due to their ability to complex microbial cell walls (Takahashi and others 1995; Cowan 1999; Zhao and others 2001; Zhao and others 2002). In recent years, the interaction of favan-3-ols, especially green tea flavan-3-ols, with the bacterial cell wall have been extensively studied for their antibacterial potential. Zhao and others (2002) demonstrated that epigallocatechin gallate (EGCG) is able to bind directly to the peptidoglycan from *S. aureus*, affecting cell integrity, and thereby reduce the tolerance of the cell to both high ionic strength and low osmotic pressure. Yoda and others (2004) studied the antibacterial activity of EGCG against various strains of *Staphylococcus* and Gram-negative rods and suggested that the different susceptibilities to EGCG of investigated bacteria were due to differences in the affinity of EGCG for their various cell wall components. However, in recent years, it has been reported that polyphenols are able to disintegrate the outer membrane, releasing LPS and increasing the permeability of the cytoplasmic membrane (Burt 2004; Nohynek and others 2006). Cloudberry, black currant, cranberry, and blueberry extracts are able to release LPS from *Salmonella enterica* serovar Typhimurium VTT E-981151 and *Salmonella enterica* serovar Infantis VTT E-97738, in a manner similar to EDTA (Nohynek and others 2006). Nohynek and others (2006) demonstrated that cloudberry and raspberry phenolic extracts are able to disintegrate the outer membrane of *Salmonella* strains by chelating divalent cations from the outer membrane or by inserting into the outer membrane with the substitution of stabilizing cations. Also, they assumed that ellagitannin from cloudberry and raspberry and ellagic acid from cranberry were responsible for the antimicrobial activity of these berries. Zhao and others (2002) suggested that EGCG binds to Gram-positive (directly) and Gram-negative (indirectly) bacteria peptidoglycan cell walls. Atomic force microscopy data from Cui and others (2012) confirmed this. They demonstrated that EGCG treatment can induce different effects on bacterial cell walls, such as aggregates in *Streptococcus mutants* and grooves in *Pseudomonas aeruginosa*, but that the major EGCG-induced morphological changes to Gram-negative bacteria cell walls depend on the release of H_2O_2. In the same study, they demonstrated that EGCG induces oxidative stress in Gram-negative bacteria. Proanthocyanidins (condensed tannins) from cranberries, grapes, and tea could interact with the various components of bacterial cell wall. Delehanty and others (2007) found that proanthocyanidins from cranberries bind and neutralize bacterial LPS, and suggested that this ability inhibits the binding of LPS to the surface of mammalian cells. Johnson and others (2008) used immobilized proanthocyanidins from whole cranberries, grape juice, black tea, and cranberry juice to capture bacterial LPS, and assumed that phenolic molecules bind to the lipid A component of the LPS. An important property of fimbriae bacteria is the adherence to the host tissue. Pili or fimbriae are small filaments that enable bacteria to adhere to the host tissue. These protein filaments are able to bind complementary carbohydrates from receptors of host cell tissue by a lectin-like mechanism. It was demonstrated that polyphenols, especially proanthocyanidins, inhibit the binding of pili to cell-specific receptors (Hisano and others 2012). One possible mechanism for this is competitive inhibition, and it was supposed that proanthocyanidins act as receptor analogs. Howell and Vorsa (1998) demonstrated that purified proanthocyanidins extracted from cranberry, at concentrations of 10 to 50 μg/mL, inhibited the adherence of P-fimbriated *E. coli* to cell surfaces. Another mechanism assumed that proanthocyanidins are able to reduce fimbrial length and density (Liu and others 2008).

Interaction with the bacterial cell membrane

Many researchers have reported on the interaction of polyphenols, especially tea catechins, with bacterial membranes (Ikigai and others 1993; Cho and others 2007; Matsumoto and others 2012). It has been reported that polyphenols interact with proteins and/or phospholipids from the lipid bilayer. In both Gram-positive and Gram-negative bacteria, interaction with membrane proteins causes disruption of the lipid bilayer, increasing membrane permeability, affecting membrane fluidity, inhibiting respiration, and altering ion transport processes (Nazzaro and others 2013). Yi and others (2014) investigated the antimicrobial effect of tea polyphenols by transmission electron microscopy, and they reported that bacterial cell membranes are damaged, including having increased outer and inner membrane permeability, disrupted cell membranes, and releasing of small cellular

molecules. Wu and others (2013a) investigated the antibacterial activity and membrane interaction of 5 flavonoids (kaempferol, quercetin, chrysin, luteolin, baicalein), 2 polymethoxyflavones (tangeritin, 5,6,7,4′-tetramethoxyflavone), and 4 isoflavonoids (daidzein, genistin, ononin, puerarin) against *E. coli* and found that the antibacterial activity decreased in the following order: flavonoids > polymethoxyflavones > isoflavonoids. They also found that flavonoids rigidified the liposomal membrane, while the other compounds increased membrane fluidity. They suggested that the antibacterial activity of flavonoids is linked to the molecular hydrophobicity and the presence of an OH group at the position 3 in the C-ring. Borges and others (2013) reported that gallic and ferulic acids induce irreversible changes in *E. coli*, *P. aeruginosa*, *S. aureus*, and *L. monocytogenes* membrane properties. They suggested that the interaction of gallic and ferulic acids with bacterial cytoplasmic membrane causes hydrophobicity changes, a decrease in negative surface charge, and local rupture and pore formation with leakage of intracellular constituents. The studies in the last decade have shown that flavonoids with an OH group at the position 3 in the C-ring decrease the membrane fluidity leading to membrane rigidification (Tsuchiya 2010; Wu and others 2013a). Many studies have used membrane models to elucidate the interaction of polyphenols with the phospholipids of the bilayer lipid membrane. Hashimoto and others (1999) studied the interaction of tea catechins with the lipid bilayer in liposome systems and reported that the affinity of the investigated catechins for the lipid bilayer decreased in the following order: epicatechin gallate > epigallocatechin gallate > epicatechin > epigallocatechin. In this paper, they observed the perturbation of membrane structure only in the case of epicatechin gallate and epigallocatechin gallate and suggested that the presence of the gallic acid residue in the catechin structure is responsible for the higher affinity for the lipid bilayer. Some researchers reported that the affinity of tea catechins for the lipid bilayer is dependent on the number of OH groups on the B-ring, the stereochemical structure of the C-ring, phenolic concentration, and external factors. Hashimoto and others (1999) found that EC had the highest lipid bilayer affinity, followed by ECG, and then by EGCG. For this reason, they concluded that increasing the number of OH groups on the B-ring of gallocatechins decreases hydrophobicity of phenolics and, therefore, their affinity for lipid bilayers. Kajiya and others (2001) reported that the affinity of *trans*-type catechins for the lipid bilayer in a liposome system was less than that of *Cys*-type catechins and that the number of the phenolics incorporated in the lipid bilayer increased with the amount of the phenolics added. Kajiya and others (2002) studied the effect of external factors on tea catechin interaction with the lipid bilayer using cell and bacteria cultures and reported the increasing of the affinity of catechins for the lipid bilayers with increasing salt concentrations, as well as the presence of another catechin. Also, they reported a decrease of inaffinity with increasing negative electric charge of the lipid bilayer. Evaluation of the antibacterial activity of the different (+)-catechin-alkyl derivatives against Gram-positive bacteria found increasing antibacterial activity with increasing carbon atom number in the alkyl chain, which peaked at a chain of 4 to 7 carbons (Kajiya and others 2004). Kajiya and others (2004) studied the injury to the lipid bilayer induced by (+)-catechin-alkyl derivatives in the liposome and reported the leakage of cellular compounds in the case of derivatives containing 4 or 5 carbons, and membrane damage in the case of derivatives with more than 5 carbons in their alkyl chain. For these reasons, Kajiya and others (2004) assumed that the lipophilicity and disrupting the activity of the studied (+)-catechin-derivatives are important factors for antibacterial activity. Investigation of the interaction of tea catechins with lipid bilayers by quartz-crystal microbalance analysis revealed that the affinity of (-)epicatechin gallate and (-)epigallocatechin gallate for 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine was approximately 1000 times higher than (-)epicatechin and (-)epigallocatechin (Kajiya and others 2002). Sun and others (2009) studied the effect of EGCG and curcumin on single giant unilamellar vesicles (GUVs) and found that both phenolics bind to the membrane lipid bilayer, but that only EGCG could solubilize lipid molecules from the bilayer, resulting in pores. By NMR spectroscopy, Uekusa and others (2007) suggested that EGCG and ECG interact with the surface of the lipid bilayer at the level of the choline residue. Possible interactions of polyphenols with cell wall components and the plasma membrane of Gram-negative and Gram-positive bacteria are shown in Figure 7.

Inhibition of biofilm formation

During slaughter and meat-handling, spoilage and pathogenic microorganisms can attach to the meat contact surface and form biofilms (Benedict 1988). Common meat spoilage bacteria (*Pseudomonas* spp., *Brochothrix thermosphacta*, and *Lactobacillus* spp.) and foodborne pathogens (*Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli*) form a biofilm in most of their habitats (Giaouris and others 2014). In the meat industry, biofilms formed by pathogenic and spoilage bacteria can be a persistent source of product contamination, leading to serious hygienic problems, and also to economic losses due to food spoilage (Van Houdt and Michiels 2010). The elucidation of the mechanisms involved in bacterial attachment to meat surfaces and the inhibition of attachment could lead to the development of new methods to reduce meat contamination by bacteria, foodborne diseases, and an increase ofmeat shelf-life. Biofilm formation requires 4 steps: (i) reversible attachment of microorganisms to solid surfaces (attraction of the cells to the surface is dependent on a balance of attractive and repulsive forces between bacteria and the surface, and of flagella-driven motility); (ii) irreversible attachment (binding of bacteria to a surface by bacterial surface polymeric structures, which include fimbrial adhesines such as 1 fimbriae, curli, type 4 pili, long polar fimbriae, and F9 fimbriae); (iii) maturation (multiplication, production of extracellular polymers, and formation of the 3D structure of the biofilm; (iv) dispersion (detachment of bacteria from biofilm and their dispersal) (Rijnaarts and others 1995; Katsikogianni and Missirlis 2004; van Houdt and Michiels 2005; Myszka and Czaczyk 2011). Many studies have reported that polyphenols can exhibit antibacterial activity via anti-biofilm agents. Borges and others (2012) studied gallic acid and ferulic acid interference with *E. coli*, *P. aeruginosa*, *S. aureus*, and *L. monocytogenes* adhesion by determining the free energy of adhesion. They found that the adhesion was disadvantaged when the bacteria were exposed to gallic acid (*P. aeruginosa*, *L. monocytogenes*, and *S. aureus*) and ferulic acid (*P. aeruginosa* and *S. aureus*). Borges and others (2012) also suggested that both phenolics inhibit bacterial motility and biofilm formation. Naringenin, quercetin, apigenin, and an aromatic ester of ferulic acid were demonstrated also to inhibit biofilm formation (Koo and others 2003; Ergün and others 2011; Borges and others 2012). Koo and others 2003 investigated the effect of apigenin on the *Streptococcus mutant* biofilm accumulation and polysaccharide production and reported the lowering of the amounts of extracellular alkali-soluble glucans, intracellular iodophilic polysaccharides, and fructan. In the last 20 y, many papers described the importance of quorum sensing

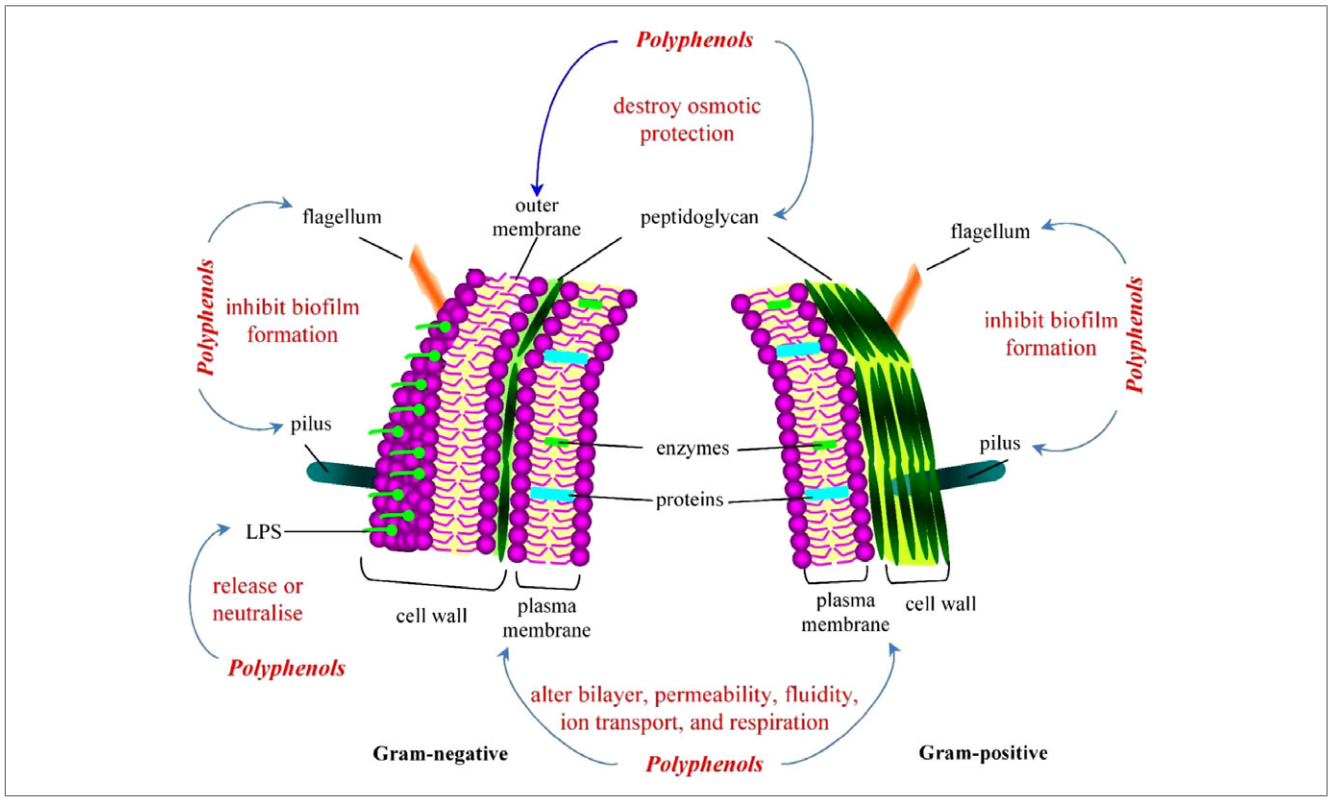


Figure 7–Possible interactions of polyphenols with cell wall components and plasma membrane of Gram-negative and Gram-positive bacteria.

(QS, cell–cell communication by extracellular chemical signals whose concentration is correlated with density of the microbial population) during the formation of bacterial biofilms (Annous and others 2009; Skandamis and Nychas 2012; Koh and others 2013; Bai and Vittal 2014; Solano and others 2014; Slobodníková and others 2016), and suggested that natural molecules and plant extracts with anti-QS and anti-biofilm activity could be used to develop a new strategy in food safety (Zhang and others 2014). Abraham and others (2011) reported the anti-QS and anti-biofilm effect of a methanolic extract obtained from *Capparis spinosa* on *S. marcescens*, *Pseudomonas aeruginosa* PAO1, *E. coli*, and *P. mirabilis.* Zhang and others (2014) reported that an extract rich in polyphenols obtained from *Rosa rugosa* tea inhibits QS of *Chromobacterium violaceum 026*, swarming motility, and biofilm formation of *E. coli K-12* and *Pseudomonas aeruginosa PAO1*, and they suggested that *Rosa rugosa* tea polyphenols can be used as anti-biofilm agents to increase shelf-life and food safety. Gopu and others (2015) tested the influence of quercetin on QS in foodborne bacteria and found anti-QS activity both *in vitro* and *in silico* studies, and they recommended the use of this polyphenol as a novel antibacterial/anti-biofilm molecule to manage foodborne pathogens. It was demonstrated that cranberry proanthocyanidins limited the motility, particular swarming motility, and reduced biofilm formation of *P. aeruginosa*, without down-regulating proteins related to flagella and type IV pili (Urley and others 2014). Urley and others (2014) also reported that a quorum-sensing inhibitor that blocked *P. aeruginosa* biofilm formation (PvdQ) had more peptide hits with proanthocyanidine treatment.

Inhibition of bacterial enzymes and substrate deprivation

Recent studies suggested the ability of polyphenols to inhibit cyclic di-AMP synthase, the enzyme that catalyzes cyclic-di-AMP biosynthesis, an important signaling molecule that controls a vast number of functions, including cell wall homeostasis (Opoku-Temeng and Sintim 2016), modulation of bacterial cell wall synthesis (Witte and others 2013; Sureka and others 2014), and grow (Huynh and others 2015). Opoku-Temeng and Sintim (2016) studied the inhibitory effect of 14 polyphenols on *B. subtilis* cyclic di-AMP synthase and reported the inhibitory effect of tannic acid, theaflavin-3′-gallate, and theaflavin-3,3′-digallate. Xiao and others (2014) investigated the targets in *E. coli* for 19 flavonoids, using comparative genomic and molecular modeling, and they identified the following enzymes as a target for the investigated flavonoids: fumarate reductase flavoprotein, dihydroorotate dehydrogenase, dihydrofolate reductase, NADH-dependent enoyl-ACP reductase, and DNA gyrase subunit. By molecular docking studies, they also revealed the importance of the 3-O-galloyl or 3-O-glycosides side chain at the flavonoid pyran ring in the mechanism of the mentioned enzyme inhibition. Bacterial hyaluronidases (hyaluronic acid lyases), produced by both Gram-positive and Gram-negative bacteria, are important virulence factors because they directly interact with host tissues or mask the bacterial surface from host-defense mechanisms (Girish and Kemparaju 2007). Flavonoids have been identified as hyaluronic acid lyases inhibitors, opening new strategies for developing natural antimicrobial agents. Hertel and others (2006) studied the inhibitory effect of flavonoids on the activity of 3 hyaluronic acid-splitting enzymes (Hyal B – from *Streptococcus agalactiae*, rHyal B – recombinant hyaluronate lyase, and Dase – hyaluronidase from bovine testes), and they reported that the inhibitory activity increased with the number of hydroxyl groups present in the flavonoid structure. Haraguchi and others (1998) studied the antibacterial mechanism of licochalcone A and C from the roots of *Glycyrrhiza inflata* and suggested that the inhibition site of these retrochalcones was

between CoQ and cytochrome *c* in the electron transport chain of the bacterial chain respiration. Scalbert (1991) suggested that tannin toxicity for bacteria is due to the ability to act directly on bacterial metabolism inhibiting the oxidative phosphorylation. Konishi and others (1993) examined the effects of some purified tannins on the NADH-ubiquinone-1-oxidoreductase activity of *Paracoccus denitrificans*, *Bacillus subtilis*, *Photobacterium phosphoreum*, and *Thermus thermophilus HB-8*, and reported their inhibitory effect.

Protein regulation

Protein synthesis may be a prime cause of the antibacterial effect of polyphenols. Ulrey and others (2014) reported that treatment of *P. aeruginosa* with cranberry proanthocyanidins down-regulated 2 proteins implicated in ATP synthesis, a cytochrome C (PA2482), hypothetical protein PA2481, proteins involved in DNA and RNA synthesis (Top A, Rp1C, and Mfd), and acid cycle proteins (subunits of acetyl-CoA carboxylase and fumarase). However, Ulrey and others (2014) also reported up-regulation of many proteins. Thus, 12 proteins related to iron siderophores or cation transporters (for example, PchD, PvdN, PhuS), 5 proteins involved in amino acid synthesis (for example, PA0335, PA2044, HutG), proteins involved in response to stress (for example, OsmC, SodM), and a hypothetical protein involved in flavonoid metabolism (PA3450) are up-regulated. Ulanowska and others (2006) used 5 commercially available flavonoids (genistein, daidzein, apigenin, naringenin, and kaempferol) to study their effects on *Escherichia coli*, *Vibrio harveyi*, and *Bacillus subtilis* cultures, and they found that in the *E. coli* culture the synthesis of DNA, RNA, and proteins was unaltered, but in the *V. harveyi* culture the nucleic acids and proteins synthesis was powerfully inhibited. Effects of flavonoids on *B. subtilis* were intermediate to those of *E. coli* and *V. harveyi*. By incorporation of specific radioactive precursors in the cells of *S. enteritidis*, Arima and others (2002) observed the inhibition of DNA synthesis induced by morin, in concentrations equal to 50 μg/mL or higher, and in a concentration of 12.5 μg/mL if in the culture medium rutin is added at a concentration of 12.5 μg/mL. Morin also inhibited RNA and protein synthesis, but rutin added did not influence the inhibition process. Apple flavonoid phloretin was reported to control *E. coli O157:H7* biofilm formation by a mechanism that implies repressing the curli genes (csgA and csgB), which are involved in fimbriae production (Lee and others 2011)

Metal iron deprivation due to chelating ability

With the exception of lactic acid bacteria, iron is an essential element for the growth of spoilage and foodborne bacteria (Andrews and others 2003; Engels and others 2009). Meat is an excellent iron source for bacteria. Heme proteins, such as myoglobin and hemoglobin, and nonheme proteins, such as transferrin and ferritin, are iron sources for the bacteria. Bacteria can also gain iron from the free heme resulting from the enzymatic and nonenzymatic degradation of heme proteins, and also the free iron ions resulting from heme degradation. Thus, limiting the level of available iron ions should, in principle, inhibit microbial growth (Kim and Shin 2009; Thompson and others 2012; Moon and others 2013). Engels and others (2009) isolated gallotannins from mango fruit (*Mangifera indica*) and, after purification, they obtained 3 pure hydrolyzable tannins (penta-, hexa-, and hepta-O-galloylglucose) with antibacterial activity against Gram-positive food spoilage bacteria and *E. coli*. Bacterial growth was restored after iron addition to the medium. Thus, they concluded that the antibacterial activity of gallotannins is due to their ability to chelate iron. Wong and Kitts (2006) evaluated antimicrobial activity of polyphenols-rich parsley (*Petroselinum crispum*) leaves and stem and cilantro (*Coriandrum sativum*) extracts against *Bacillus subtilis* and *E. coli* by determining cell damage, and found a significantly ($P < 0.05$) greater growth inhibition of both microorganisms corresponding to ferrous sequestering activity of methanol-derived stem extracts. Ulrley and others (2014) found that cranberry proanthocyanidins lower the abundance of cytochromes in *P. aeruginosa*. The antibacterial action mechanisms of polyphenols are summarized in Table 4.

Polyphenols Antimicrobials for Food Spoilage and Foodborne Bacteria in Meat

Maqsood and Benjakul (2010b) found that tannic acid could lower the psychrophilic bacteria count (PBC) in refrigerated minced beef. Lower PBCs and mesophilic bacteria counts (MBC) have been reported for ground camel meat treated with tannic acid (TA), catechin (CT), caffeic acid (CA), and gallic acid (GA) (Maqsood and others 2015). Maqsood and others (2016) studied the antimicrobial effect of tannic acid (TA), date seed extract (DSE), catechin (CT), and green tea extract (GTE) on camel meat sausages, and they found that pure phenolics (TA and CT) have the best antimicrobial activity. Zhang H and others (2016) found that rosemary ethanolic extract (RO), clove ethanolic extract (CL), and their combination (RO – CL) were able to reduce the total viable count (TVC), lactic acid bacteria (LAB), and *Pseudomonas* spp. and *Enterobacteriaceae* counts on refrigerated raw chicken meat, and that the extracts acted synergistically. Sun and others (2016) found that young apple polyphenols (YAP) could lower the aerobic bacterial count (CFU/g) in stored grass carp fillets. Rodríguez-Vaquero and others (2013) found that commercially available polyphenols (gallic and protocatechuic acids, gallic and caffeic acids, and quercetin and rutin) and 3 Argentinian red wines (Cabernet Sauvignon, Malbec, and Merlot) could protect commercial fish meat against *E. coli* and *L. monocytogenes*. Rodríguez-Vaquero and others (2013) have suggested that a combination of rutin and quercetin could be used as a fish meat preservative. Fellenberg and others (2011) added a polyphenol-rich extract obtained from quillay (*Quillaja saponaria*) to the marinade for broiler chicken and observed an improvement in the microbiological quality of the meat. Mesophilic aerobe count and total coliform count were reduced in both chicken breasts and thighs when the polyphenol-rich extract was added. Mhalla and others (2017) have reported that *Rumex tingitanus* leaf extracts, which are rich in polyphenols, inhibit the proliferation of *L. monocytogenes* in minced beef meat. Ethanolic extracts obtained from ten leafy green vegetables consumed in East Asia were added to ground beef patties, which were stored at 4 °C for 12 d. *E. coli*, *S. enterica*, *S. flexneri*, *L. monocytogenes*, *S. aureus*, and *B. subtilis* counts revealed that the studied extracts decreased the numbers of microorganisms in the patties, and it was concluded that leafy green vegetable extracts, rich in polyphenols, could be used in the meat industry as natural preservatives (Kim SJ and others 2013). Ziziphus leaf extracts, rich in e-vanillic and ellagic acids, added in beef sausages inhibited the growth of spoilage bacteria during cold storage for 14 d at 2 °C. After 14 d of cold storage, total microbial counts showed that the inhibition is dependent on extraction solvent nature and extracts concentration in sausages. Spoilage bacteria inhibition was highest for 1.0% ethanolic extract, followed by 1.0% aqueous extract, and then by 0.5% ethanolic extract (Abdulla and others 2016). Table 3 summarizes the antimicrobial activity of polyphenols in meat.

