The paradoxical effect of extra-virgin olive oil on oxidative phenomena during *in vitro* co-digestion with meat

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Abstract

Extra-virgin olive oil is an integral part of the Mediterranean diet and its consumption has been associated with a reduction risk of chronic diseases. Here we tested the potential of extra-virgin olive oil to limit the oxidative phenomena during in vitro gastro-intestinal co-digestion with turkey breast meat. The extra-virgin olive oil was particularly rich in oleuropein aglycone isomers, which represented the 66.8% of total phenolic determined with MS/MS experiments. Meals supplemented with extra-virgin olive oil equivocally affected lipid peroxidation. At low concentration (2.5% respect to meat), a significant inhibition of lipid oxidation was observed, whereas lipid peroxidation was greatly enhanced when the amount of extra-virgin olive oil was increased in the gastro-intestinal system. The inhibitory effect observed at 2.5% extra-virgin olive oil was due to the antioxidant properties of extra-virgin olive oil phenolic compounds. At high concentration, extra-virgin olive oil phenolic compounds (especially hydroxytyrosol-derivative) behaved as pro-oxidants increasing the generation of lipid hydroperoxides from meat. At the same time, the presence in the digestive system of catalyzers from meat induced the peroxidation of extra-virgin olive oil fatty acids, which was further intensified by the pro-oxidant activity of extra-virgin olive oil phenolic compounds. Our study underlined the importance of the timing and amount of consumption of extra-virgin olive oil as well as its phenolic composition in limiting the peroxidative phenomena on meat lipids during digestion.

Keywords: extra-virgin olive oil, mass spectrometry, Mediterranean diet, oleuropein, lipid peroxidation, antioxidant activity, pro-oxidant activity
1. Introduction

The traditional Mediterranean diet is likely to be the ideal dietary pattern for the prevention of digestive tract cancers and cardiovascular diseases (Barak & Fridman, 2017). The health benefits of the Mediterranean diet have been associated with the high intake of vegetable foods rich in phytochemicals such as fruits, vegetables, cereals, legumes, nuts and seeds, the moderate consumption of fermented dairy products, fish, poultry and wine and the low intake of meats (Bach-Faig et al., 2011). In addition, also the typical cooking procedures are especially effective in guaranteeing the highest nutritional value in terms of phytochemicals bioavailability and preservation of the raw materials (Pellegrini & Fogliano, 2017). Olive oil and especially extra-virgin olive oil (EVOO) represented the typical fat of Mediterranean cuisine and showed unique healthy features (Covas, 2007). Extra-virgin olive oil, produced by mechanically pressing ripe olives, contains several bioactive and antioxidant components such as polyphenols, phytosterols and vitamin E as well as monounsaturated fatty acids (Covas, 2007). Two recent studies published in the PREDIMED project showed that a Mediterranean diet supplemented with extra-virgin olive oil reduced the incidence of major cardiovascular events and cardiovascular mortality in a Mediterranean population at high cardiovascular risk respect to a low-fat Mediterranean diet (Estruch et al., 2013; Guasch-Ferré et al. 2014). Indeed, a randomized, crossover, controlled trial suggested that daily consumption of high- and medium-polyphenol olive oil decreased oxidative damage on lipids and reduced lipid cardiovascular risk factors respect to the consumption of low-polyphenol olive oil (Covas et al., 2006).