Table 4–Polyphenols as antibacterial agents – action mechanisms

Antibacterial activity mechanism	Polyphenols interaction site	Consequence	Example	Sources
Interaction with bacterial cell wall	Outer membrane (OM)	Releasing of the LPS; reduction of the tolerance of the cell to both high ionic strength and low osmotic pressure	EGCG is able to bind directly to the peptidoglycan from *S. aureus*	Zhao and others (2002)
			Cloudberry, raspberry, black currant and blueberry are able to release LPS from *Salmonella enterica* serovar *Typhimurium* VTT E-981151 and *Salmonella enterica* serovar *Infantis* VTT E-97738	Nohynek and others (2006)
	Peptidoglycan	Binding to peptidoglycan, affects cell integrity and so, the reduction of tolerance of the cell to both high ionic strength and low osmotic pressure	EGCG bind peptidoglycan from *S. aureus*	Zhao and others (2002)
			EGCG bind pepetidoglycan from Gram-positive and Gram-negative bacteria producing aggregates (*Streptococcus mutants*) and grooves (*Pseudomonas aeruginosa*)	Yoda and others (2004); Cui and others (2012)
	LPS	Binding and neutralization of LPS with inhibitory effect on LPS binding to cells	Cranberry, tea, and grapes proanthocyanidins interaction with Gram-negative bacteria	Delehanty and others (2007)
		Binding of LPS through Lipid A component	Cranberry, grape juice, green tea proanthocyanidins interaction with LPS	Johnson and others (2008)
	Fimbriae	Attachment inhibition	Purified proanthocyanidins extracted from cranberry inhibited the adherence of P-fimbriated *Escherichia coli* to cell surfaces	Howell and Vorsa (1998)
Interaction with the cell membrane	Bilayer	Irreversible changes in membrane properties and membrane integrity	Tea polyphenols produce cell membrane permeability increasing, disruption of cell membrane and releasing of small cellular molecules in *Serratia marcescens*	Yi and others (2014)
			Gallic acid and ferulic acid compromise the integrity of cytoplasm membrane of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogenes* with leakage of cellular constituents	Borges and others (2013)
Prevention and control of the biofilm	Adhesines	Inhibition of bacterial attachment	Gallic acid inhibited the adhesion of *P. aeruginosa*, *L. monocytogenes* and *S. aureus*; ferulic acid inhibited the adhesion of *P. aeruginosa* and *S. aureus*	Borges and others (2012)
	Polyphenols – Quorum sensing	Inhibit biofilm maturation	Gallic acid caused total inhibition of swimming (*L. monocytogenes*) and swarming (*L. monocytogenes* and *E. coli*) motilities; Ferulic acid caused total inhibition of swimming (*L. monocytogenes*) and swarming (*L. monocytogenes* and *E. coli*) motilities	Borges and others (2012)
			Capparis spinosa methanolic extract inhibited swimming and swarming motility of *S. marcescens*, PAO1, *E. coli* and *P. mirabilis*	Abraham and others (2011)
			Rosa rugosa tea inhibited swarming motility and biofilm formation of *Chromobacterium violaceum 026*	Zhang and others (2014)
			Cranberry proanthocyanidins, in concetration of 100 μg/mL, limited motility, particular swarming motility, and reduced biofilm formation of *P. aeruginosa*, without down-regulated proteins related to flagella and type IV pili	Urley and others (2014)
			Quercetin significantly inhibited the swimming and swarming behavior of *P. aeruginosa* and *Y. enterocolitica*	Gopu and others (2015)
			Apigenin lower the amounts of extracellular alkali-soluble glucans, intracellular iodophilic polysaccharides and,fructans in *Streptococcus mutant* biofilm	Koo and others (2003)
			Capparis spinosa methanolic extract *inhibited* exopolysaccharide production in *Escherichia coli*, *Proteus mirabilis*, *Serratia marcescens* and PAO1	Abraham and others (2011)

(Continued)

Table 4–Continued.

Antibacterial activity mechanism	Polyphenols interaction site	Consequence	Example	Sources
			Quercetin inhibited exopolysaccharide production in *K. pneumoniae*, *P. aeruginosa*,and *Y. enterocolitica*	Gopu and others (2015)
		Inhibit bacterial sporulation	Ferulic acid completly inhibit colony spreading of *S. aureus*	Borges and others (2012)
Microbial enzyme inhibition and substrate deprivation	Microbial enzymes	Decrease cyclic-di-AMP levels	Tannic acid, theaflavin-3′-gallate and theaflavin-3,3′-digallate inhibited *B. subtilis* cyclic di-AMP synthase	Opoku-Temeng and Sintim (2016)
		Decrease enzyme activity	Fumarate reductase flavoprotein, dihydroorotate dehydrogenase, dihydrofolate reductase, NADH-dependentenoyl ACP-reductase, DNA gyrase subunit from *E. coli* are target for 19 flavonoids	Xiao and others (2014)
			Hyaluronic acid-splitting enzymes Hyal B – from *Streptococcus agalactiae* – was inhibited by flavonoids	Hertel and others (2006)
			Licochalcone A and C from the roots of *Glycyrrhiza inflata* inhibited the electron transport chain of the bacterial respiration chain	Haraguchi and others (1998)
			Tannins inhibiting the oxidative phosphorylation	Scalbert (1991)
			Tannins inhibited NADH-ubiquinone-1-oxidoreductase activity of *Paracoccus denitrificans*, *Bacillus subtilis*, *Photobacterium phosphoreum*, and *Thermus thermophilus HB-8*	Konishi and others (1993)
Protein regulation	Repression/ stimulation of the bacterial genes	Down-regulated proteins	Treatament of *P. aeruginosa* with cranberry proanthocyanidins down-regulated 2 proteins implicated in ATP sinthesis, a cythocrome C (PA2482), hypotetical protein PA2481 and proteins involved in DNA and RNA synthesis (Top A, Rp1C, and Mfd), and acid cycle proteins (subunitis of acetyl-CoA carboxylase and fumarase)	Ulrey and others (2014)
			Apple flavonoid phloretin control *E. coli* O157:H7 biofilm formation by repressing curli genes csgA and csgB	Lee and others (2011)
		Up-regulated proteins	Treatament of *P. aeruginosa* with cranberry proanthocyanidins up-regulated 12 proteins related to iron siderophores or cation transporters (such as PchD, PvdN, PhuS), 5 proteines involved in amino acids synthesis (such as PA0335, PA2044, HutG), proteins involved in response to stress (such as OsmC, SodM), and a hypothetical protein involved in flavonoid metabolism (PA3450)	Ulrey and others (2014)
Metal iron deprivation due to chelating ability	Ionic iron	Iron immobilization	Gallotanins from mango (*Mangifera indica*) exhibit antibacterial activity against Gram-positive food spoilage bacteria and Gram-negative *E. coli*, due their ability to chelate iron	Engels and others (2009
			Parsley (*Petroselinum crispum*) and cilantro (*Coriandrum sativum*) polyphenols rich extracts inhibited *Bacillus subtilis* and *Escherichia coli* due to ferrous sequestering activity	Wong and Kitts (2006)
			Cranberry proanthocyanidins lower the abundance of cythocromes in *P. aeruginosa.*	Ulrley and others (2014)

Conclusions

Commercially available polyphenols and plant extracts rich in polyphenols might be useful as antioxidants and antimicrobial agents for extending the shelf-life of meat and meat products. Polyphenols can inhibit the oxidative degradation of lipids and proteins, thus maintaining the odor and improving the color stability of meat. The ability of polyphenols to interact with a variety of structural units of spoilage and foodborne bacteria, and to alter their metabolism and quorum sensing, makes them promising antimicrobial agents for meat and meat products. Geographical area

from which the plants are collected can influence the composition in polyphenols and implicitly their antioxidant and antimicrobial activity represents a barrier to the possibility of retrieving on the market and the immediate use of polyphenolic extracts in the meat industry. Further studies are now needed to clarify the synergistic/antagonistic action of various polyphenols, and to identify the best polyphenols classes, concentrations and conditions of use, so as not to negatively affect the quality parameters of meat products as well as consumer acceptability.

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Comprehensive REVIEWS in Food Science and Food Safety

Mechanisms of Oxidative Processes in Meat and Toxicity Induced by Postprandial Degradation Products: A Review

Camelia Papuc, Gheorghe V. Goran, Corina N. Predescu, and Valentin Nicorescu

Abstract: Antioxidant system loss after slaughtering, reactive species production, cell disruption, contact with oxygen and light, heme and nonheme iron, and irradiation starts up mainly by 2 related oxidative processes: lipid peroxidation and protein oxidation. Products generated in these processes are responsible for meat quality loss, and some of them are suspected to be toxic to humans. This review article is focused on reactive species implicated in oxidative processes in meat, on lipid peroxidation mechanisms, heme protein, and nonheme protein oxidation, and on some toxic oxidation and digestion products. Nonenzymatic fatty acid peroxidation is exemplified by an arachidonic acyl group, and the initiation of chain reaction can be described by 3 pathways: singlet oxygen, hydroxyl radical from the Fenton reaction, and perferrylmyoglobin. Enzymatic oxidation of fatty acids is exemplified using linoleic acid, and the main characteristics of lipoxygenase are also presented. Heme protein oxidation is described in an interrelation with lipid peroxidation and the significance for food quality is shown. For protein oxidation, 3 different mechanism types are described: oxidation of amino acid residues, oxidation of protein backbone, and reactions of proteins with carbonyl compounds from lipid peroxidation. The effects of oxidative damage on protein properties and bioavailability are also shown. At the end of each oxidative process, the postprandial toxicity induced by oxidation products and the dietary degradation products are presented. Also discussed are reports by some researchers who suggest that dietary lipid and protein oxidation products and heme iron from red meat are in part cytotoxic and/or genotoxic.

Keywords: cytotoxicity, genotoxicity, lipids, myoglobin, protein

Introduction

Meat is traditionally the skeletal muscle of ruminants, nonruminants, and poultry. Meat is a protein- and lipid-rich food that is highly perishable due to chemical and enzymatic processes occurring during cutting, mincing, irradiation, handling, packaging, storage, and cooking. Deterioration of lipids and proteins in the meat determines the development of off-odors, off-tastes, color change, and compounds of possible toxicity, which all make meat undesirable for human consumption. Production of reactive chemical species, disruption of cells, contact with oxygen and light, heme and nonheme iron presence, and gamma-irradiation can all lead to oxidative deterioration of meat and off-flavor development, initiating lipid peroxidation by a free radical chain reaction. Many of the lipid peroxidation products are volatile compounds such as aldehydes, which are responsible for off-odor (Ahn and Kim 1998; Ahn and Lee 2002), off-flavor (Dietze and others 2007), rancidity (Ahn 2002; Byrne and others 2002; Campo and others 2006), and meat color change (Guillen and Guzman 1998). Oxidation of lipids, especially of unsaturated fatty acids (PUFAs), can occur in both fresh and cooked types of meat (Min and Ahn 2005; Jo and others 2006), and it gives rise to toxic products (Kubow 1992; Esterbauer 1993). A large number of studies have suggested a link between end products of lipid peroxidation and various health conditions including atherosclerosis, neurodegenerative diseases, and cancer. The oxidation of ferrous-oxymyoglobin (Fe^{2+}) to ferric-metmyoglobin (Fe^{3+}) occurs in the presence of some reactive species (RS) and produces discoloration of meat and initiation of lipid peroxidation. Superoxide anion and hydrogen peroxide produced during oxymyoglobin oxidation can react to release the most aggressive RS, hydroxyl radical, which can initiate oxidation of other molecules such as lipids and proteins (Chaijan 2008). Protein oxidation is also considered as an important oxidative process that can cause changes in meat quality. Meat is a concentrated source of high-quality and highly digestible proteins, and it is an important source of essential amino acids. Oxidation of the meat proteins occurs at the side chain of amino acids and also of the protein backbone. Oxidation of meat proteins affects digestibility, decreases nutritional value due to oxidation of essential amino acids, and increases the risk of some diseases. In this review the basics of RS' oxidative activity, lipid and protein oxidation

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Table 1–Half-life and 1-electron reduction potential of selected reactive species.

Oxidant			
Name	Redox couple (1-electron reduction)	Half-life[a] (s)	Reduction potential[b] (E^0,V)
		Reactive oxygen species (ROS)	
Free radicals			
Superoxide radical	$O_2^{\bullet-}$, $2H^+/H_2O_2$	$>1 \times 10^{-6}$	0.94
Hydroxyl radical	HO•, $2H^+/H_2O$	$>1 \times 10^{-9}$	2.31
Perhydroxyl radical	HOO•, $2H^+/H_2O_2$	1–30	1.06
Peroxyl radical	ROO•, $2H^+$/ROOH	$>1 \times 10^{-2}$	1.00
Alkoxy radical	RO•, $2H^+$/ROH	$>1 \times 10^{-6}$	1.60
Nonradicals			
Molecular oxygen	$O_2/O_2\bullet^-$	$>1 \times 10^{-6}$	0.65
Singlet oxygen	$^1O_2$	$>1 \times 10^{-6}$	
Ozon	O_3, $2H^+/H_2O+O_2$	9×10^3	1.80
Hydrogen peroxide	H_2O_2, $2H^+/2H_2O$	10	1.77
Hypochlorous acid	HOCl, $2H^+/Cl^-$, H_2O		1.28
		Reactive nitrogen species (RNS)	
Free radicals			
Nitric oxide	NO•/NO^-	1–30	−0.80
Nitrogen dioxide	$NO_2\bullet/NO_2^-$	35 h	1.04
Nonradicals			
Peroxynitrite anion	$ONOO^-/NO_2\bullet$ $ONOO^-/NO_2^-$	$10–20 \times 10^{-3}$	1.40 1.20
		Intermediates of lipid and protein oxidation	
Free radicals			
PUFA• bis-allylic-radical	PUFA•, H^+/PUFAH	–	0.6
RS•	RS•/RS^-	–	0.92
Perferrylmyoglobin	•MbFe(IV) =O/MbFe(III)	30	–
Nonradicals			
Ferrylmyoglobin	MbFe(IV) = O/MbFe(III)	13 × 60	0.85
Transition metal ions			
Ferrous ion	Fe^{3+}(EDTA)/Fe^{2+}(EDTA)		0.12

[a]Data for half-life collected from Ostdal and others (1999), Giorgio and others (2007), and Bekhit and others (2013).
[b]Data for reduction potential collected from Koppenol and Liebman (1984), Koppenol (1990), Buettner (1993), Giorgio and others (2007), Davies and others (2008), and Winterbourn (2008).

mechanisms, and the toxicity of some products generated in the mentioned oxidative processes are presented.

Oxidative Processes: RS

Oxidative processes are chemical/biochemical reactions in which loss of electrons or hydrogen atoms or gain of oxygen occurs. In contrast, reduction processes are chemical/biochemical reactions in which gain of electrons or hydrogen atoms or loss of oxygen occurs. Because an electron donor does not exist without an electron acceptor, the oxidizing agent and the reducing agent form a redox couple. In a redox couple, the oxidant is reduced and the reductant is oxidized. The understanding of the redox reactions in biological systems is central to understanding the effects of the RSs and also the effects of the antioxidants (Kohen and Nyska 2002). In the biological systems, the term oxidant is replaced with pro-oxidant and the term reductant with antioxidant (Prior and Cao 1999; Kohen and Nyska 2002). Generally, pro-oxidants are referred to as species that promote oxidation, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), intermediates of lipid and protein oxidations, and transition metal ions (for example, ferrous ion). The oxidizing strength of an RS is given by its one-electron reduction potential (E^0) and the instability described by the half-life. E^0 is a measure of the tendency of an RS to acquire electrons and so the higher values of E^0 correspond to more potent oxidants (Table 1). The stability of an RS is reflected in its half-life, which means the time required for the amount of RS to fall to half its initial value. RSs with high half-life have high stability. Thus, it is not surprising that HO• with reduction potential 2.31 V and half life of about 1 ns, at pH 7.4 and 37 °C, is the most aggressive RS (Table 1). RSs are represented by free radicals, nonradicals, and metal ions with high reduction potential. A free radical could be an atom, a group of atoms, or a molecule that contains one or more unpaired electrons and exists for a brief period of time before reacting to be transformed into a stable species (Halliwell and Gutteridge 1990; Halliwell and Chirico 1993). An unpaired electron is an electron that singly occupies an orbital of an atom and is represented by a dot (•) in the chemical formula. Due to its unpaired electron existence, which tends to couple with another electron, a free radical is generally unstable and so is more reactive than a nonradical (Halliwell 1994; El-Beltagi and Mohamed 2013).

ROS

ROS are oxygen-centered free radicals and reactive molecules containing oxygen (Turrens 2003). Gutowski and Kowalkzyk (2013) describe oxygen-centered free radicals as containing an unpaired electron on the oxygen atom and 2 unpaired electrons in separate orbitals of its outer layer. Due to the higher reactivity, a free radical is able to abstract an electron from a neighboring molecule or cellular structure generating a new highly reactive free radical and a stable compound with 2 paired electrons. This new generated radical returns to a state with low energy by abstracting an electron from another molecule or cellular structure, and so on (Valko and others 2006). In a biological context, ROS represents the most abundant class of radical species produced by organisms (Miller and Aust 1988; Halliwell 2006). ROS were shown to

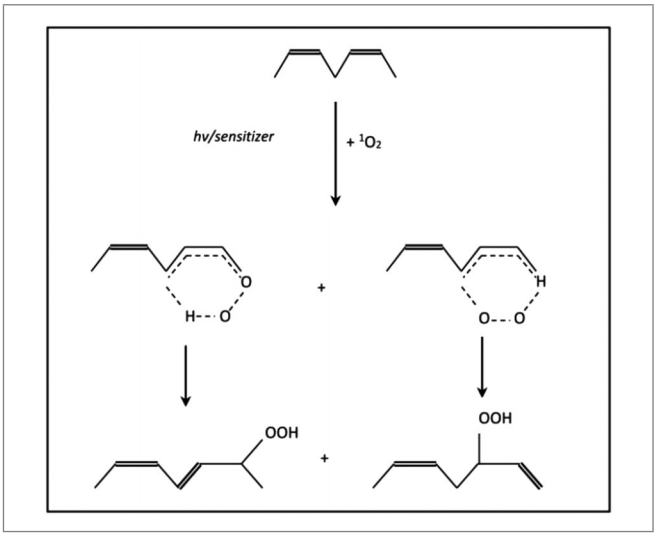

Figure 1–Conjugated and unconjugated peroxide formation from a diene fatty acid.

be produced as natural byproducts during mitochondrial respiration, the phagocyte-mediated killing of pathogens, and xenobiotic metabolism (Augusto and Miyamoto 2011).

Molecular oxygen (O_2••) and singlet oxygen ($^1O_2$)

Molecular oxygen in its ground state is a bi-radical, containing 2 unpaired electrons occupying 2 "degenerate" molecular orbitals. Because the spin of these electrons has 3 possible alignments to an external field, this configuration is referred to as "triplet oxygen." Because the electrons of the molecule are in their lowest possible energy configuration, molecular oxygen is not very reactive. Many biochemical changes occur postmortem and antemortem during the conversion of muscle in muscle food (Greaser 1986). Postmortem, the loss of the cellular integrity with time causes several physicochemical changes that affect meat stability to oxidation, such as swelling and breakage of mitochondria, which is accompanied by changes in the oxygen consumption rate (Tang and others 2005). The accessibility of oxygen to unsaturated fatty acids in membranes and the production of free radical intermediates will help accelerate the oxidative processes (Faustman and others 2010). When the 2 unpaired electrons from triplet oxygen pair up into 2 different orbitals, it results in a powerful oxidant named singlet oxygen ($^1\Delta_g$). This resulting species is very reactive because the 2 electrons with opposing spins can readily react with electron-rich molecules, such as unsaturated molecules (Turrens 2003). Photosensitizers (porphyrins, riboflavin, and myoglobin) present in raw meat, after absorption of radiant energy, especially light, induce the conversion of triplet oxygen into singlet oxygen (Afonso and others 1999; Min and Boff 2002). The reaction of the singlet oxygen with PUFAs may produce unsaturated hydroperoxides with conjugated and unconjugated double bonds (Figure 1). Min and Boff (2002) suggested that the reaction speed of singlet oxygen with PUFAs increases with unsaturation degree and is not dependent on double bond positions, such as conjugated or unconjugated.

Singlet oxygen also reacts with proteins. Very susceptible to oxidation are proteins with amino acid residues with high electron density, such as cysteine, methionine, tryptophan, tyrosine, and histidine (Davies 2012).

Superoxide radical ($O_2^{\bullet-}$) is a highly reactive compound produced when oxygen is reduced by a single electron enzymatically or nonenzymatically. In the muscle tissue, many components of electron transport chain mitochondria, such as NADPH-dependent dehydrogenase and ubiquinone, leak electrons to oxygen prematurely, forming the primary source of $O_2^{\bullet-}$ in tissues. The conversion of xanthine dehydrogenase to xanthine oxidase (Thomas 1995) and the autoxidation of hem proteins are other major sources of superoxide radical of importance (Baron and Andersen 2002). Superoxide anion is the precursor for many RS and mediates oxidation *via* an initial abstraction of labile hydrogen atoms (Turrens 2003). The most important reaction of superoxide anion is dismutation. In this reaction a superoxide radical reacts with another superoxide radical to produce hydrogen peroxide (H_2O_2), which can be reduced to water or partially reduced to the extremely reactive hydroxyl radical (HO•). The dismutation of superoxide radical may be either spontaneous or catalyzed by enzymes named superoxide dismutases. The formation of HO• is possible by H_2O_2 decomposition, catalyzed by transition metal ions in the lower valence state, such as Fe^{2+} or Cu^{+} (Fenton reactions), or by H_2O_2 reaction with superoxide radical (Haber–Weiss reaction). The oxidized transitional metals, which result from the Fenton reaction may be re-reduced by $O_2^{\bullet-}$ (Sayre and others 2005) (Figure 2).

Hydroperoxyl radical or perhydroxyl radical ($HO_2^{\bullet}$). Superoxide radical forms hydroperoxyl radical ($HO_2^{\bullet}$) at low pH (Bielski and Cabelli 1995; Halliwell and Gutteridge 1999). This is produced by protonation of superoxide radical and is more reactive than

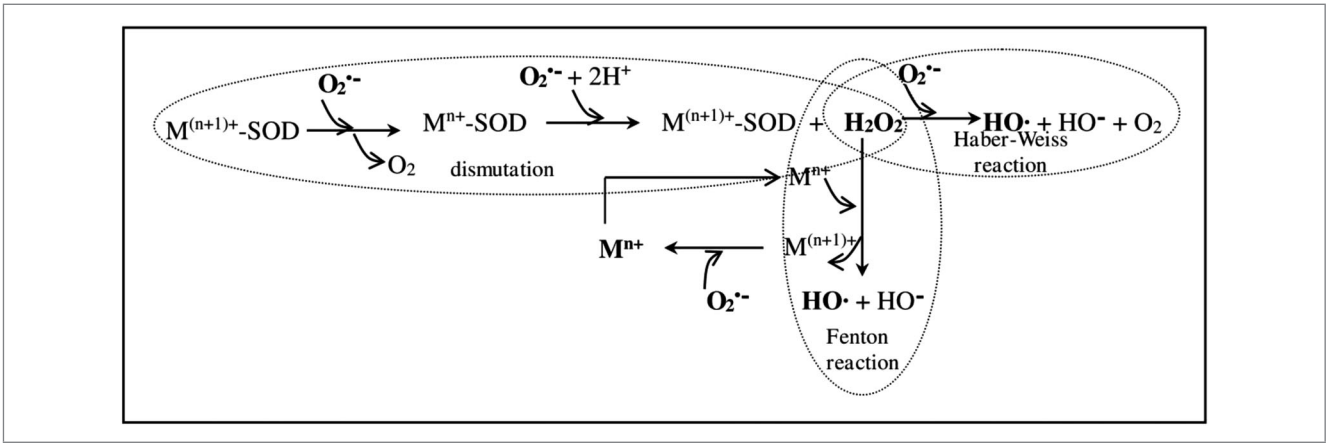

Figure 2–Superoxide anion precursor for hydrogen peroxide, hydroxyl radical, and reduced transitional metal.

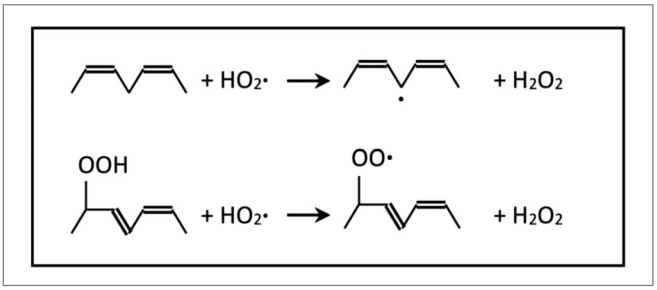

Figure 3–Initiation and stimulation of peroxidation chain reaction by hydroperoxyl radical.

superoxide radical itself (Bielski and others 1985). Less than 1% from superoxide radical produced exists in protonated form postmortem under physiological conditions (pH 7.4). After postrigor, lactic acid builds up in the muscle tissue and the pH decreases to 5.5 to 6.0 and so 10% to 20% of total $O_2^{\bullet-}$ reacts with protons and forms $HO_2^{\bullet}$ (Min and Ahn 2005). Superoxide, due to its charge, cannot pass through membranes, but hydroperoxyl radical is uncharged and it permeates into the membrane phospholipid bilayer. In the membrane, the hydroperoxyl radical can abstract a hydrogen atom from the *bis*-allylic position of a phospholipid polyunsaturated acyl radical, thereby initiating the lipid peroxidation chain (Bielski and others 1983; Choe and Min 2006). In addition, hydroperoxyl radical can stimulate the lipid peroxidation by reaction with preexisting lipid hydroperoxides to generate peroxyl radicals and hydrogen peroxide (Schaur and others 2015) (Figure 3). The hydroperoxyl radical can oxidize in one electron step other molecules (such as NADH and glyceraldehydes-3-phosphate dehydrogenase-NADH) by abstracting a hydrogen atom to form H_2O_2 (Bekhit and others 2013).

Hydroxyl radical (HO•) is the most reactive ROS formed *in vivo*. It has a half-life of 10^{-9} to 10^{-10} s (Table 1). HO• can be formed *in vivo* or *in situ* by a number of processes such as Fenton reaction (the major route), Haber–Weiss reaction, the microsomal ethanol oxidizing system (Candeias and others 1993), the reaction between the superoxide anion radical and hypochlorous acid (Folkes and others 1995), homolytic fission of water molecules (ionizing radiation) (Swallow 1984), ultraviolet radiation (Floyd and others 1988), and sonolysis of water (ultrasound) (Henglein and Kormann 1985). In addition, it can be produced by the decomposition of ozone under aqueous conditions. *In vivo*, the concentration of hydroxyl radical is zero due to its very high reduction potential. In a living cell, at the site of the formation, this powerful oxidant reacts with all surrounding molecules, such as lipids, proteins, DNA, phospholipids, sugars, and vitamins with a speed higher than the speed of the generation reaction (Min and Ahn 2005). Upon slaughter, the drop of the pH, myofibril fragmentation, and loss of cofactors compromise the cell integrity and the mechanisms that control the metal ions become nonoperant (Bekhit and others 2013). This suggests that the HO• concentration will eventually be high postmortem because the scavenging power of the antioxidant systems decreases and oxidation of lipid and proteins initiated by this powerful radical will affect the meat quality. This radical can also oxidize all amino acid residues of proteins. In biological systems, for both HO• and $^1O_2$, proteins are the major targets, consuming 65% to 70% of the available oxidants (Davies and others 2008; Pattison and others 2009; Davies 2012).

Hydrogen peroxide (H_2O_2). Under physiological conditions, hydrogen peroxide is present in aerobic cells at low levels as a result of some metabolic processes. Thus, it is produced *in vivo* by nonenzymatic and enzymatic dismutation of superoxide radical, by a range of NADH-oxidase enzymes, which should regenerate NAD^+ from NADH, accompanied by the production of hydrogen peroxide from oxygen, as well as by the peroxisomal pathway for β-oxidation of fatty acids (Chance and others 1979; Halliwell and Gutteridge 1999). The H_2O_2 is poorly reactive and cannot readily oxidize most lipids, proteins, and nucleic acids. The danger of H_2O_2 is represented by its conversion to the hydroxyl radical (HO•), the most powerful free radical, either by UV-induced homolytic fission or by interaction with transition metal ions in the lower valence state (Fenton reaction) (Halliwell 2000; Choe and Min 2006).

Ozone O_3. Ozone is formed from O_2 by the action of high-energy electromagnetic radiations and also by electrical discharges. Ozone is a little less reactive than HO• and a much more powerful oxidant, compared to oxygen, and can produce oxidative damage in many biological molecules, such as proteins, lipids, vitamins, and nucleic acids. The reaction of O_3 with aliphatic amino acids forms nitrate, ammonia, monocarbonyl, dicarbonyl, and carboxylic byproducts. In the ozonolysis of peptides and proteins, tyrosine, tryptophan, histidine, cysteine, and methionine residues are more susceptible to oxidation (Sharma and Graham 2010). The systematic studies undertaken on prosthetic groups isolated from hemoglobin, on isolated hemoglobin/methemoglobin, and on whole blood have shown that ozone action specifically directed at the prosthetic group of hemoglobin causing the disruption of heme into oxidized degradation products (Cataldo 2006). Because O_3 has a high oxidation potential, it has been shown to have a biocidal activity in red meat (Greer and Jones 1989), poultry carcass, and chill water (Sheldon and Brown 1986). However, despite biocide activity, the use of O_3 as a decontaminant for meat is not recommended because it can affect the quality of the meat. Studies undertaken to evaluate the effect of gaseous ozone treatment on beef carcass bacterial spoilage correlated to the meat quality demonstrated that ozone prevents bacterial growth, but darkens muscle color (Greer and Jones 1989). With unsaturated fatty acids, ozone reacts quickly and forms a variety of chemical species (for example, carbonyl compounds, Criegee ozonides, and aliphatic radicals) depending on the environment in which the reactions are taking place (Pryor 1993). Figure 4 shows the reaction of the 9-cis-octadecenoic acid with ozone to form aliphatic radicals and carbonyl compounds, as well as routes of trioxolanes and Criegee ozonides formation. Ozone also attacks other unsaturated compounds and produces free radicals capable of promoting lipid peroxidation.

Additionally, ozone oxidizes glutathione (GSH) to its disulfide (G–S–S–G) in a 2-electron process and can also react with thiols to produce compounds containing sulfur in its higher valence states (Pryor 1993).

Alkoxy radical (RO•) and peroxyl radical (ROO•) are carbon-centered radicals and good oxidizing agents since they have a tendency to accept electrons and thereby undergo reduction themselves, having highly positive reduction potential values (1000 to 1600 mV) (Buettner 1993; Gutowski and Kowalkzyk 2013). They can be generated by organic hydroperoxide (ROOH) decomposition induced by heat, radiation, or reaction with transition metal ions and other oxidants capable of abstracting hydrogen. In addition, biomolecule-derived ROO• and RO• can be generated from lipids, proteins, and nucleic acid oxidation (Augusto and Miyamoto 2011). These carbon-centered radicals react directly with certain biological molecules including DNA

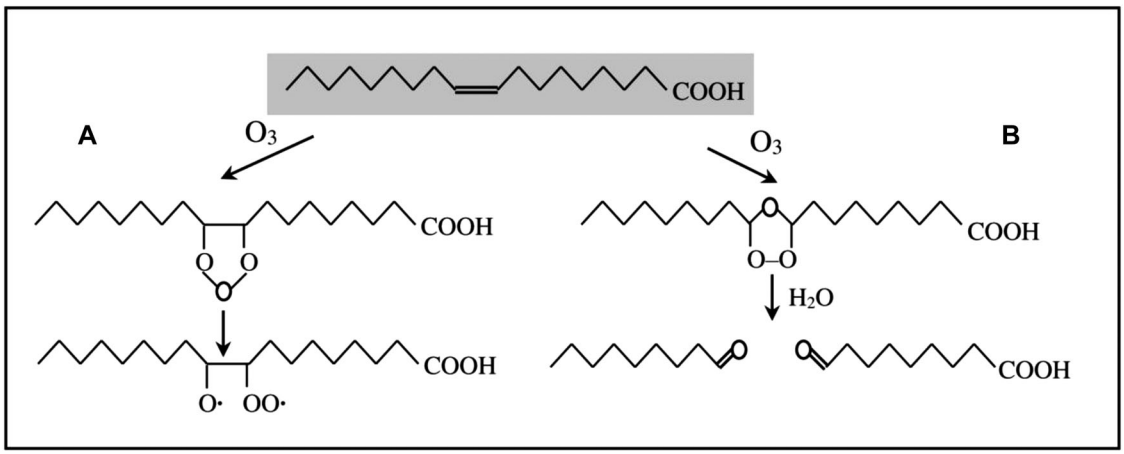

Figure 4–The reaction of 9-cis-octadecenoic acid with ozone. (A) Formation of trioxolane and diradical. (B) Formation of Criegee ozonide and carbonyl compounds.

and albumin -SH-groups. Bailey and others (2004) demonstrated that RO_2•derived from azo-initiators can induce peroxidation of lipids.

RNS

Nitric oxide (NO•) is a RNS produced in the cell from L-arginine by the action of different forms of nitric oxide synthases (NOSs) that specifically catalyze the oxidation of the substrate to N^G-hydroxy-L-arginine or to L-citrulline and NO• (Rosen and others 2002). Excess production of NO• by inducible NOSs accelerates RNS production, such as very unstable peroxynitrite anion ($ONOO^-$), which can oxidize sulfhydryls, nitrosate proteins, and nucleic acids, and cause lipid peroxidation (Radi and others 1991a, 1991b; DeFeudis 1992; Yermilov and others 1995). Studies conducted on muscle food showed that nitric oxide synthases are active only for several days of postmortem storage (Brannan and Decker 2002). Cottrell and others (2015) postulated that preslaughter events, which affect NO• production, can influence meat tenderness, potentially *via* altered muscle metabolism or the regulatory effects on the protein calpain and cathepsins. Mukherjee (2013) suggested the NO• formation by L-arginine oxidation with hydrogen peroxide as an important source of NO•. The redox activity of NO• is controversial because some researchers reported it as an initiator in chain reactions in specific conditions, while others reported it as an inhibitor. For example, Darley-Usmar and others (1992) reported that the simultaneous production of superoxide anion and nitric oxide can initiate lipid peroxidation in low-density lipoprotein. Osipov and others (2007) postulated that NO• reacts with other radicals, such as hydroxyl radical, alkoxy radicals, and peroxyl radicals, interrupting radical chain reactions. Under special conditions, NO• interacts with other free radicals to generate less reactive compounds. In reaction with HO• a nitrite anion is generated:

$$NO\cdot + HO\cdot \rightarrow NO_2^- + H^+$$

In the reaction with peroxyl radical, nitric oxide forms LOONO, followed by its decomposition to an alkoxy radical and NO_2. In a 2nd reaction, the newly generated alkoxy radical is scavenged by a 2nd molecule of nitric oxide (O'Donnell and others 1997):

$$NO\cdot + ROO\cdot \rightarrow ROONO \rightarrow RO\cdot + NO_2$$

$$NO\cdot + RO\cdot \rightarrow ROOR$$

Because nitric oxide is able to scavenge peroxyl and alkoxyl radicals, many researchers have suggested that nitric oxide may be used to control the lipid peroxidation processes in meat (Hogg and others 1993; Hogg and Kalyanaraman 1999).

NO• can bind to transitional metals, and this aspect is very important because the binding to ferrous heme complex decreases its probability of producing hydroxyl radicals in the Fenton reaction (Decatur and others 1996; Powers and others 2011). The interaction of NO• with heme-containing proteins may occur in 2 ways: (1) producing a heme iron-nitrosyl complex, or (2) participating in redox reactions, mostly as a reductant. NO• can interact with hypervalent states of hemoglobin and myoglobin (containing Fe IV and Fe V), reducing them to methemoglobin and metmyoglobin (containing Fe III) without producing nitrosyl complexes. Because NO• reduces the hypervalent states of hemoglobin and myoglobin, it is considered a veritable antioxidant and may be used to manipulate meat quality (Gorbunov and others 1995; Osipov and others 2007).

Peroxynitrite radical (ONO_2•) and peroxynitrite anion (ONO_2^-). Nitric oxide may be oxidized in 2 routes: (1) reaction with oxygen to form the peroxynitrite (nitrosyldioxyl) radical, and (2) reaction with superoxide anion to form peroxynitrite anion. Peroxynitrite anion is very unstable and acts as an oxidant and nitrating agent against many cell molecules, including DNA and proteins.

$$O=NOO\cdot \xleftarrow{O_2} NO\cdot \xrightarrow{O_2^-} O=NOO^-$$

In postmortem muscle, ONO_2^- can produce inactivation of complex I of mitochondria due to the disorders induced by S-nitrosation or Fe-nitrosylation of mitochondrial components (Orrenius and others 2007), which probably increases hydrogen peroxide production (Bekhit and others 2013). Peroxynitrite anion is suspected of accelerating meat spoilage by 2 mechanisms: (1) oxidation of ferrous ion hem containing globin proteins, leading to a color change from red to brown, and (2) peroxidation of

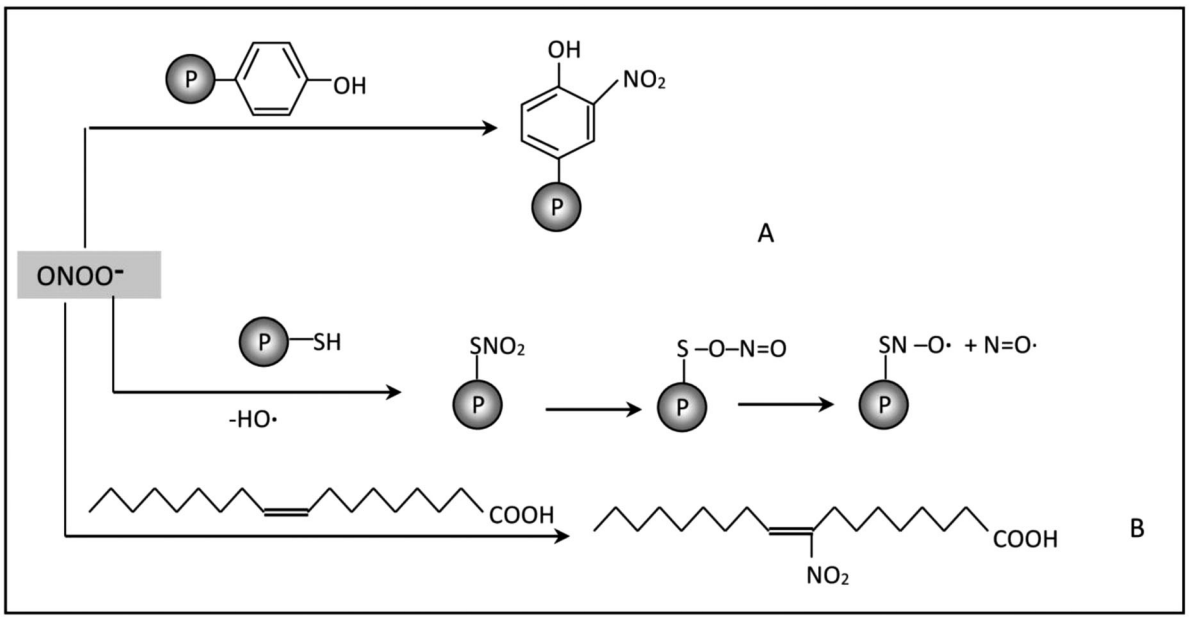

Figure 5–Interaction of peroxynitrite anion with proteins and fatty acids. (A) Protein tyrosine and protein thiol nitration. (B) Nitration of 9-cis-octadecenoic acid. P, protein residue.

unsaturated lipids, leading to flavor degradation (Vasilescu and others 2014). The interaction between peroxynitrite anion and oxyhemoglobin/deoxyhemoglobin and myoglobin results in the formation of methemoglobin and metmyoglobin (Zinchuk and Stepuro 2006). The interaction of $ONOO^-$ with proteins and lipids can induce nitrosation and oxidation of some biomolecules. Therefore, this RNS species can cause nitrosation of aromatic amino acid residues and oxidation of sulfur-containing amino acid residues, with a low probability of generating carbonyl proteins (Szabó and others 1996). Violi and others (1999) demonstrated that nitrosation of lipids induced by $OONO^-$ leads to the formation of nitrito-, nitro-, nitrosoperoxo-, and/or nitrated lipid oxidation adducts. Peroxynitrite can diffuse from extra- to intracellular compartments by 2 mechanisms in the anionic and protonated forms, causing intracellular nitration, such as nitration of hemoglobin (Denicola and others 1996, 1998). By nonenzymatic reactions, peroxynitrite anion produces, in tissues, nitration of aromatic amino acids (tyrosine and tryptophan), oxidation, nitration, and nitrosation of thiol-containing amino acid residues, and nitration of fatty acids (Kamat 2006). In Figure 5 the interactions of peroxynitrite anion with proteins and fatty acids are illustrated. Connolly and others (2002) studied the ability of peroxynitrite to induce the oxidation of myoglobin under conditions that might be expected in muscle foods, and they reported that peroxynitrite is able to induce the conversion of oxymyoglobin to metmyoglobin, and that carbon dioxide induces, to a small extent, the oxidant ability of peroxynitrite. The ability of peroxynitrite to convert myoglobin to metmyoglobin is higher in postmortem storage because the reactivity of this RNS increases when muscle pH decreases (Connolly and others 2002; Connolly and Decker 2004).

Lipid and Protein Oxidative Products

Hydroperoxides (LOOH)

Hydroperoxides are the primary products of unsaturated phospholipids, glycolipids, and cholesterol in cell membranes' oxidation. They are nonvolatile, odorless, and relatively unstable compounds. They are decomposed in unimolecular and bimolecular reactions into different free radicals able to react with other molecules or to be split off into compounds with varying flavor thresholds and molecular weights (Fennema 1999). Decomposition of hydroperoxides occurs by thermal-, photo-, or transition metal-catalyzed reactions, resulting in hydroxyl- and alkoxy radicals (Geoffroy and others 2000), which are highly reactive and may act as initiators of lipid peroxidation (Bondet and others 2000):

$$LOOH \xrightarrow{thermal/UV} HO\cdot + LO\cdot$$

$$Me^{n+} + LOOH \rightarrow LO\cdot + Me^{(n+1)+} + HO^-$$

$$Me^{(n+1)+} + LOOH \rightarrow LOO\cdot + Me^{n+} + H^+$$

Perferrylmyoglobin (•*MbFe(IV) = O*)

Metmyoglobin reacts with H_2O_2 by direct transfer of 2 oxidation equivalents from H_2O_2 to metmyoglobin, resulting in a hypervalent myoglobin species named perferrylmyoglobin (•MbFe(IV) = O) (Davies and others 1990; Davies 1991; Irwin and others 1999). Perferrylmyoglobin has a very short half-life and is rapidly autoreduced to the more stable ferrylmyoglobin (Baron and Andersen 2002) as follows:

$$MbFe\,(III) + H_2O_2 \rightarrow \cdot MbFe\,(IV) = O \rightarrow MbFe\,(IV) = O$$

Perferrylmyoglobin has pro-oxidative activities in lipid and protein systems. It can initiate lipid peroxidation by abstracting a hydrogen atom from the *bis*-allylic position of a polyunsaturated fatty acid (LH), as shown in the following reaction (Kanner and Harel 1985):

$$\cdot MbFe\,(IV) = O + LH \rightarrow MbFe\,(IV) = O + L\cdot + H^+$$

In protein systems •MbFe(IV) = O is able to induce intra- and intermolecular protein cross-linking, *via* tyrosine residue/(s) from globin, and to generate protein radicals with long half-lives, *via*

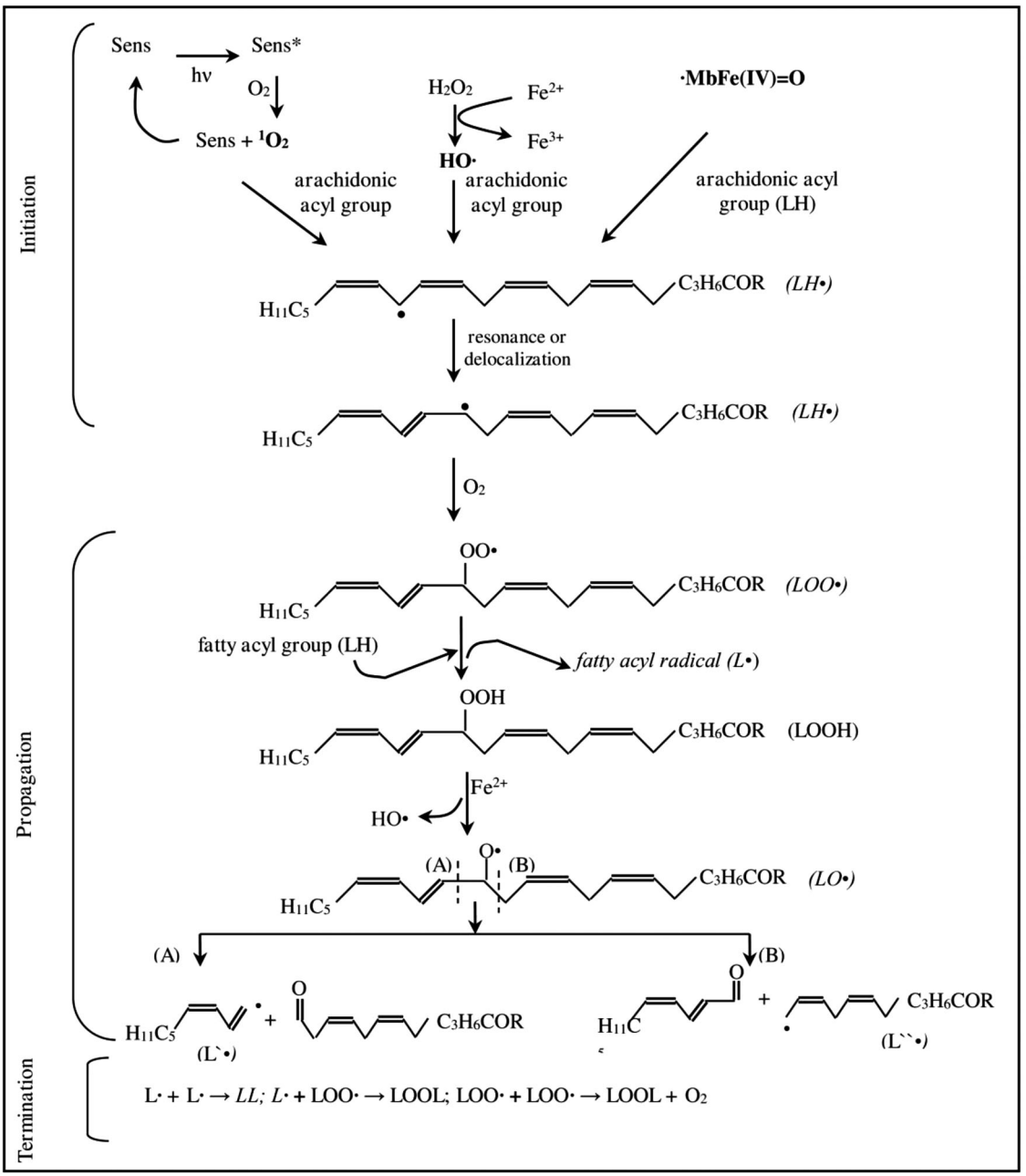

Figure 6–Arachidonic acyl group peroxidation mechanism. Initiation of lipid-derived radical chain reactions through different pathways, singlet oxygen, hydroxyl radical from Fenton reaction, and perferrylmyoglobin.

transfer of its radical to other protein molecules able to generate a new oxidation reaction (Baron and Andersen 2002).

Ferrylmyoglobin (MbFe(IV) = O)

Ferrylmyoglobin formation in muscle tissues is induced by the interactions of H_2O_2 with metmyoglobin and perferrylmyoglobin with unsaturated fatty acids (Chaijan 2008). Ferrylmyoglobin may be converted into metmyoglobin by an autoreducing reaction or in the presence of reducing agents present in muscle tissue, such as glutathione and ascorbate. The rate of autoreduction is pH-dependent. At physiological pH it is slowly reduced back to metmyoglobin (Mikkelsen and Skibsted 1995), but under the conditions found in fresh meat (pH 5.5 to 5.8), it is rapidly autoreduced to metmyoglobin (Chaijan 2008). Ferrylmyoglobin can abstract a hydrogen atom from a *bis*-allylic carbon of a PUFA-initiating lipid oxidation (Baron and Andersen 2002), or it can decompose LOOH to alkoxy or peroxyl radicals, which can undergo a new chain reaction or may be decomposed into secondary products of lipid peroxidation (Reeder and Wilson 1998, 2001).