The typical Western diet, instead, is characterized by high intake of fried foods, salty snacks, high-fat dairy products, eggs and meat and low intake of plant-based foods. Although the results are still controversial (Li et al., 2015), some studies have associated the Western dietary pattern with higher risk of colorectal tumours and cardiovascular diseases (Kesse, Clavel-Chapelon, & Boutron-Ruault, 2006). In this context, a high intake of meat (especially red meat and processed meat) has been associated with an increased risk of cancers, principally colorectal cancer, and cardiovascular
diseases (Ferguson, 2010; Micha, Wallace, & Mozaffarian, 2010). There is evidence that this risk may not be caused by meat per se, but may reflect high-fat intake, and/or carcinogens generated through various cooking and processing methods (Ferguson, 2010; Gorelik, Kanner, Schurr, & Kohen, 2013). In particular, the oxidative phenomena involving polyunsaturated fatty acids occurring during meat cooking and gastro-intestinal digestion can result in the formation of lipid oxidation products, such as lipid hydroperoxides and small reactive compounds collectively known as advanced lipoxidation end-products (ALEs), such as malondialdehyde and 4-hydroxy-2-nonenal, which might adversely affect human health following their consumption (Papuc, Goran, Predescu, & Nicorescu, 2016). Lipid hydroperoxides generated during meat cooking and especially during gastro-intestinal digestion can be absorbed in the human gastro-intestinal tract, incorporated in chylomicrons and, thus, trigger the onset and progression of atherosclerosis (Staprans, Rapp, Pan, Kim, & Feingold, 1994). Lipid hydroperoxides can also induce oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells participating in tissue injuries and in the onset and progression of degenerative diseases in humans (Wijeratne & Cuppett, 2006). ALEs are also considered highly cytotoxic, mutagenic and carcinogenic compounds, which can be also involved in the progression of atherosclerosis (Papuc et al., 2016). Strategies to inhibit the formation of lipid hydroperoxides and ALEs during gastro-intestinal digestion of meat could mitigate these health risks. Kanner and co-workers showed that red wine polyphenols inhibited the formation of lipid oxidation products during the in vitro gastric digestion of red meat and this decrease was accompanied by a reduction in the absorption of ALEs in humans (Gorelik, Ligumsky, Kohen, & Kanner, 2008a; Gorelik, Ligumsky, Kohen, & Kanner, 2008b). Other in vitro and in vivo studies demonstrated that oxidation during digestion can be reduced when meat is combined with other foods such as coffee or spices (Sirota, Gorelik, Harris, Kohen, & Kanner, 2013; Tagliazucchi, D., Verzelloni, E., & Conte, A., 2010; Van Hecke, Ho, Goethals, & De Smet, 2017).
The aim of this study was to elucidate whether extra-virgin olive oil could affect the oxidative phenomena during co-digestion with grilled turkey meat employing a harmonized basic static COST Action INFOGEST \textit{in vitro} digestive model.
2. Materials and methods

2.1. Materials

All of the digestive enzymes (α-amylase from porcine pancreas, pepsin from porcine gastric mucosa and pancreatin from porcine pancreas), phenolic standards and reagents for analytical determination were obtained from Sigma-Aldrich (Milan, Italy). The mass spectrometry reagents and solvents for phenolic compounds extraction were obtained from BioRad (Hercules, CA, USA). Turkey breast meat (pectoralis major) and extra-virgin olive oil were purchased in a local supermarket (Reggio Emilia, Italy).

2.2. Extraction of phenolic compounds from extra-virgin olive oil

Phenolic compounds from extra-virgin olive oil (EVOO) were extracted following the procedure reported in Martini, Conte, & Tagliazucchi (2017). Briefly, 15 grams of EVOO were mixed with 15 mL of a methanol/water/formic acid solution (70/28/2; v/v) and incubated for 120 minutes at 30°C in a rotary wheel. After incubation, the mixtures were centrifuged at 3000 g for 30 minutes at 4°C. When extraction was completed, the samples were stored on freezer shelves at −20°C and allowed to stand overnight for lipid precipitation and separation (Lentza-Rizos, Avramides, & Cherasco, 2001). After that, two fractions were obtained: the liquid supernatant (phenolic-rich fraction) and the solid pellet (fat fraction). Both the fractions were stored at -20°C until analysis.

2.3. Total phenolic content and antioxidant activity determination in extra-virgin olive oil phenolic-rich fraction

Total phenolic content was determined on the EVOO phenolic-rich fraction using the Folin-Ciocalteau assay and gallic acid as standard (Singleton, Orthofer, & Lamuela-Raventos, 1999). Data were expressed as mg of gallic acid equivalent per 100 g of EVOO.