Transition Metals

Transition metals with 2 or more valence states exert a pro-oxidant activity in meat. The main transition metal existing in skeletal muscle tissue is iron, one of the strongest pro-oxidants. Red meat is a rich source of heme iron and nonheme iron that may initiate lipid peroxidation via HO• generated in the Fenton

reaction (Ahn and Kim 1998). During processing, meat transition metals may enter into meat due to the contact of meat with metal equipment surfaces or ingredients (Faustman and others 2010). Two main oxidative mechanisms are described for the pro-oxidant activity of transition metals: (1) the reaction of ferrous iron with H_2O_2 to generate the highly oxidizing hydroxyl radical, and (2) decomposition of lipid hydroperoxides in free radicals able to initiate or propagate lipid peroxidation (Faustman and others 2010). The redox state of transition metals in the exhibit of pro-oxidant activity is more important than the concentration. Therefore, ferrous ion (Fe^{2+}) is a powerful pro-oxidant, compared with ferric ion (Fe^{3+}), which exhibits the pro-oxidant activity in the presence of a reducing agent (for example, ascorbic acid) (Aruoma and others 1989; Halliwell and Gutteridge 1990). Both ferric and ferrous ions may be catalysts in the breakdown of lipid hydroperoxides to form hydroperoxide-derived free radicals, but the catalytic activity of ferrous ion is significantly higher than the catalytic activity of ferric ion (O'Brien 1969); moreover, the alkoxy radical is more reactive with the abstraction of a labile hydrogen atom than the peroxyl radical (Min and Ahn 2005).

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO\cdot + HO^{-}$$

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO\cdot + H^{+}$$

In raw and cooked meat, iron ion released from hem may be a strong catalyst in lipid peroxidation (Min and Ahn 2005). Low pH enhances the ability of transition metals to be active in lipid peroxidation and promotes the release of iron ion from heme (Reeder and Wilson 2001) and, in addition, at low pH there is also a greater likelihood of hemoglobin subunits to dissociate and thus lose the heme (Kristinsson and Hultin 2004).

Lipid Oxidation

Nonenzymatic fatty acid peroxidation

Lipid peroxidation along with other postmortem oxidative processes is decisive for meat quality. Lipid peroxidation affects raw meat under 3 headings: (1) safety—secondary lipid peroxidation products expected to be toxic for human; (2) bioavailability—oxidation of essential fatty acids which will decrease nutritional value; and (3) sensory quality—flavor and color changed in an undesirable manner, as well as appearance of a typical odor and brown color. Contact with molecular oxygen, light, myoglobin presence, and the absence of a vitamin defense mechanism represents a favorable condition for the development of lipid peroxidation in meat. The high tendency of unsaturated fatty acids, especially polyunsaturated fatty acids, to rapidly oxidize is important in further biochemical changes that have undesirable effects, such as rancidity and color deterioration (Wood and others 2003). The unsaturated fatty acyl group of phospholipids from muscle fiber membranes (sarcolemma, mitochondria, and sarcoplasmic reticulum) is the 1st to be oxidized (Morrissey and others 1998; Shleikin and Medvedev 2014). Turkki and Campbell (1967) have studied the distribution of phospholipids in 2 beef muscles and suggested that in both muscles lecithin accounted for about 62%, cephalins for 30%, and sphingomyelins for less than 10%. The polyunsaturated fatty acid/saturated fatty acid ratio (PUFAs/SFAs) in food muscle is different in ruminants and nonruminants. Enser and others (1996) reported a PUFAs/SFAs ratio of 0.58 in pork, 0.11 in beef, and 0.15 in lamb. In addition, the ratio PUFAs (n-6)/PUFAs (n-3) is lower in loin muscle of ruminants (2.1 in beef and 1.3 in lamb) than in nonruminants (7.2 in pork) (Enser and others 1996). In beef, lamb, and pork loin steaks, purchased from 4 supermarkets, linoleic acid (18:n-6) was the major PUFA, 2.4%, 2.7%, and 14.2%, respectively, and arachidonic acid was the major fatty acid in the C20-C22 PUFAs, 0.63%, 0.64%, and 2.21%, respectively (Enser and others 1996). Due to the presence in high concentration in raw meat, free and esterified arachidonic and linoleic acids are the most important components of lipoproteins and the membrane bilayer, which may be oxidized after slaughtering during cutting, mincing, irradiation, packaging, and storage. After slaughter, various changes promote lipid oxidation accompanied by flavor modification, for example, pH decline, the release of iron from heme pigments, and the inactivation of some antioxidant enzymes (Morrissey and others 1998). Because unsaturated fatty acid levels are higher in pork, compared to the ruminants, the generation of lipid oxidation products leading to off-odors, off-flavors, and color changes is higher in pork (Enser and others 1996; Wood and others 2003). Nonenzymatic lipid peroxidation is a free radical chain reaction which consists of 3 major steps: initiation, propagation, and termination. Initiation starts by the attack of any RS that has a high enough one-electron reduction potential (E^0) to abstract a highly reactive hydrogen atom from a lipid molecule to form a lipid radical. The most reactive hydrogen atoms in lipids are those found in the methylene group attached to 2 carbon–carbon double bonds, named *bis*-allylic position (Blanksby and Ellison 2003). Blanksby and Ellison (2003) estimated the relative bond strengths of the various R-H bonds in arachidonic acid and found the following relationship between bond enthalpy (DH_{298}) and R-H bond position in the acid: DH_{298} (carboxylic acid, O-H) = 112 > DH_{298} (vinyl, C-H) = 111 > DH_{298} (methylene, C-H) = 99 > DH_{298} (α-carbonyl, C-H) = 94 > DH_{298} (allylic, C-H) = 88 kcal mol^{-1} > DH_{298} (*bis*-allylic, C-H) = 80 kcal/mol. The relationship found for the bond enthalpy estimates emphasizes that the hydrogen atoms most susceptible to abstraction are those in the allylic positions. Min and Ahn (2005) suggest that the rate of lipid radicals' formation increases exponentially with the number of allylic groups and is not dependent on the lipid chain length. The most notable initiators in lipid oxidation are ROS, especially HO•. Venkataraman and others (2004) estimated that the initiator in lipid peroxidation may be a free radical that has a one-electron reduction potential greater than 0.6 V. This would include HO• (the most potent radical), alkoxy (RO•), peroxyl (ROO•), perhydroxyl (HOO•), and nitrogen dioxide (NO_2•) radicals, but not the nitric oxide (NO•) radical. An alternative to initiation by free radical with reduction potential greater than 0.6 V is the initiation with iron in the form of iron-oxygen complexes, such as perferryl ion or ferryl ion. Whereas the ratio $[O_2]/[H_2O_2]$ under physiological conditions is high, approximately 10^3, the very low quantity of HO• produced by the reaction of iron with preexisting H_2O_2 made from HO• elicited from this reaction is an insignificant initiator of chain reaction in lipid peroxidation. Some researchers claim that the major initiators of biological free radical oxidations are iron-oxygen complexes, which are oxidizing species produced in the reaction of loosely bound Fe^{2+} with dioxygen molecule (Qian and Buettner 1999; Schafer and others 2000). Schafer and others (2000) used enriched leukemia cells (K-562 and L1210 cells) with docosahexaenoic acid (DHA) to study iron-mediated lipid peroxidation by 2 routes: (1) *via* iron-oxygen complexes, and (2) *via* Fenton reaction. The results obtained made them propose that the main route for the initiation of the free-radical chain in lipid oxidation is *via* iron-oxygen complexes. In addition, H_2O_2 can mediate the degradation of heme proteins to release free iron from the prosthetic group or to convert heme iron to perferryl

Table 2–Lipid and protein oxidation products in meat.

Compound classes	Examples	*References*
Lipid oxidation products		
Nonenzymatic fatty acids peroxidation		
Hydroperoxides	Allyl hydroperoxides	*Bielski and others* (1983); Choe and Min (2006)
Carbonyls (ketones and aldehydes)	Propanal, pentenal, malondialdehyde (MDA), hexanal, 2-hexenal, 4-heptenal, 2,4-heptadienal, 1,5-octadien-3-one, 2-nonenal, 2,6-nonadienal, 4-hydroxy-2-trans-nonenal (HNE), and 2,4,7-decatrienal	Hsieh and Kinsella (1989); *Frankel* (1993); *Min and Ahn* (2005)
Alcohols	1-Octen-3-ol, 1,5-octadien-3-ol, 2,5-octadien-1-ol	*Hsieh and Kinsella* (1989); *Frankel* (1993); *Min and Ahn* (2005)
Hydrocarbons (alkanes, alkenes, dienes)	Hexane, octane, pentadecane, 1-butene, 2-butene, 1-hexene, 1-heptene, 1-octene, 1-nonene, tetradecene, heptadecene and hexadecadiene	*Bielski and others* (1983); *Schreiber and others* (1994); *Du and others* (2002)
Furans	2-Penthyl furan	*Bielski and others* (1983); *Ruiz and others* (1999)
Enzymatic fatty acids peroxidation		
Stereoisomeric fatty acid hydroperoxides	15(S)15-Hydroperoxyicosa-5, 8, 11, 13-tetraenoate (5Z, 8Z, 11Z, 13 E) (15-HPETE), and 13(S)13-hydroperoxyoctadeca-9, 11-dienoate (9Z, 11E) (13-HPODE)	*Ivanov and others* (2010); *El-Beltagi and Mohamed* (2013)
Non-enzymatic oxidation of cholesterol		
Cholesterol hydroperoxides	5-, 6- and 7-Hydroperoxycholesterols	*Choe and Min* (2006); *Orczewska-Dudek and others* (2012)
Hydroxycholesterols	7α-Hydroxycholesterol, 7β-hydroxycholesterol, 20α-hydroxycholesterol, and 25-hydroxycholesterol	*Orczewska-Dudek and others* (2012)
Ketocholesterols	7-Ketocholesterol	*Pie and others* (1991); *Novelli and others* (1998); Orczewska-Dudek and others (2012)
Epoxycholesterols	5,6-Epoxycholesterol	*Orczewska-Dudek and others* (2012)
Enzymatic oxidation of cholesterol		
Oxysterols	7α-Hydroxycholesterol, 20-α-hydroxycholesterol, 22R-hydroxycholesterol, 22S-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, (25R)-26-hydroxycholesterol, and 27-hydroxycholesterol	*Mariutti and others* (2008); *Brown* (2009)
Protein oxidation products		
Heme proteins oxidation		
Heme proteins with Fe(III)	Metmyoglobin	*Mancini and Hunt* (2005); *Alderton and others* (2003); *Bekhit and Faustman* (2005)
Oxidation at the side chain of amino acids residues		
Sulfur-containig amino acid residues oxidation products	Cysteine sulfenic acid, cysteine sulfinic acid, cysteine sulfonic acid, methionine sulfoxide, methionine sulfone, dimethyl trisulfide, and cross linked myofibrillar proteins by disulfide bonds	Patterson and Stevenson (1995);*Vogt* (1995); *Claiborne and others* (2003); *Rowe and others* (2004); *Turell and others* (2008); *Xiong and others* (2009); *Lund and others* (2011); *Zakrys-Waliwander and others* (2012)
Tyrosine residues oxidation products	Cross-linked proteins by dityrosine	*Morzel and others* (2006); *Zhang and others* (2013)
Tryptophan residues oxidation products	Kynurenine and N-formylkynurenine	*Friedman and Cuq* (1988)
Histidine residues oxidation products	2-Oxo-histidine, asparagine, and aspartic acid residues	*Stadtman and Berlett* (1997); *Kowalik-Jankowska and others* (2004)
Lysine residues oxidation products	α-Aminoadipic semialdehyde (AAS) and α-aminoadipic acid (AAA), and Schiff bases	*Utrera and others* (2012)
Oxidation of protein backbone		
Protein oxidation products	Backbone protein hydroperoxide, intra- or intermolecular cross-linked proteins, and imines	*Davies* (2012); *Stadtman and Berlett* (1997); *Soladoye and others* (2015); *Davies* (2016)
Protein fragmentation products	Substituted amides, protein carbonyl derivatives, and peptides	*Schuessler and Schilling* (1984); *Berlett and Stadtman* (1997)
Condensation reaction between proteins and aldehydes resulted from lipid oxidation		
Protein oxidation products	Intra- or inter-cross-linked proteins, and Schiff bases	*Szweda and others* (1993); *Uchida and others* (1994); *Shen and others* (1996); *Refsgaard and others* (2000)

Table 3–Lipid and protein oxidation products in meat, and their effects on sensory properties and acceptability of meat.

Compound classes	Effects on sensory properties and acceptability of meat	*References*
	Lipid oxidation products	
Hydroperoxides	Decrease nutritional value due to essential fatty acids oxidation	*Enser and others* (1996); *Wood and others* (2003);
Carbonyls	Off-odor, off-flavor, rancidity, meat color change	*Ahn and others* (2000); *Guillen and Guzman* (1998); *Schreiber and others* (1994); *Ahn and Lee* (2002); *Byrne and others* (2002); *Campo and others* (2006); *Dietze and others* (2007)
Hydrocarbons and alcohols	Undesirable flavor	*Schreiber and others* (1994)
Cholesterol oxidative products	Decrease nutritional value of meat due to the implications of COPs in the modulation of lipid metabolic processes	*Sasaki and others* (2010); *Boselli and others* (2012)
	Protein oxidation products	
Metmyoglobin	Meat discoloration	*Mancini and Hunt* (2005); *Suman and others* (2014); *Wongwichian and others* (2015)
Oxidized proteins	Decrease digestibility, nutritional value, tenderness, and juiciness	*Pattison and Davies* (2001); *Rowe and others* (2004); *Huff-Lonergan and Lonergan* (2005); *Pattison and others* (2012); *Zhang and others* (2013); *Soladoye and others* (2015)
Sulfur volatile compounds resulted from amino acid residues (dimethyl trisulfide, carbon disulfide)	Off-odor	*Patterson and Stevenson* (1995); *Ahn* (2002)

radical, which is rapidly reduced to ferryl radical (Baron and Andersen 2002). More studies were undertaken to determine in which form iron is responsible for the catalysis of the lipid peroxidation in meat and meat products. In experimental models with various meat species, such as beef and fish, many researchers have indicated that iron from meat heme proteins plays a crucial role in catalysis of lipid peroxidation (Rhee and Ziprin 1987; Min and Ahn 2005; Min and others 2008; Gheisari and others 2010; Shleikin and Medvedev 2014). Rhee and Ziprin (1987) studied lipid peroxidation, myoglobin concentration, and total pigment in pork, beef, and chicken raw muscles during 2 to 6 d of storage and they concluded that the lipid peroxidation intensity was dependent on animal species and muscle type. Rhee and Ziprin (1987) showed that beef was more susceptible to lipid peroxidation than pork and chicken muscles and suggested a relationship between myoglobin concentration/total pigment and the intensity of the lipid peroxidation process in the stored muscles among the 3 species. Grunwald and Richards (2006) have studied the mechanism of heme protein-mediated lipid oxidation in raw and heated washed muscle using experimental models that involved hemoglobin and myoglobin variants and have concluded that released hemin is of primary importance in promoting lipid oxidation. PUFAs from meat and meat products may be easily oxidized following exposure to light in the presence of oxygen and a photosensitizer, due to activation of diatomic oxygen to singlet oxygen. Singlet oxygen may be an initiator of lipid peroxidation due to its ability to abstract labile hydrogen from the *bis*-allylic group (Kubow 1992). Therefore, the main pathways to free radical chain reaction initiation in lipid peroxidation are the hydroxyl radical, iron-oxygen complexes, and singlet oxygen. Figure 6 shows the peroxidation of the arachidonic acyl group (LH) from a phospholipid initiated by singlet oxygen, hydroxyl radical, and perferrylmyoglobin. In the propagation step, fatty acid radical, which resulted from the initiation step, reacts with dioxygen to form a hydroperoxy radical. The resulting radical can abstract a hydrogen atom from another unsaturated fatty acyl group (LH) and produce a new lipid radical (L•) and fatty acyl hydroperoxide (the 11-hydroperoxide of the arachidonic acyl group, in our case), as shown in Figure 6. In the presence of nonheme Fe^{2+}, lipid hydroperoxide is decomposed to an alkoxy radical by the homolytic cleavage of the covalent bond between oxygen atoms from the hydroperoxide group, which has lower bond energy (44 kcal/mol) than the bond between oxygen and hydrogen of hydroperoxide (90 kcal/mol) (Hiatt and others 1968). The resulting alkoxy radical is cleaved by homolytic β-scission of the carbon–carbon bond on either side of the carbon atom connected to oxygen (A and B positions in Figure 6) and gives rise to a huge range of volatile and nonvolatile compounds such as carbonyls (for example, ketones and aldehydes), alcohols, hydrocarbons (for example, alkane, alkene, dienes), and furans which all contribute to an undesirable flavor in raw meat (Table 2 and 3). It was suggested that hydrogen from the *bis*-allylic methylene group is required for the reaction between an unsaturated fatty acid and a hydroperoxy radical (Bielski and others 1983; Schreiber and others 1994; Ruiz and others 1999; Du and others 2002; Min and Ahn 2005). Reaction rate constants of hydroperoxy radical with linoleic, linolenic, and arachidonic acids are 1.2×10^3, 1.7×10^3, and 3.1×10^3 M^{-1}/s, respectively, which shows that the increases in the numbers of *bis*-allylic methylene groups increase the reaction rate (Bielski and others 1983; Aikens and Dix 1991; Min and Ahn 2005; Choe and Min 2006).

Oleic acid, an acid without a *bis*-allylic methylene group, did not react with hydroperoxy radical (Bielski and others 1983). The peroxyl radicals and alkoxy radicals are the major propagators of lipid peroxidation. Because in PUFAs there is always more than one *bis*-allylic position, it is possible for more than one lipid-free radical to be formed upon initiation; the same goes for many peroxyl and alkoxy radicals and many primary and secondary peroxidation products. The last step of the lipid peroxidation is the termination process in which the radicals L•, LO•, and LOO• react with each other or with other free radicals to form nonradical stable products. Due to the structural diversity, PUFA peroxidation can generate a huge number of different secondary peroxidation products that cause rancidity and other off-flavors. Thus, n-6 fatty acid peroxidation leads to hexanal, 2-octenal, l-octen-3-ol, 2-nonenal, and 4-hydroxy-2-trans-nonenal (HNE), while n-3 fatty acids lead to propanal, 2-pentenal, 2-hexenal, 4-heptenal, 2,4-heptadienal, 2,4,7-decatrienal, l,5-octadien-3-ol,

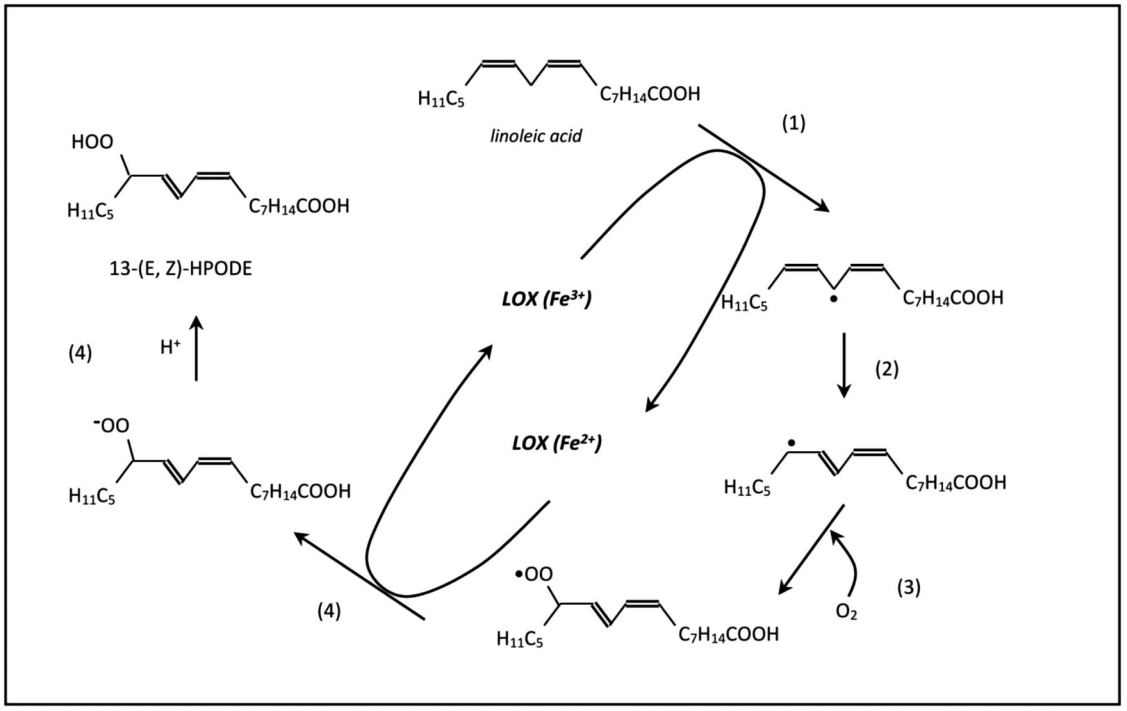

Figure 7–Formation of 13-(E, Z)-HPODE by the action of lipoxygenase: (1) abstraction of a labile hydrogen from a *bis*-allylic methylene group and reduction of Fe^{3+} ion bound enzyme to Fe^{2+}; (2) delocalization of a double bond and formation of a conjugated diene; (3) dioxygenation of the lipid radical and formation of peroxy radical; (4) peroxy radical reduction *via* Fe^{2+} bound enzyme and the protonation of the peroxy anion.

2,5-octadien-l-ol, l,5-octadien-3-one, and 2,6-nonadienal (Hsieh and Kinsella 1989; Frankel 1993; Min and Ahn 2005) (Table 2).

Enzymatic oxidation of fatty acids

Lipoxygenases (LOXs) are iron-containing oxidases (Kuhn and others 2005) that catalyze the stereo-specific dioxygenation of PUFAs containing at least one 1-cis, 4-cis-pentadiene system (Ivanov and others 2010), such as linoleic, linolenic, or arachidonic acid. Eicosapentaenoic acid (EPA) and DHA can also be dioxygenated by lipoxygenases to form corresponding hydroperoxides (Wang and others 1991). The conventional nomenclature classifies animal LOXs considering the specific positions at which they produce dioxygenation of arachidonic acids, such as 5-LOX, 8-LOX, 11-LOX, 12-LOX, or 15-LOX (Ivanov and others 2010). For example, 15-LOX catalyzes the oxidation of arachidonate in position 15 to give 15(S)15-hydroperoxyeicosa-5,8,11,13-tetraenoate (5Z, 8Z, 11Z, 13E) (15-HPETE) and linoleate to give 13(S)13-hydroperoxyoctadeca-9, 11-dienoate (9Z, 11E) (13-HPODE) (Table 2). Enzymatic oxidation of linoleates gives exclusively 13-HPODE; while the free radical-mediated oxidation gives 4 racemic products (El-Beltagi and Mohamed 2013). Oxygenation of fatty acids *via* lipoxygenase consists of 4 consecutive elementary reactions: (1) abstraction of a labile hydrogen from a *bis*-allylic methylene group and reduction of Fe^{3+} ion-bound enzyme to Fe^{2+}; (2) delocalization of a double bond and formation of a conjugated diene—during this elementary reaction, the radical electron is dislocated either in the direction of the methyl end of the fatty acid ([+2] rearrangement) or in the direction of the carboxylate ([−2] rearrangement) (Ivanov and others 2010); (3) dioxygenation of the lipid radical and formation of peroxy radical; and (4) peroxy radical reduction *via* Fe^{2+}-bound enzyme and the protonation of the peroxy anion (Figure 7).

Several researchers have reported the presence of a lipoxygenase in microsomal fractions isolated from beef, pork, and turkey muscle, which can explain the oxidative instability of muscle tissues (Kanner and Harel 1985; Grossman and others 1988; Gata and others 1996). Grossman and others (1988) demonstrated 15-LOX activity in chicken muscle by the examination of [C^{14}] arachidonic acid oxidation products. 15-LOX partially purified by affinity chromatography by Grossman and others (1988) was stable during frozen storage at −20 °C for 12 mo. After this storage period, the enzymes regain, in part, the enzymatic activity to induce a slight decrease in substrate (cis, cis-1,4-pentadiene fatty acids) concentration. The results published by Grossman and others (1988) suggest that 15-LOX may catalyze the oxidation of cis, cis-1,4-pentadiene fatty acids from chicken meat during frozen storage. Min and others (2008) have studied the susceptibility of meats from different animal species—chicken (breast and thigh), pork, and beef—to lipid oxidation and found that, after 7 d of storage, only in raw beef were TBARS values increased significantly, which suggest that lipid oxidation is stimulated by high iron levels and high lipoxygenase-like activities. Disruption of muscle cells, inactivating antioxidant systems, degradation of heme accompanied by iron liberation, the formation of ferrylmyoglobin by the interaction of myoglobin with H_2O_2, and HO• production by the Fenton reaction generate PUFA oxidation in a similar manner as does lipoxygenase (Min and others 2008). Min and others (2008) reported a higher lipoxygenase-like activity in raw beef loin and chicken thigh than in chicken breast and pork loin, and they suggested that lipoxygenase-like activity is highly related to heme iron content in meat. Gata and others (1996) purified

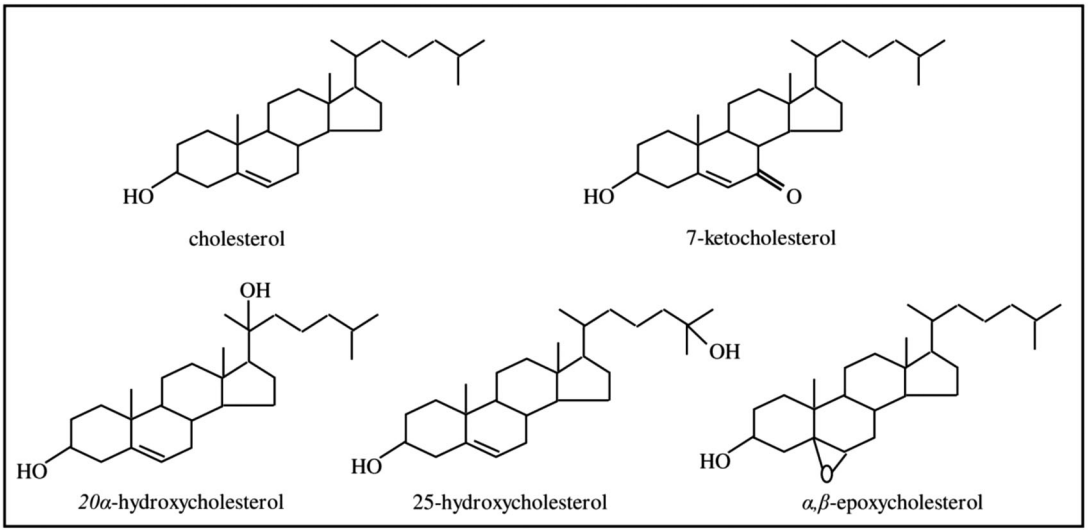

Figure 8–The structure of cholesterol and the main autoxidation products present in muscle food.

and characterized the lipoxygenase from Iberian pig muscle and concluded that the enzyme had a high affinity for linoleic acid as a substrate. The incubation of purified enzyme with the linoleic acid generated 2 main reaction products, identified by direct-phase HPLC: 9-hydroperoxy octadecadienoic acid and 13-hydroperoxy octadecadienoic acid in the ratio 45:55.

Cholesterol Oxidation

Nonenzymatic oxidation of cholesterol

Cholesterol (5α-cholesten-3β-ol) is a component of cell membranes. Many researchers have reported cholesterol oxidation under certain conditions, such as high temperature, long-term storage, electromagnetic radiation exposure (Boselli and others 2010, 2012; Cardenia and others 2012, 2013). Cholesterol oxidation products (COPs) can be formed by nonenzymatic oxidation (autoxidation) or by enzymatic reactions. The cholesterol in a membrane environment may be attacked more readily than PUFAs by ROS. The main autoxidation cholesterol products present in food muscle are 7-ketocholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol, and α,β-epoxycholesterol (Orczewska-Dudek and others 2012) (Table 2). Figure 8 shows the structure of cholesterol and the main autoxidation products present in food muscle tissue. The enzymatic oxidation of cholesterol is carried out by several enzymes that are mainly from the cytochrome P450 family of oxygenases. Cholesterol autoxidation is started frequently at the methylene group from the allylic position (C-7) by a free radical to give a cholesteryl radical, which reacts rapidly with dioxygen to give 7α- or 7β- peroxyl radical. The formed radical stabilizes, abstracting a hydrogen from the allylic carbon of another lipid molecule forming primary COPs, which are isomers of 7-hydroperoxycholesterol (7α- or 7β-OOHCh). These 7-hydroperoxycholesterols can further convert into 7α-hydroxycholesterol (7α-OHCh) and 7β-hydroxycholesterol (7β-OHCh), which are commonly found in food. Formation of 5,6α-epoxycholesterol and 5,6β-epoxycholesterol in minced meat were reported during meat refrigeration at 4°C in the presence of oxygen and it is supposed to be due to the interaction between cholesterol and some ROS, such as singlet oxygen and lipid peroxy radical (Orczewska-Dudek and others 2012). COPs are detected at trace levels in fresh meat, but during storage and after irradiations, COP levels increase because the generation of free radicals also increases, due to PUFA oxidation (Hwang and Maerker 1993b; Lee and others 2001). Xu and others (2011) investigated interactions of stearic, oleic, linoleic, and α-linolenic acids during oxidation of cholesterol, and they concluded that the surrounding fatty acids affect cholesterol oxidation. They reported that the fatty acids accelerated the oxidation of cholesterol for the first 60 min at 180 °C and then their pro-oxidant activity became weaker, excepting α-linolenic acid which exerted an inhibitory effect after 120 min. Hwang and Maerker (1993a) found increased COPs levels in stored beef, pork, and veal after γ-irradiation with a dose of 10 kGy. During storage, COPs increased significantly in chicken meat with storage time, and the presence of long-chain PUFAs accelerated cholesterol oxidation (Li and others 1996). Excessive oxidation of cholesterol in food muscle affects the nutritional quality due to the implications of COPs in the modulation of lipid metabolic processes, such as linoleic acid desaturation and cholesterol metabolism (Sasaki and others 2010; Boselli and others 2012), but it can be prevented using adequate packaging systems that eliminate exposure to oxygen and light (Orczewska-Dudek and others 2012).

Because 7-ketocholesterol is found in relatively high concentrations in raw meat stored by freezing (Pie and others 1991; Novelli and others 1998), and its content is matrix-dependent (Lercker and Rodriguez-Estrada 2000), it has been proposed as a marker of cholesterol oxidation (Park and Addis 1985). In the last few years, researchers have reported the presence of COPs in fresh meat and have suggested that transparent films used in packing meat do not prevent cholesterol oxidation because they are permeable for light and oxygen, and the cholesterol oxidation process in meat is promoted by hem protein photocatalysis (Rao and others 1996; Lercker and Rodriguez-Estrada 2000).

Enzymatic oxidation of cholesterol

Some enzymes, such as monooxygenase, dioxygenase, dehydrogenase, and oxidases, oxidize cholesterol to oxysterols. The COPs, 7α-hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol, 20α-hydroxycholesterol, (25R)-26-hydroxycholesterol, 22R-hydroxycholesterol, and 27-hydroxycholesterol, are produced by the enzymatic oxidation of cholesterol

(Brown 2009). Mariutti and others (2008) have reported the presence of 22R-hydroxycholesterol, 24S-hydroxycholesterol, and 22S-hydroxycholesterol in chicken meat (Table 2). The main lipid oxidation products identified in meat and their effects on sensory properties and acceptability of meat are summarized in Table 3.

Postprandial Lipid Peroxidation Product-Induced Toxicity

The toxic effects of dietary lipid peroxidation products have attracted much interest in the last several years (Kubow 1990, 1992; Frankel 1993; Kanner 1994, 2007; Miller and others 1998; Halliwell 2000, 2002; Macovei and others 2015). The primary peroxidation product-induced toxicity is considerably lower than secondary peroxidation product-induced toxicity (Kubow 1992). The toxicity of lipid peroxides is controversial. Bergan and Draper (1970) suggested a low absorption of lipid hydroperoxides in the gut and also some alterations before and during the absorptive process. Kowalski and others (1990) suggest that exogenous reduced glutathione (GSH) plays an important role in the protection against absorption of lipid peroxides in the small intestine. GSH seems to be taken up by the intestinal brush border membrane and used in intracellular reactions to metabolize the peroxidized lipids ingested, and thereby prevents their transport to the contra-luminal side (Kubow 1992). Earles and others (1991) have reported that 13-hydroxyoctadecadienoic aid (13-HODE), an oxidation product generated in the enzymatic oxidation of linoleic acid, could be oxidized by an NAD^+-dependent dehydrogenase, present in the rat colon mucosa, to produce 2,4-dienone and 13-oxooctadecadienoic acid. The formation in the colon of the dienone may be a protective mechanism because it is much less reactive and, in this way, the oxidized fatty acid is protected from the lipid autoxidative cascade (Kubow 1992). Kanazawa and Ashida (1998) have reported that the absorption in the body of the linoleic acid hydroperoxides (LA-OOHs) is dose-dependent. At small intragastric doses (6.5 mumol), LA-OOHs were not transported to the intestine, but at large doses (200 or 800 mumol) there was a partial leakage of LA-OOHs to the intestine. Thirty minutes after treatment, the composition of products found in the gastric lumen was: 27% unchanged LA-OOHs, 9.7% epoxyketones, 3.5% hydroxyls (LA-OHs), 2.4% decomposed aldehydes, and 13% unknown products; 25% was incorporated into the gastric tissue, and the other 6.4% was traced in the intestinal lumen and tissue as aldehydes, which resulted from decomposition of the LA-OOHs. Kanazawa and Ashida (1998) suggested that LA-OOHs are decomposed to aldehydes in the stomach, which are then partly absorbed by the body. Several researchers have advanced the idea that oxidized lipids from food promote atherosclerosis risk because after intestinal absorption they are incorporated into the very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and chylomicrons and are transported *via* the lymphatic system to the blood (Staprans and others 1993, 1994, 1996; Palinski and others 1994; Steinberg 1997) (Table 4). Khan-Merchant and others (2002) evaluated the effect of dietary oxidized fatty acids on atherosclerotic lesion development and plasma cholesterol concentration in mice, and they suggested that oxidized fatty acids promote atherosclerosis only in the presence of dietary cholesterol in LDL receptor knockout mice. The role of lipid hydroperoxides in mediating cellular and molecular events involved in some degenerative intestinal disorders, with particular emphasis on colorectal cancer, was also investigated. The primary autoxidation products of PUFAs were reported to stimulate DNA synthesis and to induce ornithine decarboxylase activity in the colonic mucosa, indicating enhancement of tumorigenesis (Bull and others 1984, 1988; Earles and others 1991) (Table 4). Studies on the relationship between lipid oxidation product uptake from food and cancer risk have advanced the hypothesis that DNA is susceptible to oxidation product action and peroxyl radicals are able to cause depurination, depyrimidination, and base modifications in DNA (Marquez-Ruiz and others 2008). These studies suggest that mutations at a higher frequency may contribute to the initial steps of tumorigenesis (Kanazawa and others 2002). The oxidation of unsaturated fatty acids in meat causes a significant production of dietary advanced lipid oxidation end products (ALEs). Studies related to the hypothesis that the ALEs are cytotoxic and highly mutagenic compounds show that they block the Watson–Crick base pairing region (De Bont and van Larebeke 2004). After digestion of oxidized food, a part of ALEs are absorbed into the lymph or directly into the bloodstream. ALEs from the diet absorbed in the small intestine, and then transported into circulation, appear to act as injurious chemical substances that activate an inflammatory response, which affects the circulatory system and various organs such as liver, kidneys, lungs, and the intestine itself (Kanner 2007). Kanazawa and others (1985) heated [^{14}C] linoleic acid to 37 °C for 1 wk and then separated from the mixture the linoleic acid, the hydroperoxide of linoleic acid, and the secondary products of peroxidation. After feeding Wistar rats, separately by gavage, with these peroxidation products, data showed significant increase of the radioactivity in their livers, moderate increase of liver enzyme activity in the serum, and a slight hepatocellular hypertrophy indicating damage to the hepatocytes. Experimental animal studies have also supported the hypothesis that ingestion of ALEs along with foods may lead to LDL oxidation *in vivo*, accompanied by an increase of atherogenicity and foam cell formation (Esterbauer 1993). Malondialdehyde (MDA), the major ALE that is produced from arachidonic acid and long-chain PUFAs through nonenzymatic and enzymatic processes, has received much attention because it is a bifunctional aldehyde that can react with many biomolecules, including DNA and proteins, to form a variety of adducts (Kubow 1990; Addis and Warner 1991). Several researchers have reported MDA mutagenicity and carcinogenicity (Basu and Marnett 1983; Draper and Hadley 1990; Niederhofer and others 2003). Exposure of red blood cells (RBCs) to exogenous MDA in a physiological environment induces early redox unbalance followed by a decrease of the oxyhemoglobin level, membrane structural and functional damages, and fragmentation and aggregation of fragments (Tesoriere and others 2002). Tesoriere and others (2002) have suggested that the exposure to MDA causes a quickly occurring oxidative stress in the cells and potentiates oxidative cascades on RBCs, causing their dysfunction. Oral toxicity studies after administering malondialdehyde to Swiss mice have shown increased incidence of total neoplasms and neoplastic lesions in the liver, in a dose-dependent relationship (Siu and Draper 1978). Malondialdehyde can react with DNA, resulting in deoxyguanosine (M1G), deoxyadenosine (M1A), and deoxycytidine (M1C) adducts, all suspected to be mutagenic in mammalian cells and carcinogenic in rat livers (Rajinder and others 2001). Marnett (1999) reported important tissue-to-tissue variations in the levels of M1G, and increases of M1G levels with age, and the content of unsaturated fatty acids in the diet. The polarity of COPs is generally higher than cholesterol, and for this reason the lymphatic absorption mechanism of dietary COPs could be similar to the mechanism involved in cholesterol

Table 4–Lipid and protein oxidation products in meat. Examples and consequences on human health.

Compound classes	Consequences on human health	*References*
	Lipid oxidation products	
Oxidized lipids	Promote atherosclerosis risk	*Staprans and others* (1993, 1994, 1996); *Palinski and others* (1994); *Steinberg* (1997); *Khan-Merchant and others* (2002)
Lipid hydroperoxides	Enhancement of tumorigenesis	*Bull and others* (1984, 1988); *Earles and others* (1991)
Advanced lipid oxidation end products (carbonyls, alcohols, hydrocarbons, and furans), especially MDA	Cytotoxic and highly mutagenic and carcinogenic compounds	*Basu and Marnett* (1983); *Draper and Hadley* (1990); *Earles and others* (1991); *Marnett* (1999); *Rajinder and others* (2001); *Kanazawa and others* (2002); *Niederhofer and others* (2003); *De Bont and van Larebeke* (2004); *Marquez-Ruiz and others* (2008)
	Activate inflammatory response	*Kanner* (2007)
	Damage to the hepatocytes	*Kanazawa and others* (1985)
	Increase of the atherogenicity and foam cell formation	*Esterbauer* (1993)
	Dysfunction on red blood cells	*Tesoriere and others* (2002)
Cholesterol oxidative products	Promoting atherosclerosis, cancer and damage to cell membranes	*Staprans and others* (1998); *Phillips and others* (2001); *Pietras and others* (2012)
	Proteins oxidation products	
Metmyoglobin	Heme released from heme proteins may be suspected of increasing the risk of colorectal cancer	*Cross and others* (2003); *Hebels and others* (2010); *Bastide and others* (2011); *Gilsing and others* (2013); *Jakszyn and others* (2013)
Digestion-resistant oxidized proteins	Affect colon epithelial renewal and homeostasis	*Kim and others* (2013)
	Intestinal tumorigenesis	*Le Leu and others* (2007)
Bacterial degradation compounds derived from digestion resistant oxidized proteins	*Ammonia* – involved in tumor promotion	*Visek* (1979); *Hughes and Rowland* (2000)
	Phenolics – reacts with nitrite-forming mutagenic p-diazoquinone and can also stimulate N-nitrosation of dimethylamine *via* production of p-nitrosophenol	*Yokoyama and Carlson* (1981)
	Acyclic amines – precursors of N-nitroso compounds, which are potentially carcinogenic	*Hughes and Rowland* (2000)
	Cyclic amines – role in the etiology of colon cancer in humans	*Ohgaki and others* (1991); *Delfino and others* (2000); *Tavan and others* (2002)
	N-nitroso compounds – carcinogenic and mutagenic effects	*Silvester and others* (1997); *Hughes and Rowland* (2000)
	Hydrogen sulfide – an irritant implicated in ulcerative colitis, suspected to be carcinogenic	*Moore and others* (1997); *Attene-Ramos and others* (2007)
Oxidized amino acids	*Ortho- and meta-tyrosine* – may be misincorporated into proteins in the place of phenylalanine forming damaged protein in mammalian cells	*Rodgers and others* (2002); *Gurer-Orhan and others* (2006); *Klipcan and others* (2009); *Dunlop and others* (2013)
	Aminoadipic acid (AAA) – toxic effects on retinal glial cells and cerebral astrocytes	*Ishikawa and Mine* (1983); *Brown and Kretzschmar* (1998)
	Kynurenine and N-formylkynurenine – potential carcinogens	*Friedman and Cuq* (1988)

absorption (Morin and Peng 1992), but they are able to pass through the intestinal epithelium much faster than cholesterol does (Emanuel and others 1991) and could enter the blood flow with exogenous cholesterol and endogenous cholesterol (biliary cholesterol) as constituents of lymph chylomicrons (Linseisen and Wolfran 1998). COPs in the diet could constitute a health risk for promoting atherosclerosis, cancer, and damage to cell membranes (Pietras and others 2012) (Table 4). Staprans and others (2003) studied the effect of α-epoxy cholesterol in the diet on circulating lipoproteins in human serum and concluded that it contributes to lipoprotein oxidation. Staprans and others (1998) fed rabbits with a diet containing 0.33% cholesterol, of which 5% was oxidized cholesterol, and they found that this diet enriched in oxidized cholesterol produced a 100% increase in the earliest lesions seen within atherosclerosis in the aorta and suggested that oxidized cholesterol is a risk factor for atherosclerosis. Phillips and others (2001) suggested that 7-ketocholesterol, one of the major oxysterols found in oxidized low-density lipoproteins (OxLDL) and in atherosclerotic plaque, plays a role in atherosclerotic obstructive arterial diseases. The consequences of lipid oxidation products on human health are summarized in Table 4.

Heme Protein Oxidation

The meat heme proteins responsible for color are myoglobin, hemoglobin, and cytochrome *c*. The main heme protein found in well-bled muscle tissue is myoglobin, representing 70% to 95% of the total heme protein in meat (Nam and Ahn 2002). Myoglobin oxygen reserve for living muscle is a noncirculating pigment that binds oxygen carried by the hemoglobin. This pigment provides the red meat color and plays a decisive role in the curing reactions. Hemoglobin is the major heme protein found in circulating blood and can also contribute to meat color because it is present in a small amount after slaughter. The hemoglobin level in meat is strongly influenced by the thoroughness of exsanguination at slaughter. Inefficient and inadequate bleeding during exsanguination causes the retaining of a greater amount of hemoglobin in the muscle tissue (Miller 1994). Due to the low level of cytochrome *c* in meat, this heme protein influences to a very small degree the meat color.

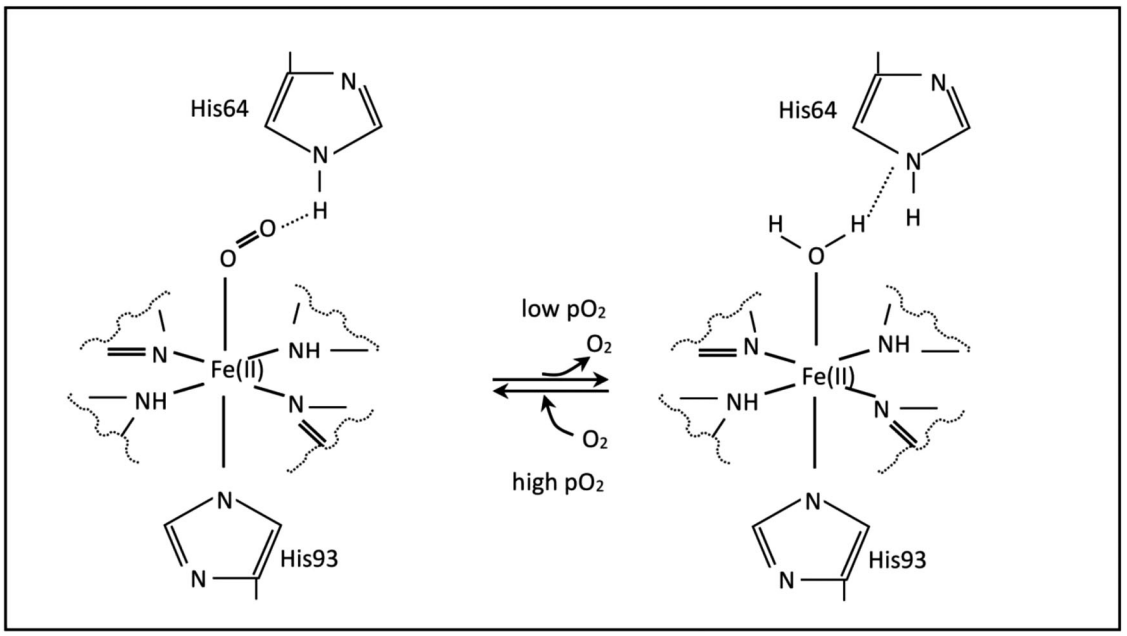

Figure 9–The 6 bonds realized by Fe(II) in oxymyoglobin with protoporphyrin IX, histidine residue (His93) of globin, and molecular oxygen (O_2).

Myoglobin structure and autoxidation

Myoglobin is a cytoplasmic heme protein consisting of a polypeptide chain (154 amino acids), named globin, and a heme prosthetic group (red pigment) found in the hydrophobic pocket of the globin. The central part of heme contains iron linked to the 4 pyrrole nitrogen atoms of protoporphyrin IX. The iron can form 2 additional coordinate bonds, one on each side of the heme plane, in the 5th and 6th coordination sites. The 5th coordination site is occupied by proximal histidine (His93) from the polypeptide chain. The 6th coordination site could be occupied by diatomic ligands such as O_2, NO, and CO. In oxymyoglobin and MbFe(II)O_2, oxygen-binding occurs at the 6th coordination site, and there is also a coordinate bond between O_2 and distal histidine (His64) of globin (Figure 9). Deoxymyoglobin [deoxyMbFe(II)] occurs when no oxygen is found at the 6th coordination site and the heme iron is ferrous (Figure 9). Oxymyoglobin has a bright red color and deoxymyoglobin has a purplish-red (purplish-pink) color. The color of deoxymyoglobin is typically associated with the color of vacuum-packaged meat products and of muscle immediately after cutting (Mancini and Hunt 2005). Oxidation of Fe(II) from both myoglobin ferrous derivative to Fe(III) results in metmyoglobin formation [MbFe(III)] accompanied by meat discoloration (Table 3). The ligand coordinated at the 6th coordination site and the iron oxidation state dictate myoglobin color and thereby meat color. Metmyoglobin formation depends on numerous factors, including oxygen partial pressure, temperature, pH, loss of tertiary structure of globin, the activity of mitochondrial enzymes and content of their substrates, microbial load, and light illumination (Nam and Ahn 2002; Mancini and Hunt 2005).

Brantley Jr and others (1993) studied the oxidation of both native and mutant myoglobins and proposed 2 pathways for autoxidation of myoglobin. The disruption of the hydrogen bond between oxygen bound to Fe(II) by a coordinate bond and the hydrogen atom from the distal histidine (His64) is the initial step of the 2 pathways for autoxidation of myoglobin.

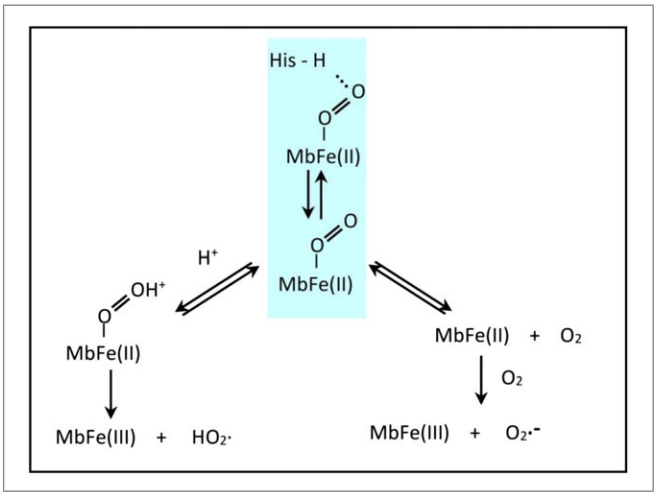

Figure 10–Oxidation of oxymyoglobin and deoxymyoglobin to metmyoglobin.

The 1st pathway shows the formation of MbFe(III) from MbFe(II)O_2 by a proton-mediated mechanism: the proton diffuses into the globin pocket and protonates the bound O_2, then 1 electron is removed from ferroprotoporphyrin IX, and perhydroxyradical and MbFe(III) are generated. The 2nd pathway occurs at a low partial pressure of O_2 and suggests the direct reaction of deoxymyoglobin and O_2, accompanied by the transfer of an electron from the ferroprotoporphyrin IX to the oxygen and formation of a superoxide radical (Figure 10).