The total antioxidant properties of EVOO phenolic-rich fraction were analyzed by using five different assays. The radical scavenging ability was assayed by using the ABTS assay according to
For the determination of the Fe$^{3+}$ reducing ability, a protocol based on the ferric reducing/antioxidant power (FRAP) assay was utilized (Benzie & Strain, 1999). The capacity to scavenge hydroxyl radical and superoxide anion were evaluated according to the methods reported by Martini, Conte, & Tagliazucchi (2017). The results were expressed as μmol of trolox equivalent/mmol of phenolic compounds. The Fe$^{2+}$-chelation ability of EVOO phenolic–rich fraction was evaluated by the ferrozine assay (Karama & Pegg, 2009).

**2.4. Identification and quantification of phenolic compounds by liquid chromatography**

**electrospray ionization ion trap mass spectrometer (LC-ESI-IT-MS)**

Phenolic-rich fraction of EVOO was analyzed on a HPLC Agilent 1200 Series system equipped with a C18 column (HxSil C18 Reversed phase, 250×4.6 mm, 5 μm particle size, Hamilton company, Reno, Nevada, USA) as reported in Mena, Cirlini, Tassotti, Herrlinger, Dall’Asta, & Del Rio (2016). The mobile phase consisted of (A) H$_2$O/formic acid (99.9:0.1, v/v) and (B) acetonitrile/formic acid (99.9:0.1, v/v). The gradient started at 1% B for 1 min then linearly ramped up to 40% B in 13 min. The mobile phase composition was raised up to 99% B in 13 min and maintained for 2 min in order to wash the column before returning to the initial condition. After passing through the column, the eluate was split, and 0.3 mL/min was directed to an Agilent 6300 ion trap mass spectrometer. Negative ESI-MS parameters were the same as reported in Martini et al. (2017). Identification of phenolic compounds in all samples was carried out using full scan, data-dependent MS$^2$ scanning from m/z 100 to 1500 and selected reaction monitoring.

Phenolic compounds were quantified in hydroxytyrosol equivalents with the exception of luteolin that was quantified as luteolin equivalents.

**2.5. In vitro digestion of grilled turkey breast meat**

Turkey breast meat (average size of 10x15x0.4 cm) was cooked on a grill at 140°C for 5 min until complete cooking was achieved. After cooking, the meat was cooled on ice and stored at -80°C.
overnight. Frozen meat was then homogenized in a laboratory blender and divided in portions of 5 g. Grilled and homogenized turkey meat was in vitro digested following the protocol previously developed within the COST Action INFOGEST (Minekus et al., 2014). Simulated salivary, gastric, pancreatic and bile fluids were prepared according to Minekus et al. (2014). To simulate the oral phase, 5 g of homogenized grilled turkey breast meat were mixed with 5 mL of simulated salivary fluid containing 150 U/mL of porcine α-amylase and incubated for 5 min at 37°C in a rotating wheel (10 rpm). The gastric phase was carried out by adding 10 mL of simulated gastric fluid to the bolus. The pH was adjusted to 2.0 with HCl 6 mol/L and supplemented with porcine pepsin (2000 U/mL of simulated gastric fluid). The gastric bolus was then incubated for 120 min at 37°C in a rotating wheel (10 rpm). The intestinal digestion was carried out by adding 10 mL of pancreatic fluid and 5 mL of bile fluid to the gastric bolus, adjusting the pH to 7.0 and supplemented with pancreatin. The chyme was further incubated for 120 min at 37°C in a rotating wheel (10 rpm). For each digestion, aliquots were taken after 0 and 5 minutes of salivary digestion, after 30, 60, 90 and 120 minutes of gastric digestion and after 30, 60, 90 and 120 minutes of intestinal digestion. The digested samples were immediately cooled on ice and frozen at –80°C for further analysis. The digestions were performed in triplicate. In addition, a control digestion, which included only the gastro-intestinal juices and enzymes and water in place of meat, was carried out to consider the possible impact of the digestive enzymes and fluids in the subsequent analysis.