A continuous metmyoglobin conversion to the myoglobin deoxy-forms (Baron and Andersen 2002) occurs *in vivo*, due to an NADH-dependent enzyme named metmyoglobin reductase, also known as NADH-dependent metmyoglobin reducing system, cytochrome *b5* MetMb reductase, diaphorase, or aerobic and anaerobic reducing system (Bekhit and Faustman 2005). Hayashi

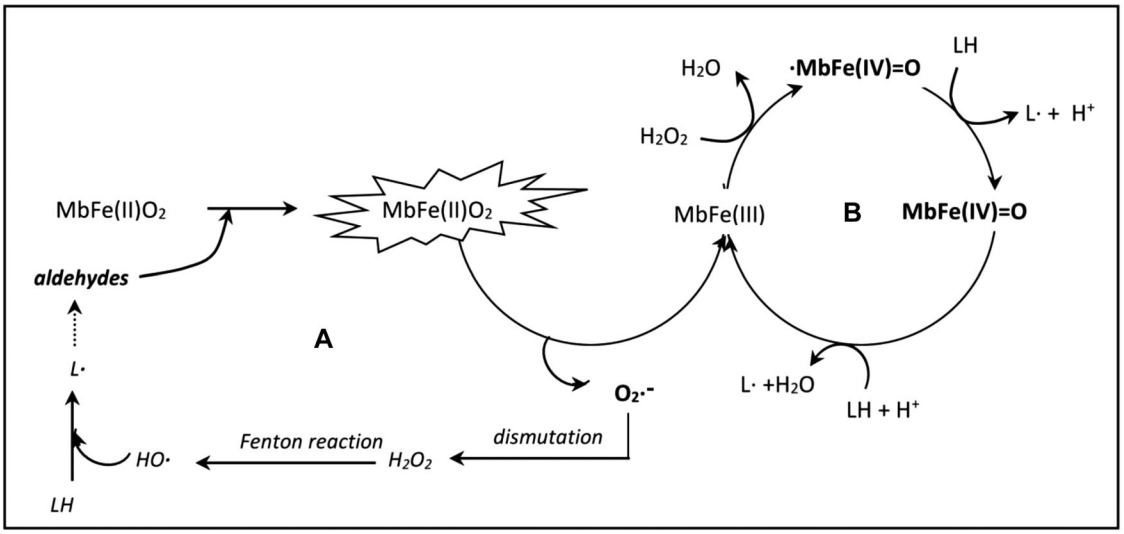

Figure 11–Possible interrelations between lipid oxidation and myoglobin oxidation. (A) Aldehydes resulting from lipid oxidation alter oxymyoglobin stability by increasing oxymyoglobin oxidation and metmyoglobin formation; superoxide anion is prooxidant for lipids. (B) Ferrylmyoglobin and perferrylmyoglobin are initiators in the reaction mechanism.

and others (1973) reduced metmyoglobin using an NADPH-generating system (NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase), an electron-mediating system (ferredoxin and ferredoxin-NADP reductase), and an oxidoreductase such as catalase or peroxidase. Tang and others (2005) studied the mitochondrial reduction of metmyoglobin. They reported an increase of metmyoglobin reduction which depended on mitochondrial density and succinate concentration and it decreased with time postmortem. After slaughter, enzymatic systems involved in metmyoglobin reduction are continuously depleted as time progresses, thus the meat color is affected. Belskie and others (2015) reported that succinate and NAD can generate NADH in bovine tissue postmortem by reverse electron flow, which can be used to reduce metmyoglobin by 2 pathways: electron transport-mediated and metmyoglobin reductase NADH-dependent. The discoloration of red meat is the result of oxymyoglobin (cherry red color) from fresh red meat oxidation to metmyoglobin (brown color). In freshly cut meat, the primary pigment is oxymyoglobin, while a thin layer of the metmyoglobin exists at a certain depth in the meat, followed by the deoxymyoglobin (Livingston and Brown 1981); thus, discoloration of meat is related to the amount of surface area covered by metmyoglobin (Mancini and Hunt 2005). Recent studies have demonstrated a relationship between myoglobin stability and lipid peroxidation. Oxymyoglobin susceptibility to oxidation increases in the presence of 4-hydroxy-2-nonenal (4-HNE) (Lynch and Faustman 2000) by covalent modification at the histidine residues (Alderton and others 2003). Monahan and others (2005) investigated lipid oxidation and oxymyoglobin oxidation in bovine muscle homogenates subjected to bubbles, with and without oxygen additions, and they suggested that a probable cause of oxymyoglobin oxidation in muscle tissue is lipid oxidation-induced oxygen depletion. Elroy and others (2015) described the effects of 4-HNE on the nonenzymatic reduction of bovine, porcine, and equine-purified metmyoglobin, and they reported that decreased nonenzymatic reduction of myoglobins and their rates of reduction were determined by the species (beef > horse > pork). Elroy and others (2015) preincubated myoglobin from 3 species with 4-HNE and reported adducts of 4-HNE with myoglobin and suggested that the compound that resulted in the covalent binding of 4-HNE to histidine residue of myoglobin decreased the ability of heme iron (III) to function as electron acceptor. Early studies showed the effects of intermediates of glycolysis and the tricarboxylic acid cycle on myoglobin redox stability, and they reported oxymyoglobin increase in beef strip loin (Purohit and others 2014) and postmortem generation of NADH involved in metmyoglobin reduction in beef (Mancini and others 2004). Many researchers agree that the processes of lipid oxidation and myoglobin oxidation in meat can be independent of each other but can be interrelated too. Thus, secondary lipid peroxidation products (aldehydes) affect oxymyoglobin stability by increasing the susceptibility of oxymyoglobin to oxidation, and the metmyoglobin resulting from this process has prooxidant activity in lipid oxidation (Lynch and Faustman 2000). Figure 11 shows possible interrelations between lipid oxidation and myoglobin oxidation. Suman and others (2014) have suggested that lipid oxidation-induced myoglobin oxidation is more critical to beef color than to pork color due to the structural differences in the polypeptide chain structure; the number of aldehyde-protein adducts generated in the reaction of histidine residues with aldehydes is greater in beef myoglobin than in pork myoglobin, and so secondary lipid peroxidation products accelerate the formation of metmyoglobin in beef to a greater extent than in pork. Wongwichian and others (2015) described the relationship between myoglobin and lipid oxidation in oxeye scad (*Selar boops*) and reported that these 2 oxidative processes are competitive and each process seems to stimulate the other.

Postprandial Heme Iron-Induced Toxicity

Due to the acidic pH in the stomach and the proteolytic enzyme activity in the small intestine, globin is denaturated and then digested, and heme is released from heme proteins. Concentrated heme released from meat pigments is poorly absorbed and much of the ingested heme is passed into the colon. It has been shown that after ingestion of red meat, but not of chicken or fish (which have low heme contents), heme could be recovered from the feces (Schwartz

and Ellefson 1985). Heme may be suspected of increasing the risk of colorectal cancer associated with red meat consumption (Cross and others 2003) (Table 4). The mechanisms implicated in the induction of colorectal cancer by heme are poorly understood. One hypothesis is based on the catalytic effect of heme iron on: (1) the formation of carcinogenic endogenous N-nitroso compounds (NOCs) and (2) the generation of cytotoxic and genotoxic aldehydes by lipid peroxidation (Bastide and others 2011). Hebels and others (2010) suggested that some NOCs may be direct alkylating agents of DNA, whereas others need to be metabolically activated before becoming very reactive intermediates causing a mutation to DNA base pairs. Furthermore, both heme iron and NOCs may promote the formation of ROS that may induce genetic mutations, which produce an inflammatory response and increased cytotoxicity in the colon. Experimental studies in rats with chemically induced colon cancer have shown that dietary heme iron and red meat promote aberrant crypt foci, causing a putative precancer lesion (Pierre and others 2003, 2004, 2008; Bastide and others 2011). In the last decade, a meta-analysis of prospective cohort studies, which included hundreds of thousands of individuals, has been carried out. All studies suggested that consumption of red and processed meat is associated with cancer risks. These cohort studies suggested that heme iron intake is associated with an increased risk of colorectal tumors, and that an alkylating rather than an oxidative DNA-damaging mechanism is involved in heme-induced colorectal carcinogenesis. Jakszyn and others (2013) demonstrated a potential association between higher intakes of heme iron and the risk of esophageal adenocarcinoma. Gilsing and others (2013) studied the interrelationship between dietary heme iron and the risk of colorectal cancer with specific mutations in Kristen-ras (KRAS) and adenomatous polyposis coli (APC), and they observed a positive association between heme iron intake and risk of colorectal cancer with the activating G > A mutation in KRAS and overall G > A mutation in APC.

Another mechanism that explains the association between high intake of red meat and colorectal cancer involves the ability of heme to be nitrosated to become a nitrosating agent under the anaerobic conditions found in the small intestine. Nitrosyl heme at the side of nitrosothiols can N-nitrosate the amines and amides produced by bacterial decarboxylases to generate carcinogenic NOCs (Hooda and others 2014). This mechanism is supported by the research of Martin and others (2015) who used a factorial design to study the role of the intestinal microbiota in the process of colon carcinogenesis in rats and observed that the intestinal microbiota is involved in the heme-induced promotion of colorectal carcinogenesis.

Protein Oxidation

Protein oxidation mechanisms in foods, especially in fresh meat and meat products, have been very little studied (Zhang and others 2013). Protein oxidation in postmortem muscle tissue subjected to RS action is a free radical chain reaction. Given the complexity and the diversity of molecules, protein oxidation occurs by routes of greater intricacy and generates a larger variety of oxidation products than seen with lipid peroxidation (Lund and others 2011; Soladoye and others 2015) (Table 2). Protein oxidation in meat could be the result of the direct action of RS or could be initiated by the secondary products of lipid oxidation (Lund and others 2011). The changes induced by protein oxidation in meat could decrease amino acid residue bioavailability, and also protein digestibility, and lead to loss of essential amino acids, which would negatively affect their capacity to nourish the body with the meat proteins (Lund and others 2011) (Table 3). The most oxidative processes occur at: (1) the side chains of amino acid residues (for example, oxidation of thiol groups, hydroxylation of aromatic rings, formation of hydroperoxides, and formation of carbonyl groups) (Stadtman 1990) to cause solubility loss, essential amino acid loss, and an increase of susceptibility to aggregation (Rowe and others 2004); and (2) the backbone of a protein to produce changes in the local spatial arrangement of the atoms of the polypeptide chain, fragmentation, aggregation, and polymerization of the proteins (Pattison and Davies 2001; Pattison and others 2012; Zhang and others 2013; Soladoye and others 2015).

Oxidation at the side chain of amino acid residues

SH-containing amino acid residues are the most susceptible to oxidation due to the high reactivity of the thiol group (Zhang and others 2013). The oxidation of the thiol group occurs by 2 major pathways: (1) free radicals oxidize SH groups to generate thiyl radicals, which can react with other thiols/thiolates to form disulfide bonds or react with O_2 to produce thiyl peroxy radicals (RSOO•); and (2) a nonradical RS (for example, H_2O_2, $^1O_2$) forms with thiol group sulfur-containing acids, which can be the subject of further reactions. For example, the cysteine residue generates, in the reaction with hydrogen peroxide, unstable sulfenic acid (CysSOH), sulfinic acid ($CysSO_2H$), and sulfonic acid ($CysSO_3H$) (Claiborne and others 2003), which can produce oxyacids by hydrolysis reactions or compounds with disulfide bonds by reacting with another thiol group (Turell and others 2008) (Table 2). Figure 12 shows the schematic pathways of cysteine residue oxidation by the 2 mechanisms described above. In pork patties, during chilled storage, proteins lose thiols up to 9 d and form cross-linked myosin disulfide after 12 d (Nieto and others 2013). This reaction is often accompanied by solubility loss and increased susceptibility to protein aggregation (Rowe and others 2004).

Similarly, methionine residues can be easily oxidized by various RS. The major product resulting from methionine oxidation is methionine sulfoxide (Met-SO$^-$), which can further be the subject of oxidation to generate methionine sulfone (Met-SO) (Vogt 1995) (Table 2). Figure 13 shows the oxidation of methionine residues by $^1O_2$.

The oxidized residues of cysteine and methionine may be reduced back to initial residues by antioxidant enzymes, such as methionine sulfoxide reductases or thioredoxin NADPH-dependent reductases (Stadtman and Berlett 1997; Levine and others 2000), but after slaughter, enzymatic systems involved in reduction are continually depleted as time progresses. This is because μ-calpain and m-calpain enzymes, located within the muscle cell and involved in protein degradation and containing both histidine and cysteine residues at their active sites, may be susceptible to inactivation induced by RS such as oxygen, ozone, superoxide anion, hydrogen peroxide, hypochlorous acid, and peroxynitrite (Huff-Lonergan and Lonergan 2005). As the postmortem period progresses, the biochemical processes are accompanied by considerable increase of oxidation conditions that produce the oxidation of sulfur-containing amino acid residues, thus limiting the proteolytic activity of calpains which affect the water-holding capacity (Huff-Lonergan and Lonergan 2005). Sulfur-containing amino acids are very susceptible to oxidation by gamma-irradiation. Irradiation can produce radiolytic products of water, such as hydroxyl radical (Shuryak and Brenner 2010), which migrates to the side chain of amino acids. Patterson and Stevenson (1995) found that dimethyl trisulfide,

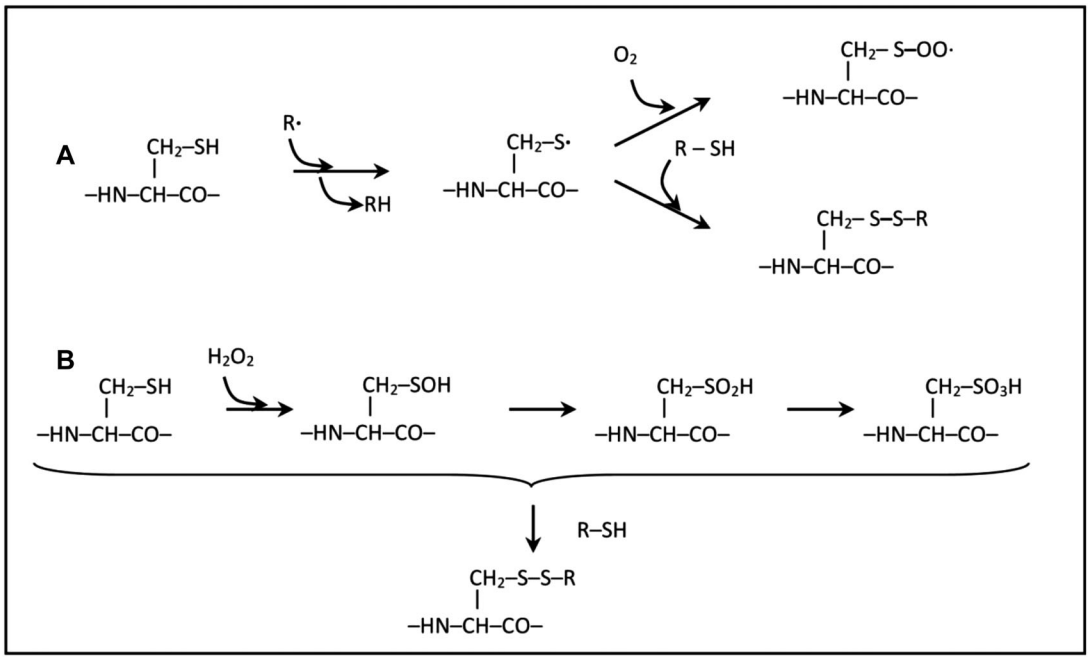

Figure 12–The oxidation of cysteine residue by 2 pathways: (A) Oxidation with a free radical. (B) Oxidation with a nonradical (for example, H_2O_2).

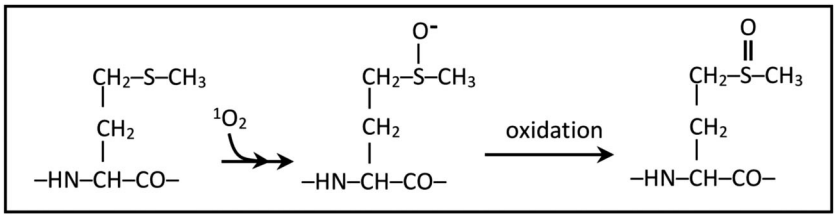

Figure 13–The oxidation of methionine residue by singlet oxygen.

which results from sulfur-containing amino acid residues, is the most active off-odor compound in irradiated chicken meat. Ahn (2002) studied the production of volatile compounds generated from amino acid homopolymers by irradiation and concluded that sulfur compounds resulting from the conversion of the side chains of methionine and cysteine are the most important volatile compounds for off-odor produced in irradiated meat and also that these compounds are very reactive and can react with other compounds around them (Table 3). Formation of disulfides is one of the most common oxidation product occurrences at the side chain of SH-containing amino acid residues, and both intra- and intermolecular protein cross-links can be formed. Many researchers have reported myofibrillar protein cross-linking through disulfide bonds (Xiong and others 2009; Lund and others 2011; Zakrys-Waliwander and others 2012; Nieto and others 2013) (Table 2). Formation of protein disulfide cross-linking of the myosin heavy chain (MHC) was correlated with reduced tenderness in pork (*longissimus dorsi* muscle) during chilled storage (Lund and others 2011). During meat storage the speed of loss of thiol depends on packaging atmosphere and type of musculature. Jongberg and others (2014) reported that protein thiol concentrations in chicken breast and thigh decreased significantly between 5 and 9 d of storage in high-oxygen atmosphere packaging compared with nonoxygen storage due to MHC disulfide cross-linking; thiol loss and protein cross-link formation were more pronounced in chicken thighs compared with breasts. Compared with other amino acids, sulfur-containing amino acids are the most susceptible to irradiation, and the changes induced by the electromagnetic radiations take place at more than one site of the amino acid side chain (Ahn and Lee 2002). Oxidative chemical changes induced by irradiation are not only dependent upon the structure and state of proteins, but also on irradiation conditions such as dose, temperature, presence of oxygen, and the nature of surrounding molecules (Ahn and others 2000; Zhu and others 2004; Shuryak and Brenner 2010). Another amino acid susceptible to oxidation at the side chain is tyrosine (Table 2). Due to the presence of the hydroxyl group on the benzene ring, this amino acid is more reactive than phenylalanine, and adjacent tyrosine residues in the same protein chain/different protein chains are oxidatively coupled, forming dityrosine, which can lead to intra- and interlinked proteins (Zhang and others 2013) (Figure 14).

Morzel and others (2006) oxidized myofibrillar proteins isolated from *longissimus dorsi* muscle by a hydroxyl radical-generating system and reported formation of cross-linked proteins by dityrosine and disulfide bridge formation, and they suggested that myosin was the most susceptible to oxidation (Table 2). During food storage, in the presence of oxygen or other oxidizing agents, tryptophan residues can be converted to N-formylkynurenine and

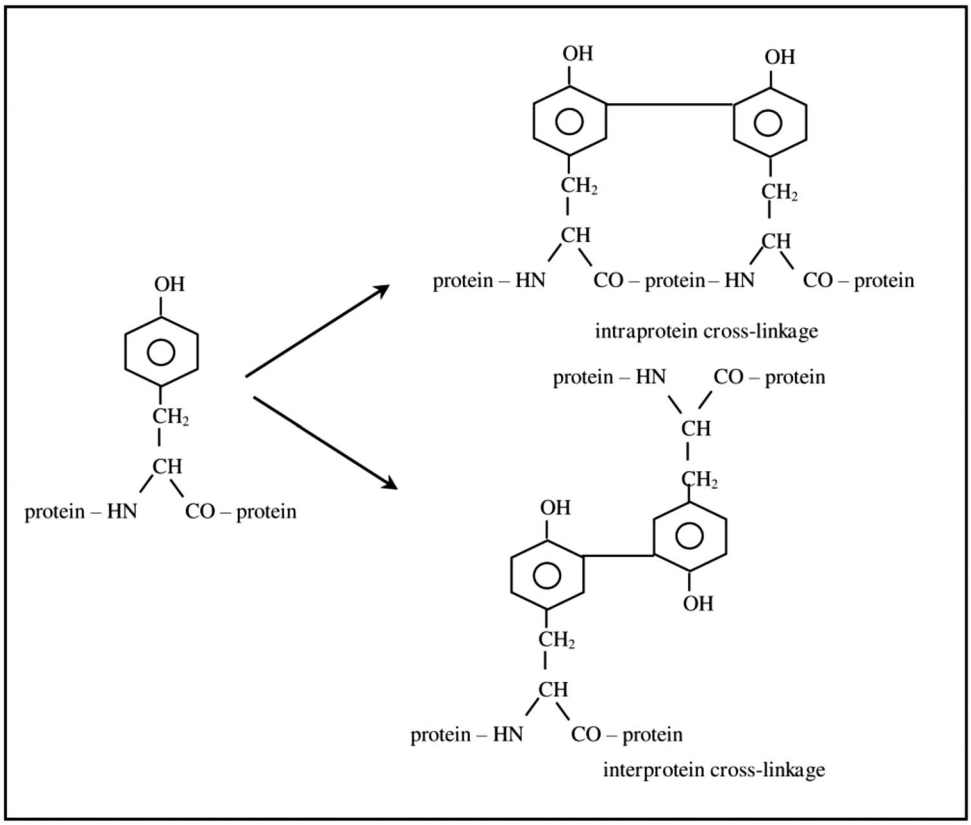

Figure 14–Formation of intra- and interprotein cross-linkages by tyrosine oxidation.

kynurenine, which are potentially toxic compounds (Friedman and Cuq 1988) (Table 2). In the presence of hydrogen peroxide and Cu^{2+} ions, histidine residues can undergo oxidative processes forming 2-oxo-histidine, asparagine, and aspartic acid residues (Stadtman and Berlett 1997; Kowalik-Jankowska and others 2004) (Table 2). During chilled storage of meat and meat products, lysine residues form oxidation products, such as α-aminoadipic semialdehyde (AAS) and α-aminoadipic acid (AAA) and Schiff bases (Utrera and others 2012) (Table 2). According to the research conducted on pork patties subjected to chilled storage, Utrera and others (2012) suggested that the loss of tryptophan and the formation of specific lysine oxidation products are important reliable protein oxidation markers in meat.

Oxidation of protein backbone

Oxidation of protein backbone can be initiated by ROS, produced either from the metal-catalyzed decomposition of H_2O_2 or radiolysis of water. ROS abstracts a hydrogen atom from an amino acid α-carbon site, resulting in the formation of a carbon-centered radical (Stadtman and Berlett 1997; Soladoye and others 2015). Under anaerobic conditions, 2 such carbon-centered radicals react with each other to form an intra- or intermolecular protein cross-linkage. In the presence of oxygen, carbon-centered radicals are converted into the corresponding alkylperoxyl radicals. These peroxyl radicals, in turn, undergo an additional reaction that produces hydroperoxides (Davies 2012) (Figure 15).

Another transforming pathway of alkylperoxyl radical is decomposition to imines, followed by the backbone protein fragmentation by hydrolysis to generate amides and α-keto-acyl derivatives (Figure 16A). Protein hydroperoxides can be decomposed in reactions catalyzed by transitional metal ions forming backbone protein alkoxy radicals that can be subject to the backbone protein fragmentation in order to generate substituted amides and protein carbonyl derivatives (Davies 2016) (Table 2) (Figure 16B). Protein backbone fragmentation can be the result of ROS attack of glutamyl, aspartyl, and prolyl side chain (Berlett and Stadtman 1997). Schuessler and Schilling (1984) reported that a protein subjected to radiolysis cleaves into a number of peptides that is approximately equal to the number of prolyl residues, and they suggested that oxidation of prolyl residues causes peptide bond cleavage. Davies (1996) suggested that during protein fragmentation in the presence of oxygen, α-carbon alkoxy radicals are the most probable reaction intermediates. Protein backbone fragmentation generates carbonyl and imine derivatives (Table 2). Carbonyl derivatives arise from the condensation reaction between proteins and aldehydes formed by lipid oxidation. The dialdehydes that result from lipid oxidation can react with the ε-amino group of lysine residues located in the same protein or with the ε-amino group of lysine residues located in different protein chains leading to intra- or intercross-linking of proteins (Shen and others 1996) (Table 2).

Reactions of proteins with carbonyl compounds from lipid oxidation

Carbonyl compounds that result from lipid oxidation react with proteins by 2 common pathways: (1) condensation of saturated and unsaturated carbonyl compounds with amino group from amino

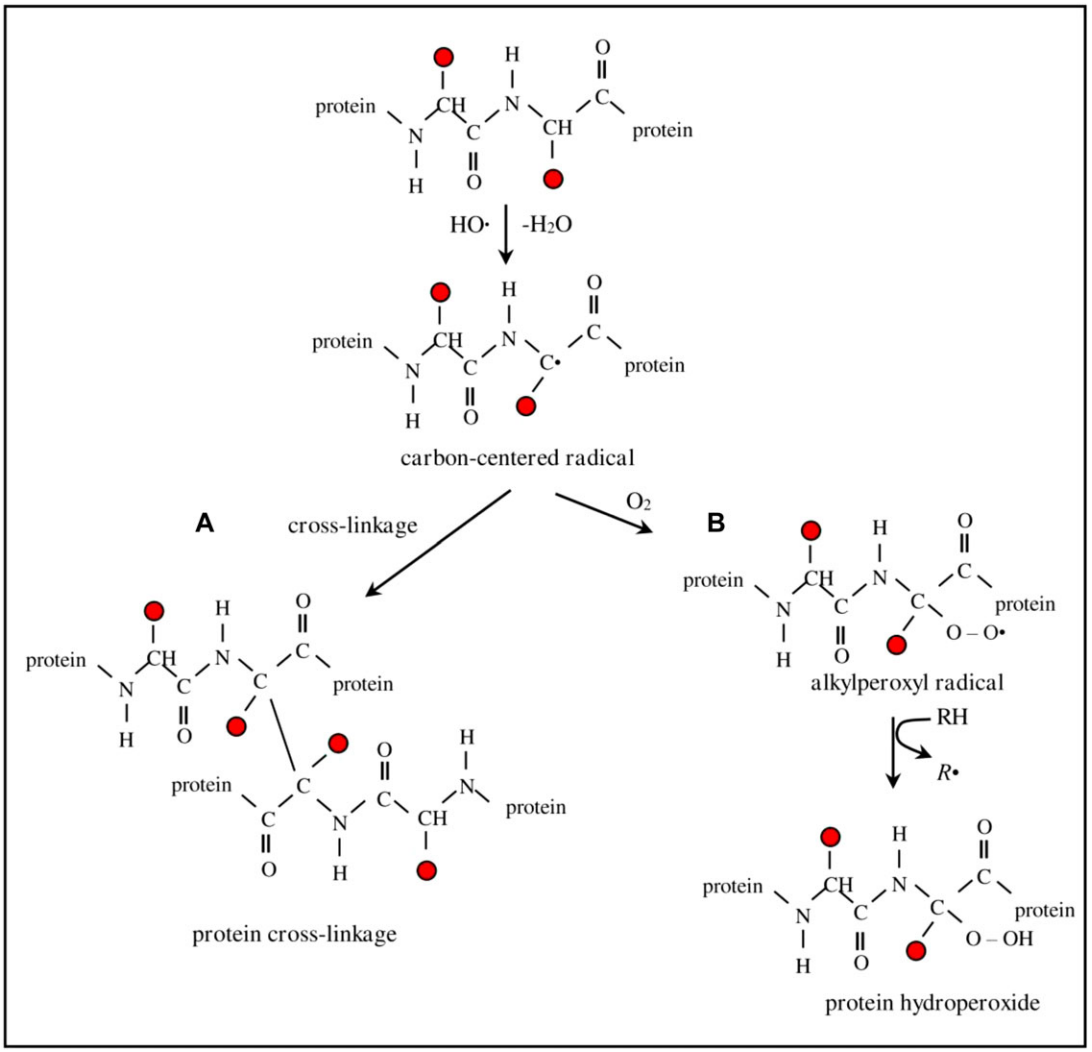

Figure 15–Possible mechanisms of oxidation of backbone protein. (A) Formation of an interprotein cross-linkage. (B) Formation of a backbone protein hydroperoxide. ●, amino acid residue.

acid residues to form Schiff base adducts; and (2) Michael-type addition of nucleophile groups from amino acid residues (for example, imidazole ring of histidine, -SH group of cysteine or methionine, and lysine ε-amino group) to α, β-unsaturated aldehydes (Refsgaard and others 2000). 4-HNE is an α- and β-unsaturated aldehyde that may be involved in both pathways. Thus, 4-HNE may react by a condensation reaction with amino groups of proteins forming Schiff bases and with cysteine, histidine, and lysine residues by Michael addition reactions (Szweda and others 1993; Uchida and others 1994; Refsgaard and others 2000). Due to the structural diversity of proteins and 4-HNE reactivity, the reactions between 4-HNE and proteins can generate a variety of adducts and proteins cross-linked because Michael addition products may be involved in the subsequent condensation to form Schiff adducts (Table 2). For example, Michael addition of 4-HNE to cysteine, histidine, or lysine may be followed by condensation with an amino group to form a Schiff base (Requena and others 1996). Two MDA protein adducts were identified in proteins: (1) Schiff base adduct of MDA to the ε-amino group of lysine residues; and (2) *bis*-Schiff base diimine cross-link formed by the condensation reaction of MDA with 2 ε-amino groups of lysine residues, resulting in either intra- or intermolecular cross-linking proteins (Requena and others 1996). Figure 17 shows the structures of 2 adducts of MDA to protein (A) and the structures of products in Michael addition reactions of 4-HNE with the cysteine thiol group, histidine imidazole ring, and ε-amino group of lysine (B). Protein oxidation-induced changes can influence protein properties, solubility, hydrophobicity, water-holding capacity, and, consequently, reduce meat quality because it decreases tenderness and juiciness. Oxidative damage to proteins can also affect the digestibility and nutritional value of proteins. Formation of protein cross-linkage is followed by protein aggregation and it is known that aggregates are more resistant to proteolytic enzymatic degradation. Weak oxidation of proteins could increase the availability of corresponding proteases (Davies 1991) because it is highly possible to change protein hydrophobicity (Zhang and others 2013). Irradiation, a useful tool for improving meat safety, induces protein oxidation and, consequently, decreases meat tenderness. Rowe and others (2004) reported that early postmortem irradiation of fresh beef steaks increased oxidation of both sarcoplasmic and

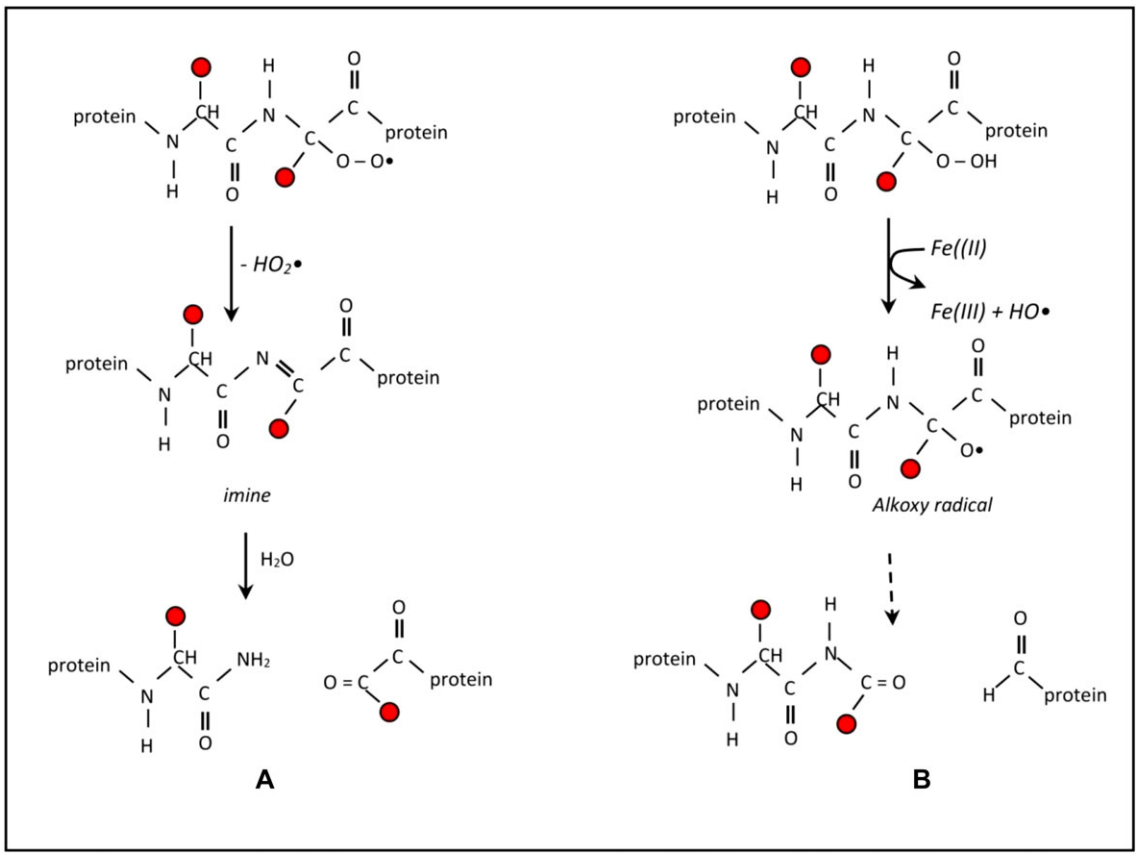

Figure 16–(A) Decomposition of backbone protein hydroperoxyl radical to imine and backbone fragmentation by hydrolysis. (B) Decomposition of backbone protein hydroperoxide to the alkoxy radical and backbone fragmentation. ●, amino acid residue.

myofibrillar proteins, and protein carbonyl content increased in irradiated steaks, compared with nonirradiated steaks at 0, 1, 3, and 7 d after irradiation. In Table 2 are summarized the most important protein oxidation products identified in meat, while their effects on sensory properties and acceptability of meat are presented in Table 3.

Postprandial Protein-Induced Toxicity

Meat proteins represent an important source of energy and essential amino acids for humans. During passage through the digestive tract, proteins are hydrolyzed by proteases into amino acids or small peptides, which are absorbed into the bloodstream. During storage, mincing, salting, irradiation, exposure to light, and cooking, RS can also induce alterations to meat nutritional value and oxidation product formation, and such end products are suspected to be toxic (Table 3 and 4). Very little is known about oxidized protein toxicity. Most difficulties arise due to the structural diversity of these compounds. Formation of aggregates and change in surface hydrophobicity affect the interaction between proteins and proteolytic enzymes; absorption of oxidized amino acids can increase the pool of oxidized amino acids in the cells and cause their misincorporation during protein synthesis, thus generating modified proteins. Recent studies have shown that oxidation decreases susceptibility of skeletal muscle myofibrillar proteins to proteolytic enzyme action. Morzel and others (2006) exposed oxidized myofibrillar proteins isolated from *longissimus dorsi* muscle to proteolysis in an experimental model which involved generation of hydroxyl radicals, and they reported a direct and quantitative relationship between the degree of protein oxidation and decrease of proteolytic susceptibility. Rutherfurd and others (2014) examined the effect of oxidized proteins from 7 dietary sources on the true ileal amino acid digestibility (TIAAD), and they suggested the existence of an equilibrium between 2 processes: (1) protein denaturation accompanied by increased digestion and (2) formation of indigestible peptides accompanied by decreased digestion. The decrease of protein digestibility increases the amount of protein substrate for colonic microbial enzymes, with possible ill effects on human health. Thus, Kim and others (2013) reported that the increases in the bacterial degradation compounds derived from amino acids could affect colon epithelial renewal and homeostasis. Le Leu and others (2007) investigated the effect of dietary digestion-resistant potato protein on colonic fermentation processes and the quantitative relationship between indigestion, protein level, and intestinal tumorigenesis, and they reported that potato protein alone increased protein fermentation products, the incidence, and a number of small intestinal neoplasms including adenocarcinomas. The distribution of protein fermentation products in the colon segments differs. Researchers have shown that the levels of protein bacterial metabolites are higher in the descending (distal) colon as compared to the ascending (proximal) colon, suggesting that protein fermentation is more predominant in the distal colon (Hughes and others 2000). Studies have shown that the products generated in the colon by bacterial enzyme degradation from undigested dietary substrates include toxic ammonia,

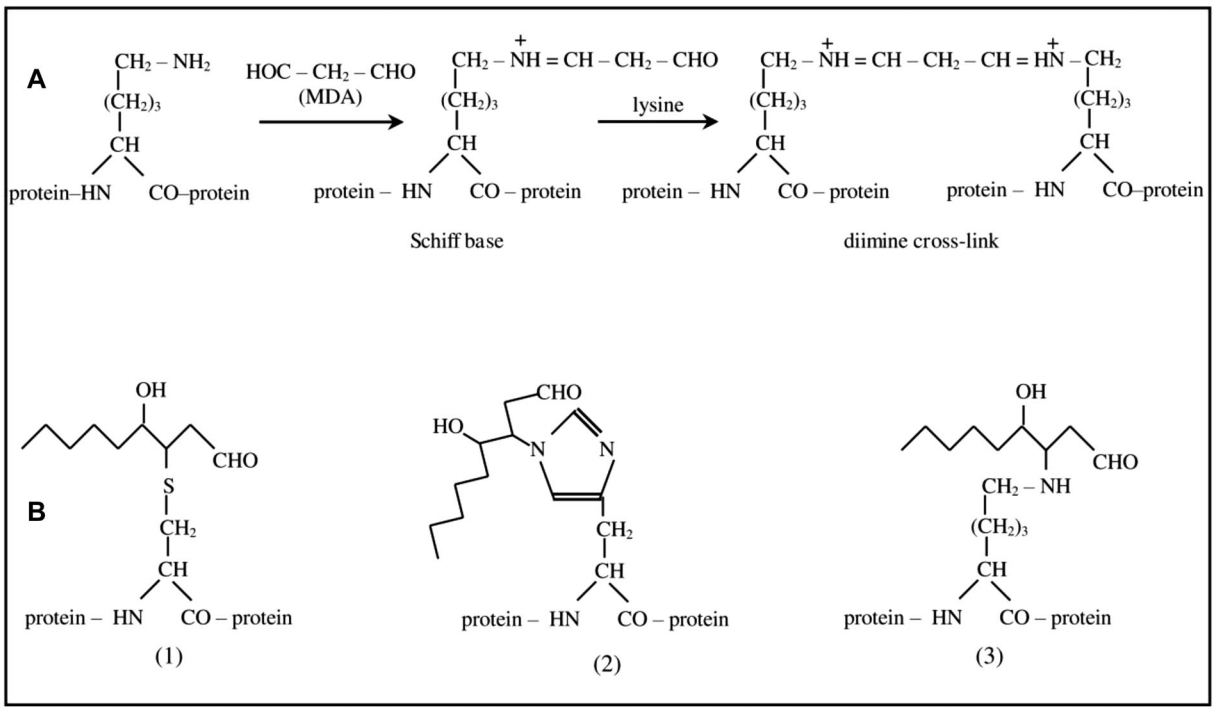

Figure 17–(A) Malondialdehyde adducts and cross-links with lysine residues in protein. (B) Products of Michael addition reactions of 4-HNE with (1) cysteine, (2) histidine, and (3) lysine residues in protein.

phenols, cresols, N-nitroso compounds, and sulfides (Hughes and Rowland 2000). Ammonia released from amino acid deamination suggests that it seems to be involved in tumor promotion by several mechanisms, such as alteration of the morphology and metabolism of intestinal cells, effect on the pattern of DNA replication, and reduction of life expectancy of intestinal cells (Visek 1979; Hughes and Rowland 2000). Phenolic compounds are formed by bacterial degradation of aromatic amino acids, absorbed in the colon, detoxified by the liver through conjugation, and then excreted in the urine, especially as p-cresol (MacFarlane and others 1986). But it has been shown that phenol reacts with nitrite-forming mutagenic p-diazoquinone and can also stimulate *N*-nitrosation of dimethylamine *via* the production of p-nitrosophenol (Yokoyama and Carlson 1981). Amines (such as tyramine, pyrrolidine, piperidine, cadaverine, putrescine, and histamine) formed in the large intestine *via* hydrolysis and decarboxylation (MacFarlane and Cummings 1991) could exert toxic effects because they are precursors of *N*-nitroso compounds, which are potentially carcinogenic (Hughes and Rowland 2000). Cyclic amines formed from heterocyclic amino acid decarboxylation, such as when meat is cooked at a high temperature (Gross and others 1993), have been shown to be carcinogenic (Delfino and others 2000). One of the most toxic heterocyclic amines is 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoline, formed in small quantities when meat is grilled or fried. It has been shown that this amine induces tumors at various sites in rodents, including the large intestine, suggesting that it could play a role in the etiology of colon cancer in humans (Ohgaki and others 1991; Tavan and others 2002). *N*-nitrosation reactions of amines occur in the stomach too. In the acid conditions of the stomach, nitrite easily reacts with secondary amines to form *N*-nitroso compounds (Flint and Wallace 2010). Many *N*-nitroso compounds that result from these reactions are known to exert carcinogenic and mutagenic effects (Silvester and others 1997; Hughes and Rowland 2000). Breast cancer risk is associated with the heterocyclic aromatic amines which are formed when meat is cooked at a high temperature; however, before binding to DNA and to initiate carcinogenesis, heterocyclic amines require enzymatic activation (Delfino and others 2000). Another toxic compound found in the human large intestine is hydrogen sulfide. This is the end product in the reactions catalyzed by enzymes biosynthesised by sulfate-reducing bacteria, and an important substrate for these enzymes is dietary sulfur-containing protein (Magee and others 2000). Hydrogen sulfide is an irritant that may cause ulcerative colitis and is suspected to be carcinogenic. Several studies have shown its ability to perturb cellular homeostasis, to modulate gene expression involved in cell-cycle progression, and to initiate both inflammatory and DNA repair responses (Moore and others 1997; Attene-Ramos and others 2007). Recent studies have demonstrated the formation of ortho- and meta-tyrosine from the interaction of HO• with phenylalanine in tissues (Nair and others 1995; Davies 2005). Meta-tyrosine can be misincorporated into proteins in the place of phenylalanine by eukaryotic phenylalanyl-tRNA synthetases (Gurer-Orhan and others 2006; Klipcan and others 2009) and it has been reported to be toxic to cultured CHO (Chinese-hamster ovary) by a pathway that could involve the misincorporation of this oxidized amino acid into proteins. An alternative mechanism for oxidative stress and tissue injury during aging and disease could be due to the incorporation of meta-tyrosine into proteins during protein synthesis (Gurer-Orhan and others 2006). Aminoadipic acid is an intermediate in the metabolism of lysine and has toxic effects on retinal glial cells (Ishikawa and Mine 1983) and cerebral astrocytes (Brown and Kretzschmar 1998). Youngman and others (1992) have suggested that accumulation of oxidized products could be a major factor in cellular aging. In their

study, the accumulation of oxidized proteins in rats was compared between animals consuming protein-restricted diets and animals consuming calorically restricted diets, and it was concluded that accumulation of oxidized proteins during oxidative stress, as induced by exposure to ^{137}Cs, was reduced in animals on the protein-restricted diet. Little is known about absorption of oxidized amino acids that result from protein digestion and enter into the bloodstream, about their transport into cells, and about their misincorporation into human proteins, thus affecting human health. Rodgers and Shiozawa (2008) suggested that analogous amino acids (oxidized amino acids) may fool the cellular mechanism involved in protein synthesis; they can be misincorporated into the growing polypeptide chain during chain elongation, thereby generating damaged proteins in mammalian cells. *In vitro* studies undertaken in experimental models, using a range of mammalian primary cells and cell lines, have shown that some oxidized amino acids can be incorporated into proteins *via* protein synthesis when the medium is deficient in the parent amino acid (Rodgers and others 2002, 2004) or even under standard culturing conditions when there exists a competition between the oxidized amino acid the parent amino acid (Rodgers and others 2002). Rodgers and others (2002) have suggested that, in mammalian cells, the aromatic oxidized amino acids (*m*-tyrosine and 3,4-dihydroxyphenylalanine) and aliphatic oxidized amino acids (leucine hydroxide and valine hydroxide) can also be incorporated into proteins during the elongation stage of protein synthesis. Misincorporation of oxidized amino acids in protein affects all protein structural possibilities. As an example, Dunlop and others (2013) demonstrated that the misincorporation of the nonprotein amino acid β-N-methylamino-L-alanine (BMAA) into human proteins in the place of serine caused protein misfolding and then proper protein aggregation. In a study about misincorporation of amino acid analogs into proteins, Rodgers and Shiozawa (2008) concluded that, although misincorporation represents extreme cases, an association between damaged proteins generated by the misincorporation of amino acid analogs from diet and autoimmune symptoms is debatable.

Conclusions

After slaughter, meat tissues lose their antioxidant defense and more complex oxidative processes will emerge. Lipid, myoglobin, and protein oxidation are the major oxidative processes in uncured meat. Lipid oxidation occurs by enzymatic or nonenzymatic reactions and is influenced by both intrinsic and extrinsic factors, such as meat fatty acid composition, pro-oxidant and antioxidant levels, pH, and storage condition. Myoglobin oxidation leads to discoloration of red meat and it is influenced by oxygen concentration, pH, and the presence of aldehydes generated by lipid oxidation. Lipid and myoglobin oxidations are related in meat because superoxide anion generated by myoglobin oxidation is pro-oxidant for lipids. Metmyoglobin and perferrylmyoglobin are initiators in the chain reaction, and secondary products of lipid peroxidation (aldehydes) increase myoglobin oxidation. Protein oxidation in meat can be induced directly by RS and indirectly by secondary products of lipid peroxidation. The main oxidative modifications of protein occur at the side chains of amino acid residues or at the backbone of a protein causing fragmentation, aggregation, conformational changes in the secondary and tertiary structure, and polymerization of the protein. Secondary products of dietary lipid oxidation could constitute a health risk for atherosclerosis, cancer, as well as structural and functional damage to the cell membranes. Heme and heme-iron released from myoglobin in the digestive tract could account for the increased colorectal cancer risk associated with red meat consumption. Meat protein oxidation can induce meat nutritional value alteration and toxic oxidized amino acid formation, which could be misincorporated into nascent proteins.

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The Influence of Solid-to-Solvent Ratio and Extraction Method on Total Phenolic Content, Flavonoid Content and Antioxidant Properties of Some Ethanolic Plant Extracts

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The purpose of this study was to determine to what extent plant material, solid-to-solvent ratio, and extraction method influence the content of total phenolics and flavonoids and the antioxidant activity of ethanolic extracts obtained from dried fruits of dog-rose, sea buckthorn and hawthorn. The extractions were performed by maceration, Soxhlet and ultrasound-assisted methods, with 60% ethanol, and the solid-to-solvent ratios used were 1/5 and 1/10 (w:v). For each extract it was determined the total phenolic content (TPC), the flavonoid content (FC), the ability to scavenge DPPH•, Fe^{3+} reducing power, and also the ability of chelating Fe^{2+}. The highest total phenolics and flavonoids contents were found in dog-rose fruits extracts. Maceration and ultrasound-assisted extraction methods led to the highest concentrations of phenolics and flavonoids, and solid-to-solvent ratio 1/10 (w:v) was the most effective. The extracts of dog-rose fruits showed the highest DPPH• scavenging activity and Fe^{3+} reducing power, and the hawthorn fruits extracts registered the highest capacity of chelating Fe^{2+}.

Key words: phenolics, flavonoids, solid-to-solvent ratio, extraction method

In the last decades, physicians and patients have looked for natural drugs, non toxic remedies, suitable to the organism [1]. The researchers suggested that compounds with antioxidant activity are able to remove the excess of free radicals in the body, and thus to prevent or to cure diseases caused by oxidative stress such as cancer, degenerative diseases and cardiovascular diseases. Due to the antioxidant properties and health benefits, the researchers turned their attention to plants rich in phenolics used in traditional medicine [2 - 5]. The researchers are interested in phenolic extracts ability to scavenge free radicals, and also on the influence of the methods and conditions of extraction on the antioxidant activity of the extracts [6, 7]. Phenolics can be extracted from fresh, frozen, or dried plant material with different solvents such as methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plant materials, often with different proportions of water [6].

The nature of the extraction solvent, the solid-to-solvent ratio and the extraction method determine the amount and type of extracted phenolics, and also the antioxidant activity of the extracts. The most used solvents for the phenolics extraction are methanol and methanol-water mixtures, but very good results can be obtained with ethanol and ethanol-water mixtures. Because the ethanol is not toxic, and the mixture polarity can be set by adjusting the ethanol/water ratio, some authors [8] recommend the use of this solvent for the extraction of phenolics.

Furthermore, the concentration of total phenolics and the antioxidant activity of the extracts depend on the solid-to-solvent ratio; the increase of solvent volume increases both the extraction efficiency and the price of the extract, and decreasing the solvent volume lowers the extraction efficiency due to saturation effects, but decreases the cost price.

In this study, we aimed to investigate in which way plant material, solid-to-solvent ratio, and extraction method influence the total phenolics and flavonoids content as well as the antioxidant activity of ethanolic extracts obtained from dried fruits of dog-rose *(Rosa canina),* sea buckthorn *(Hippophae rhamnoides)* and hawthorn *(Crataegus monogyna).*

Experimental part

Extractions

Dog-rose, sea buckthorn and hawthorn fruits were harvested from the geographical area of Slanic Prahova, Romania, dried in the dark and in airflow, and then grounded. Extraction of phenolics was performed with 60% ethanol at 50°C for 3 hours. The extractions were carried out by maceration, Soxhlet and ultrasound-assisted methods, for two solid to solvent ratios: 1/5, and 1/10 (w:v), respectively. The extraction by maceration was performed using a Thermolab-GFL 1092 shaking water bath. The extraction by Soxhlet method was carried out using a VELP Scientifica extractor. The ultrasound-assisted extraction method was performed with an Elmasonic S 80 H equipment, at 60 Hz.

Determination of total phenolic content (TPC)

For the determination of TPC, the method with Folin Ciocalteu reagent was used [9]. A volume of 500 µL of plant extract was pipetted into a 10 mL test tube which contained 7.0 mL distilled water. Then, 0.5 mL Folin Ciocalteu reagent were added and the reaction mixture was vortexed and left to stand for 2 min. In the end, 2.0 mL of 20 % (w/v) Na_2CO_3 solution were added. After 20 min, the absorbance was measured at 725 nm using V670 UV-VIS Jasco spectrophotometer. The results were expressed as mg gallic acid equivalent/g dry weight (mg GAE/g DW). All analyses were performed in triplicate.