2.6. In vitro co-digestion of grilled turkey breast meat with extra-virgin olive oil, extra-virgin olive oil phenolic-rich fraction or extra-virgin olive oil fat fraction

In the co-digestion experiments EVOO was added to the grilled and homogenized turkey breast meat in proportion of 2.5%, 5% and 10% respect to meat (w/w). After that, the in vitro digestions were carried out as reported above. Specific control digestions, in which meat was replaced with water, were carried out to check the possible impact of EVOO in the subsequent analysis.
Further experiments were carried out to gain more information about the effect of EVOO polyphenols or fatty acids on the oxidative phenomena during in vitro co-digestion with meat. These co-digestions were carried out as reported above but replacing EVOO with the corresponding amount of EVOO phenolic-rich fraction or EVOO fat fraction.

2.7. Determination of lipid hydroperoxides
Lipid hydroperoxides were extracted by 10-fold dilution in methanol HPLC grade under slow stirring for 60 min (Tagliazucchi et al., 2010). After centrifugation at 3000g for 15 min at 4°C, the hydroperoxides in the supernatants were determined with the FOX assay (Nourooz-Zadeh, 1999) at 560 nm adapted to a microplate reader. The FOX reagent contained 250 μmol/L of ammonium ferrous sulfate, 100 μmol/L xylenol orange, 25 mmol/L H$_2$SO$_4$, and 4 mmol/L BHT in 90% (v/v) methanol HPLC grade. For the assay, 60 μL of extracted sample were added to 140 μL of FOX reagent and incubated for 30 minutes at room temperature. The hydroperoxides content was expressed in nanomol H$_2$O$_2$ equivalents per g of meat.

2.8. Determination of advanced lipoxidation end-products
Advanced lipoxidation end-products were quantified as thiobarbituric acid-reactive substances (TBA-RS) on digested samples as reported by Buege & Aust (1978). Briefly, 80 μL of digested sample was added to 200 μL of water, 120 μL of trichloroacetic acid 50% and 200 μL of thiobarbituric acid solution (0.75% in 0.5 N HCl). The mixture was incubated for 30 min in boiling water, cooled, and then centrifuged at 10000g for 5 min at 20°C. The TBA-RS in the supernatant was determined at 532 nm, and the results were expressed as nanomol malondialdehyde (MDA) equivalent per g of meat.

2.9. Statistics
All data are presented as mean ± SD for three replicates for each prepared sample. Univariate analysis of variance (ANOVA) with Tukey’s post-hoc test was applied using Graph Pad prism 6.0 (GraphPad Software, San Diego, CA, U.S.A.) when multiple comparisons were performed. The differences were considered significant with $P < 0.05$. 
3. Result

3.1. Total phenolic content, phenolic profile and antioxidant properties of extra-virgin olive oil

Extra-virgin olive oil (EVOO) was characterized for its content in total phenolic compounds, individual phenolic compounds as well as for its antioxidant properties. The total amount of phenolic compounds extracted from EVOO was 127.8 ± 2.5 mg of gallic acid equivalent/100 g of EVOO. The phenolic profile of EVOO was investigated using a non-targeted procedure through LC-ESI-MS/MS experiments. The mass spectrum data along with peak assignments and retention time for the identified phenolic compounds are described in Table 1. This approach allowed the tentative identification of 33 compounds (Table 1). The total amount of phenolic compounds identified by MS/MS experiments was 98.9 ± 1.8 mg/100 g of EVOO, which represented the 77.4% of total phenolic compounds determined with the Folin-Ciocalteau assay. Considering the individual phenolic compounds, the EVOO used in our study was particularly rich in oleuropein aglycone and ligstroside aglycone. The sum of the amount of the oleuropein aglycone isomers represented the 51.7% and the 66.8% of total phenolic determined with the Folin-Ciocalteau assay and the MS/MS experiments, respectively. Instead, the amount of ligstroside aglycone isomers was the 21.6% and the 27.9% of total phenolic determined with the Folin-Ciocalteau assay and the MS/MS experiments, respectively. In addition, two non-phenolic compounds, namely elenolic acid (m/z 241; MS² ion fragments at m/z 209, 165, 139, 127, 121) and hydroxyelenolic acid (m/z 257; MS² ion fragments at m/z 225, 195, 137) were identified in the EVOO phenolic-rich extract.