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Determination of flavonoids content (FC)

For the determination of FC, the method with aluminium chloride was used [10]. The determination of total flavonoids in extract samples started by mixing 1 mL of sample solution with 0.3 mL of 5 % $NaNO_2$ in a 10 mL test tube. After 5 min, 0.3 mL of 10 % $AlCl_3$ were added to the solution by mixing in a vortex. After 6 min of reaction, the solution was neutralized with 2 mL of 1 M NaOH. The reaction mixtures were brought to volume with distilated water. These solutions were once more mixed in a vortex and transferred to a glass cuvette. The absorbance was measured using V670 UV-Vis Jasco spectrophotometer at 510 nm. The concentration of flavonoids was expressed as mg catechin equivalent/g dry weight (mg CE/g DW). All analyses were performed in triplicate.

Analysis of phenolics by thin-layer chromatography (TLC)

5.0 μL of the extracts and standard quercetin, rutin and kaempferol flavonoids, at a concentration of 0.1% in ethanol, were applied to 0.2 mm thick silica gel plates. The following TLC system was used: ethyl acetate:methyl ethyl ketone:formic acid:water (57:27:5:10, v/v/v/v); spraying with NP/PEG as follows: 5% (v/v) ethanol NP (diphenylboric acid 2-aminoethyl ester) followed by 5% (v/v) ethanol PEG 4000 (polyethylene glycol 4000); visualization under UV light at 366 nm [11].

Determination of the antioxidant activity of the extracts

Determination of DPPH radical scavenging activity of the extracts

To assess the ability of the extracts to scavenge 1,1-diphenyl-2-picrilhidrazil (DPPH•) synthetic radical, a *photocolorimetric* method was used [12]. Briefly, 0.3 mL of extract were mixed with ethanolic solution containing DPPH radical (0.004 g/100 mL, 2.7 mL). The mixture was vigorously shaken and left to stand for 30 min in the dark. The annihilation of DPPH radical was determined by measuring the absorbance of the mixture at 517 nm. The results were expressed as % Inhibition. All analyses were performed in triplicate.

Determination of the Fe^{3+} reducing power of the extracts

Fe^{3+} reducing power of the extracts was evaluated by a photocolorimetric method [13]. An aliquot of 0.5 ml plant extract was mixed with 1 mL phosphate buffer (0.2M, *p*H 6.6) and 1 mL 1% $K_3[Fe(CN)_6]$, shaken well and incubated at 50°C for 20 min. After incubation, 1 mL TCA (10%) was added to stop the reaction. The mixture was centrifuged at 3000 rpm for 10 min. 1.5 mL supernatant, 1.5 mL distillated water and 0.1 mL $FeCl_3$ (0.1%) were mixed and incubated for 10 min. The results were expressed as absorbance at 700 nm. All analyses were performed in triplicate.

Determination of the Fe^{2+} chelating activity of the extracts

The ability of plant phenolics to chelate Fe^{2+} ions was determined photocolorimetrically [14]. In this assay, the plant phenolics bind Fe^{2+} ion, using iron (II) sulphate as ion donor. 0.85 mL of the plant extract were mixed with 1.5 mL of Tris-HCl buffer (0.1M, *p*H 7.4), followed by the addition of 1.5 mL of 500μM iron (II) sulphate. The mixture was left to stand at room temperature of 5 min. 0.15 mL of 0.25% aqueous 1,10-phenanthroline were added. The absorbance of the solution was read at 510 nm against blank. The results were expressed as % Chelation. All analyses were performed in triplicate.

Results and discussions

Determination of TPC and FC

The comparative results on TPC and FC of investigated plants depending on solid/solvent ratio and extraction method are presented in Table 1. The TPC in plant materials was in range of 6.30-32.52 mg GAE/g DW and FC ranged between 2.10-23.82 mg CE/g DW.

The TPC and FC varied depending on the plant material, solid-solvent ratio and extraction method. The TPC found in rosehip was significantly ($p < 0.05$) higher compared to the levels found in sea buckthorn and hawthorn fruits; TPC mean value for rose-dog fruits was with 13.04 mg GAE/g DW higher than the one found in sea buckthorn fruits and with 13.55 mg GAE/g DW higher than the one found in hawthorn fruits. The FC found in rosehip was significantly ($p < 0.05$) higher than the ones found in sea buckthorn and hawthorn, FC mean values found in rosehip being with 12,01 mg CE/g DW higher than the ones found in the sea buckthorn fruits and with 9.07 mg CE/g DW higher than that found in hawthorn fruits (table 1).

Rosehip TPC values registered in this study are comparable to previ-ous findings which reported values between 78 mg GAE/100 g DW [15] and 9600 mg/100 g DW [16]. Some authors [17], reported, for different varieties of *Rosa canina* from Transilvania, a TPC that ranged between 326 and 575 mg GAE/100 g frozen pulp. The FC found in rosehip was higher compared to the level reported in some studies, 41 mg QE/100 g dry fruit [18]. These results show a high variability in the content of flavonoids in the dog-rose fruits. Other studies [17] also reported for different varieties of *Rosa canina* from Transilvania, FC values that ranged between 101.3 and 163.0 mg/100 QE g frozen pulp.

In a study on frozen pulp of sea buckthorn fruits, it was reported a TPC of 14.408 mg GAE/g and a FC of 6.794 mg RE/g [19]. In another study, TPC and FC in sea buckthorn fruits from 6 growers in Central Europe were determined and there were found values that ranged between 8.62 and 14.17 g GAE kg^{-1}FM, and between 4.18 and 7.97 g RE kg^{-1} FM, respectively [20].

Hawthorn fruits showed an important content of phenolics. The found values varied depending of extraction method and solid/solvent ratio. The TPC found in hawthorn fruits had values that ranged between 6.91 and 10.89 mg GAE/g DW. These values were lower compared to those reported by another study, 28.30 mg/g DM [21]. FC values ranged in hawthorn fruits from 5.23 to 7.78 mg CE/g DW, values close to those obtained for FC reported by the same study [21], 8.77 QE/g DW. In another study, phenolics and flavonoids from different parts of hawthorn fruits were extracted; TPC value in pulp was 122.6 mg GAE/100g DW, while in peel was 123.35 mg GAE/100 g DW, while reported FC values were 60.89 mg RE/100g DW, and 71.24 mg RE/100g DW, respectively [22].

Effect of solid/solvent ratio on phenolic compounds extraction

Compared to solid-to-solvent ratio of 1/5 (w:v), 1/10 (w:v) ratio was found to be favorable for phenolic compounds extraction (table 1). The use of 1/10 (w:v) solid-to-solvent ratio showed TPC mean values higher with 14.02 mg GAE/g DW for rosehips, with 3.81 mg GAE/g DW for sea buckthorn fruits, and with 2.92 mg GAE/g DW for hawthorn fruits, compared to the mean values registered in case of 1/5 (w:v) solid-to-solvent ratio.

When the 1/10 (w:v) solid-to-solvent ratio was used, the FC mean values were higher with 9.55 mg CE/g DW for rosehips, with 1.51 mg CE/g DW for sea buckthorn fruits, and with 2.22 mg CE/g DW for hawthorn fruits, compared to 1/5 (w:v) solid/solvent ratio. The TPC and FC found in all studied vegetal materials were significantly ($p < 0.05$) higher when the 1/10 (w:v) solid-to-solvent ratio was used,

Extraction method	Solid/solvent ratio (w:v)	TPC (mg GAE/g DW)	FC (mg CE/g DW)
Dog-rose fruits (rosehips)			
Maceration	1/5	17.52 ± 2.11	12.83 ± 1.28
Soxhlet	1/5	7.28 ± 0.66	5.32 ± 0.93
Ultrasound	1/5	20.66 ± 2.17	13.82 ± 1.88
Maceration	1/10	32.20 ± 2.33	21.40 ± 2.93
Soxhlet	1/10	22.78 ± 1.52	15.41 ± 1.33
Ultrasound	1/10	32.52 ± 3.68	23.82 ± 2.18
Sea buckthorn fruits			
Maceration	1/5	7.40 ± 0.66	2.78 ± 0.51
Soxhlet	1/5	6.30 ± 0.48	2.10 ± 0.54
Ultrasound	1/5	7.96 ± 0.85	3.10 ± 0.41
Maceration	1/10	13.08 ± 1.86	5.02 ± 0.52
Soxhlet	1/10	8.25 ± 0.94	3.10 ± 0.81
Ultrasound	1/10	11.75 ±1.19	4.39 ± 0.51
Hawthorn fruits			
Maceration	1/5	7.52 ± 0.68	5.26 ± 0.52
Soxhlet	1/5	6.91 ± 0.70	5.26 ± 0.45
Ultrasound	1/5	7.01 ± 0.63	5.23 ± 0.85
Maceration	1/10	10.32± 1.98	7.53 ± 0.63
Soxhlet	1/10	9.00 ± 0.87	7.10 ± 0.69
Ultrasound	1/10	10.89 ±1.07	7.78 ± 0.65

Table 1
TOTAL PHENOLIC CONTENT (TPC) AND FLAVONOIDS CONTENT (FC) OF PLANT MATERIALS, DEPENDING ON SOLID/ SOLVENT RATIO AND EXTRACTION METHOD

compared to 1/5 (w:v) solid-to-solvent ratio.

These results were consistent with mass transfer principles where the driving force for mass transfer is considered to be the concentration gradient between the solid and the solvent [23]. Higher solid-to-solvent ratio increases the concentration gradient, leading to an increased diffusion rate of the compounds from the extracted solid material into the solvent, but also determines the increasing of the necessary period of time to achieve equilibrium. Solid-to-solvent ratio could significantly affect the equilibrium constant and characterize the relationship between yield and solvent use as a steep exponential increase followed by a steady state to give the maximum yield [24]. Similar observations were reported also by other researchers. In a study on the effect of solid to solvent ratio on the extraction efficiency of phenolic compounds from *Aquilaria crassna* [25], it was fount that the TPC increased significantly when the solid-to-solvent ratio was increased from 1/10 to 1/20, and insignificantly when the solid-to-solvent ratio was increased to 1/60. In a study on extracted phenolic compounds from olive leaves [26], it was reported the increasing of extraction efficiency of total phenols until a solid-to-solvent ratio of 1/8, and constant extraction efficiency to a solid to solvent ratio of 1/10.

Effect of extraction method on phenolic compounds extraction

The TPC found in analyzed vegetal materials was significantly dependent ($p<0.05$) to the extraction method. The mean values obtained for the extractions carried out by maceration method were significantly ($p<0.05$) higher (with 4.59 mg GAE/g DW) compared to the mean values obtained after using the Soxhlet method (table 1). The TPC mean value found in vegetal materials extracted by ultrasound-assisted method was significantly higher (with 5.05 mg GAE/g DW) compared to Soxhlet method and insignificantly ($p>0.05$) higher compared to maceration method (with 0.46 mg GAE/g DW). FC found in vegetal materials extracted by maceration method and ultrasound-assisted method were significantly higher ($p<0.05$) compared to Soxhlet method, the mean values being higher with 2.76 mg CE/g DW, and 3.31 mg CE/g DW, respectively. The differences between the mean values registered for FC after ultrasound-assisted and maceration extraction methods were insignificant (0.55 mg CE/g DW; $p>0.05$).

Ýlbay Z. *et al.* (2013) extracted phenolics from dog-rose fruits by Soxhlet and ultrasound-assisted methods (with ethanol 50%), in different extraction conditions, and they reported TPC values that ranged from 20.23 to 31.37 mg GAE/g DW, and 41.52 mg GAE/g DW to 51.18 mg GAE/g DW, respectively [7]. In our study, the TPC found in dog-rose fruits by Soxhlet method was much lower than the one found in another study that reported a level of 62.79 mg GAE/g DM [27]. This difference could be explained by the different conditions of extraction used in that study (40°C for 24 h) [27].

Analysis of the phenolics by TLC

Figure1 shows the chromatographic profile of the ethanolic extracts obtained from dog-rose fruits, sea buckthorn fruits and hawthorn fruits by maceration, Soxhlet and ultrasound-assisted methods, for solid-to-solvent ratio of 1/5 and 1/10. The comparative analysis showed that the chromatographic profile of the extracts depended only on the vegetal material. The extracts obtained from the same vegetal material contained the same phenolics, regardless of the extraction method and solid-to-solvent ratio (table 2).

Antioxidant activity of the ethanolic extracts

Antioxidant activity of phenolic compounds derives from the ability of donating hydrogen atoms or electrons to reactive radicals [28] and to be effective as metal chelators [29]. Table 3 shows the antioxidant activity of ethanolic extracts depending on their TPC.

DPPH radical scavenging activity

The ability of the extracts to scavenge DPPH synthetic radical was significantly dependent ($p<0.05$) on the vegetal material and the solid-to-solvent ratio, and insignificantly

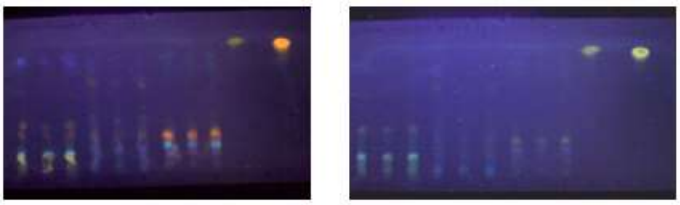

Fig. 1. Comparative thin layer chromatography (TLC) for phenolics (λ excitation 366 nm) Vegetal material: 1 – dog-rose, 2 – sea buckthorn, 3 – hawthorn; The extraction method used: a – maceration, b – Soxhlet, c – ultrasounds

dependent ($p>0.05$) on the extraction method. The highest DPPH radical scavenging activity was found for dog-rose fruits extracts, the mean values obtained being significantly ($p<0.05$) higher compared to the mean values obtained for sea buckthorn (by 10.21%) and hawthorn fruits (by 4.15%). In the case of ethanolic extracts obtained by 1/10 (w:v) solid-to-solvent ratio, it was found a DPPH radical scavenging activity significantly ($p<0.05$) lower compared to the one registered for 1/5 (w:v) solid-to-solvent ratio, excepting for rosehip extract obtained by maceration. The results obtained for all extracts are due to the lower concentration of phenolic compounds in the extracts obtained by 1/10 (w:v) solid-to-solvent ratio compared to those obtained by a 1/5 (w:v) solid-to-solvent ratio. In the case of 1/10 (w:v) solid-to-solvent ratio, dog-rose fruits extracts obtained by Soxhlet method registered a TPC concentration higher than the one obtained when 1/5 (w:v) solid-to-solvent ratio was used, but a lower DPPH radical scavenging activity was found. Some authors [30] suggested that dog-rose fruits phenolics have prooxidant activity at higher concentrations and that is why DPPH radical scavenging activity did not show an increasing trend at high concentrations.

Fe^{3+} reducing power of the extracts

Fe^{3+} reducing power of the ethanolic extracts was significantly dependent ($p<0.05$) on the plant material and on the solid-to-solvent ratio; the extraction method insignificantly influenced ($p>0.05$) the reducing power of the extracts. Fe^{3+} reducing power of rosehip ethanolic extract was significantly ($p<0.05$) higher compared to the one of sea buckthorn and hawthorn fruits extracts, the mean values being higher with 0.14 and 0.18 (A700nm), respectively. The reducing power of the extracts registered for the 1/5 solid-to-solvent ratio was significantly higher

Fluorescence (UV)	Maceration	Soxhlet	Ultrasound	Maceration	Soxhlet	Ultrasound
	Rf					
	Solid/Solvent 1:5 (w:v)			*Solid/Solvent 1:10 (w:v)*		
Dog-rose fruits						
light Blue	0.02	0.02	0.02	0.02	0.02	0.02
Yellow	0.09	0.09	0.09	0.09	0.09	0.09
light Blue	0.16	0.16	0.16	0.16	0.16	0.16
Orange	0.23	0.23	0.23	0.23	0.23	0.23
Orange	0.30	0.30	0.30	0.30	0.30	0.30
Yellow	0.37	0.37	0.37	0.37	0.37	0.37
Blue	0.87	0.87	0.87	0.87	0.87	0.87
Sea buckthorn fruits						
light Blue	0.02	0.02	0.02	0.02	0.02	0.02
Orange	0.13	0.13	0.13	0.13	0.13	0.13
light Blue	0.18	0.18	0.18	0.18	0.18	0.18
Orange	0.26	0.26	0.26	0.26	0.26	0.26
Yellow	0.38	0.38	0.38	0.38	0.38	0.38
Orange	0.68	0.68	0.68	0.68	0.68	0.68
light Blue	0.81	0.81	0.81	0.81	0.81	0.81
Hawthorn fruits						
Orange	0.05	0.05	0.05	0.05	0.05	0.05
Orange	0.11	0.11	0.11	0.11	0.11	0.11
light Blue	0.23	0.23	0.23	0.23	0.23	0.23
Orange	0.26	0.26	0.26	0.26	0.26	0.26
Blue	0.77	0.77	0.77	0.77	0.77	0.77
Blue	0.86	0.86	0.86	0.86	0.86	0.86

Table 2
RESULTS OF TLC ANALYSIS OF THE ETHANOLIC EXTRACTS OBTAINED FROM DOG-ROSE FRUITS, SEA BUCKTHORN FRUITS AND HAWTHORN FRUITS

Extraction method	Solid/Solvent ratio (w:v)	TPC (mg GAE/mL extract)	DPPH (% Inhibition)	Fe^{3+} reducing power (A700nm)	Fe^{2+} chelating activity (% Chelation)
Dog-rose fruits					
Maceration	1/5	3.50 ± 0.42	93.22 ± 4.19	0.31 ± 0.01	7.79 ± 0.65
Soxhlet	1/5	1.46 ± 0.13	91.06 ± 4.27	0.32 ± 0.01	6.17 ± 0.51
Ultrasound	1/5	4.13 ± 0.43	93.11 ± 4.18	0.38 ± 0.02	28.73 ± 2.79
Maceration	1/10	3.22 ± 0.23	93.63 ± 4.21	0.24 ± 0.01	20.78 ± 1.96
Soxhlet	1/10	2.28 ± 0.15	89.83 ± 4.00	0.20 ± 0.00	27.60 ± 1.67
Ultrasound	1/10	3.25 ± 0.37	91.37 ± 4.08	0.23 ± 0.02	25.16 ± 2.22
Sea buckthorn fruits					
Maceration	1/5	1.48 ± 0.13	91.26 ± 4.08	0.19 ± 0.01	12.01 ± 1.05
Soxhlet	1/5	1.26 ± 0.10	87.40 ± 3.87	0.15 ± 0.01	11.20 ± 0.97
Ultrasound	1/5	1.59 ± 0.17	90.65 ± 4.04	0.16 ± 0.02	17.69 ± 1.63
Maceration	1/10	1.31 ± 0.19	75.03 ± 3.22	0.09 ± 0.00	49.19 ± 4.96
Soxhlet	1/10	0.83 ± 0.09	78.62 ± 3.40	0.11 ± 0.00	53.41 ± 4.40
Ultrasound	1/10	1.18 ± 0.12	68.04 ± 2.90	0.12 ± 0.01	25.65 ± 2.40
Hawthorn fruits					
Maceration	1/5	1.50 ± 0.14	90.75 ± 4.05	0.14 ± 0.00	32.31 ± 1.17
Soxhlet	1/5	1.38 ± 0.14	89.10 ± 3.96	0.13 ± 0.00	31.50 ± 2.08
Ultrasound	1/5	1.40 ± 0.13	90.44 ± 4.03	0.13 ± 0.01	44.81 ± 2.49
Maceration	1/10	1.03 ± 0.20	86.64 ± 3.82	0.08 ± 0.02	63.64 ± 3.49
Soxhlet	1/10	0.90 ± 0.09	84.50 ± 3.71	0.07 ± 0.02	62.10 ± 3.32
Ultrasound	1/10	1.09 ± 0.11	85.92 ± 3.79	0.07 ± 0.01	65.26 ± 2.66

Table 3
ANTIOXIDANT ACTIVITY OF PHENOLIC EXTRACTS DEPENDING ON TOTAL PHENOLIC CONTENT

($p<0.05$) compared to the one registered for 1/10 ratio, due to the higher concentration of phenolic compounds in the extracts.

Fe^{2+} chelating activity of the extracts

The ability of chelating Fe^{2+} ions by the tested ethanolic extracts was significantly dependent ($p<0.05$) on the plant material, the solid-to-solvent ratio, and the extraction method used. The hawthorn fruits ethanolic extracts showed a Fe^{2+} chelating activity significantly higher compared to those of dog-rose fruits (with 30.57%) and sea buckthorn fruits (with 21.75%). Generally, ethanolic extracts obtained for 1/10 solid-to-solvent ratio showed a Fe^{2+} chelating ability significantly ($p<0.05$) higher than the extracts obtained for the 1/5 ratio. For the 1/5 solid-to-solvent ratio, the ability to chelate Fe^{2+} of alcoholic extracts obtained by ultrasound-assisted method was significantly ($p<0.05$) higher than of the ones obtained by maceration and Soxhlet methods; the mean value found for the ultrasound-assisted method was with 14.12% higher compared to that registered for Soxhlet method, and with 13.04% higher than that found for maceration method.

Correlations

Phenolic compounds are widely studied for their antioxidant properties, although the term antioxidant has a broad range of meanings. Antioxidant activity refers to both the ability of phenolic compounds to prevent damage from reactive oxygen species (ROS) (such as through radical scavenging) or to prevent generation of these species (by binding iron) [29]. Radical scavenging activity of phenolic compounds is due to their ability to act as reducing agents, hydrogen or electrons donors and singlet oxygen quenchers. In this study, the correlation between TPC and FC, TPC/FC and the antioxidant activity were determined by using linear correlations. The correlations found between TPC and FC, for the extracts obtained from the three studied vegetal materials were strong positive: dog-rose fruits ($r = 0.973$), sea buckthorn fruits ($r = 0.979$) and hawthorn fruits ($r = 0.957$). DPPH radical scavenging activity was weakly positively correlated to TPC for the sea buckthorn fruits extracts ($r = 0.479$) and hawthorn fruits ($r = 0.466$); there was no correlation between TPC/FC and DPPH• scavenging activity for dog-rose fruits extract. These results indicate that DPPH radical scavenging activity of each extract could be related not only to the concentration of phenolic hydroxyl groups, but very important is the phenolic compounds structure. Phenolic compounds included tannins, flavonoids, phenolic acids and other compounds that have phenolic structure. Flavonoids are not always phenolic compounds; this is dependent on the position of OH radical into the flavonoid structure - only flavonoids that have OH radicals in A and/or B ring are characterized as phenolic compounds. Also, phenolic acids have a lower antioxidant activity than flavonoids [31]. In previous studies on the correlations of phenolic compounds and DPPH•, the scavenging activity showed that the phenolics were involved differently ($r = 0.792$; $r = -0.772$) or no correlation were found [32]. Other

authors [30] observed a negative correlation with the metal ion chelating activity and DPPH• % inhibition at higher concentration of phenolics from dog-rose fruits.

Also, they reported that in dog-rose fruits extracts, the DPPH radical scavenging activity did not show an increasing trend at higher concentrations, moreover, the scavenging activity decreased at higher concentration [30]. The ability of Fe^{2+} chelating activity and TPC was weakly positively correlated (r = 0.462) for the dog-rose fruits extracts, weakly negatively correlated (r = - 0.570) for the sea buckthorn fruits extract, and strongly negatively correlated (r = - 0.684) for the hawthorn fruits extract. These results demonstrate the molecular heterogeneity of the extracts, Fe^{2+} chelating activity being dependent of phenolic compounds structure. Metal chelating potency of phenolic compounds depends upon their unique phenolic structure and to the number and location of the hydroxyl groups [33]. In other studies, correlations between ferrous ion chelating ability and TPC were reported as being significantly positive (r = 0.978) [34], insignificantly negative (r = - 0.412) [35] or no correlations [36]. Between FC and Fe^{3+} reducing power of the extracts was found a strong positive correlation for the sea buckthorn fruits extracts (r = 0.654) and hawthorn fruits (r = 0.818), but there were no correlations for the dog-rose fruits extracts. In other studies on the flavonoids content and the Fe^{3+} reducing power, there were reported positive correlations (r = 0.974) for the ethanolic extracts, but there were not observed any correlations for the tamarillo aqueous extracts [37].

Between the solid-to-solvent ratio and the ability of chelating ferrous ions were found strong positive correlations for dog-rose fruits (r = 0.842) and hawthorn fruits (r = 0.980), and weak positive correlations for sea buckthorn fruits (r = 0.553). Between the ability of chelating ferrous ions and ferric ions reducing power were found strong negative correlations for sea buckthorn fruits (r = - 0.712) and hawthorn fruits (r = - 0.772), and no correlations were found for dog-rose fruits.

Conclusions

Dog-rose fruits had a TPC and a FC significantly higher than sea buckthorn and hawthorn fruits. By the maceration and ultrasound-assisted methods were found TPC and FC values significantly higher than those obtained by Soxhlet method. The reducing properties of the extracts significantly depended on the vegetal material and the solid-to-solvent ratio. The 1/10 (w:v) solid-to-solvent ratio was more favorable for phenolics and flavonoids extraction than 1/5 (w:v) ratio. Rosehips ethanolic extracts showed DPPH• scavenging activity and Fe^{3+} reducing power significantly higher than those of the sea buckthorn and hawthorn fruits extracts. Hawthorn fruits extracts had the highest ability of chelating Fe^{2+}, significantly higher compared to the ones registered for sea buckthorn and dog-rose fruits extracts.

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