EVOO phenolic compounds were able to scavenge hydroxyl radical (569.5 ± 40.5 μmol of trolox equivalent/mmol of phenolic compounds), superoxide anion (2569.2 ± 194.7 μmol of trolox equivalent/mmol of phenolic compounds) and the ABTS organic nitro-radical (1218.3 ± 134.8 μmol of trolox equivalent/mmol of phenolic compounds). There was no evidence of chelation of Fe²⁺ by EVOO phenolic-rich extract. Finally, EVOO phenolic compounds were able to reduce ferric iron to ferrous iron (187.0 ± 17.4 μmol of trolox equivalent/mmol of phenolic compounds).
3.2. Effect of extra-virgin olive oil on lipid oxidation during co-digestion with turkey breast meat

Figure 1A shows the cumulative amount of lipid hydroperoxides during in vitro digestion of turkey breast meat and during co-digestion of turkey breast meat with different amounts of EVOO. In the control digestion without meat, no reactivity with the FOX assay was detected (data not shown). The level of lipid hydroperoxides measured during the digestion of turkey breast meat remained constant during the 5 minutes of salivary digestion ($P > 0.05$). After 30 minutes of the gastric digestion, the level of lipid hydroperoxide significantly increased ($P < 0.05$) and then remained constant during the subsequent 90 minutes of gastric digestion (Figure 1A). The transition from gastric to pancreatic treatment significantly increased the lipid hydroperoxides value from $406.1 \pm 37.8$ nmol H$_2$O$_2$/g of meat at the end of gastric digestion to $2127.1 \pm 142.8$ nmol H$_2$O$_2$/g of meat after 30 min of pancreatic digestion ($P < 0.001$) (Figure 1A). Subsequently, the amount of lipid hydroperoxides decreased gradually reaching the value of $361.9 \pm 52.4$ nmol H$_2$O$_2$/g of meat after 120 min of the intestinal incubation. As reported in Figure 1B, the amount of TBA-RS tended to increase significantly ($P < 0.001$) during the first 60 minutes of gastric digestion of turkey breast meat, after that it did not change significantly during the remaining time of digestion. In the control digestion without meat, no reactivity with the TBA-RS assay was detected (data not shown).

When turkey breast meat was co-digested with 2.5% of EVOO we found a decrease in the amount of generated lipid hydroperoxides both during gastric and pancreatic digestion (Figure 1A). At the end of the gastric digestion, the amount of lipid hydroperoxides generated in presence of 2.5% of EVOO was $162.7 \pm 12.1$ nmol H$_2$O$_2$/g of meat (inhibition of 59.9%) whereas at the end of the pancreatic digestion the amount of lipid hydroperoxides dropped to a value near to zero ($23.5 \pm 7.0$ nmol H$_2$O$_2$/g of meat, which resulted in a inhibition of 93.5%). The TBA-RS production during co-digestion with 2.5% of EVOO was inhibited by 33.5% at the end of the gastric digestion and 34.4% at the end of the pancreatic digestion (Figure 1B). Surprisingly, when the amount of EVOO was increased in the digestive system we found an unexpected increase in the amount of lipid hydroperoxides (Figure 1A) at all times of digestion. The increase in lipid hydroperoxides was
dependent on the concentration of EVOO (Figure 2). Whereas, the amount of generated TBA-RS was higher in the sample co-digested with EVOO at 5% or 10% respect to turkey breast meat (P<0.05) after 30 minutes of gastric digestion, but we did not find significant differences between the digested turkey breast meat and the turkey breast meat co-digested with 5% or 10% of EVOO during the remaining time of digestion (Figure 1B and Figure 2).

3.3. Effect of extra-virgin olive oil phenolic compounds on lipid oxidation during co-digestion with turkey breast meat

When turkey breast meat was co-digested with the EVOO phenolic-rich extract at the same concentration as found in 2.5% EVOO, a strong inhibition in both lipid hydroperoxides and TBA-RS formation was revealed (Figure 3). At the end of the digestion, the formation of lipid hydroperoxide was totally inhibited whereas the inhibition recorded by the determination of TBA-RS accumulation was 30.8%. Results were quite similar to the inhibition observed after co-digestion of turkey breast meat with 2.5% EVOO. However, co-digestion of turkey breast meat with EVOO phenolic-rich fraction at the same concentrations found in 5% and 10% EVOO determined an increase in the concentration of lipid hydroperoxides, which was dependent on phenol concentration (Figure 3). The increase in lipid hydroperoxides concentration due to 5% and 10% EVOO phenolic-rich fraction was lower respect to the increase observed in presence of 5% and 10% EVOO. On the contrary, TBA-RS production was inhibited by the addition of phenolic-rich fraction to turkey breast meat at the same concentrations found in 5% and 10% EVOO (Figure 3).

3.3. Effect of extra-virgin olive oil fat fraction on lipid oxidation during co-digestion with turkey breast meat

To demonstrate a possible involvement of EVOO triglycerides in the enhancement of the lipid peroxidation observed at high EVOO concentrations, 10% EVOO was in vitro digested without meat. As reported in Figure 4A, in vitro digestion of 10% EVOO resulted in a lipid hydroperoxides
amount at the end of the digestion of 94.15 ± 1.3 nmol H$_2$O$_2$/g of meat which was not significantly different with the value measured at the beginning of the digestion (102.70 ± 11.5 nmol H$_2$O$_2$/g of meat). No TBA-RS formation was recorded during the digestion of EVOO alone (Figure 4B). However, when EVOO fat fraction was co-digested with meat, an increased formation of lipid hydroperoxides and TBA-RS respect to the digestion of meat alone was observed (Figure 4A and B).
Lipid peroxidation of poly-unsaturated fatty acids is an oxidative phenomenon, which ultimately may result in the formation of toxic compounds. A large number of studies have suggested a link between products of lipid peroxidation, such as lipid hydroperoxides and lipid oxidation end-products and various health conditions including atherosclerosis, neurodegenerative diseases, and cancer. Meat, which contains high concentrations of iron catalysers and poly-unsaturated fatty acids such as linoleic, linolenic, arachidonic, and docosahexaenoic acids, is particularly sensitive to lipid oxidation (Tirosh, Shpaizer, & Kanner, 2015). Lipid hydroperoxides and lipid oxidation end-products may be already present in meat but, more interestingly, they may be generated during its gastro-intestinal digestion (Kanner & Lapidot, 2001). In addition, lipid peroxidation proceeds rapidly when the raw meat structure is disrupted such as after cooking and mastication (Papuc et al., 2017). The main pathways to free radical chain reaction initiation in lipid peroxidation during gastro-intestinal digestion of meat involves the formation of the hydroxyl radical (HO•) through Fenton reaction, the production of perhydroxy radical (HOO•) via generation of superoxide anion (O$_2$•-) by reaction between ferrous iron with dissolved oxygen and the generation of perferrylmioglobin-containing peptides (Figure 5) (Carlsen & Skibsted, 2004; Oueslati, de La Pomélie, Santé-Lhoutellier, & Gatellier, 2016; Papuc et al., 2017). All of these reactive species are able to abstract a hydrogen from lipids generating a fatty acyl radical (L•), which in turn reacts with dissolved oxygen to form a hydroperoxyl radical (LOO•). The resulting radical can abstract a hydrogen atom from another unsaturated fatty acyl group (LH) and produce a new fatty acyl radical (L•) and a lipid hydroperoxide (LOOH). In the presence of ferrous iron (Fe$^{2+}$), lipid hydroperoxide can decompose to an alkoxy radical (LO•), which can undergo cleavage giving rise to a huge range of volatile and non-volatile compounds, collectively known as advanced lipoxidation end-products (Papuc et al., 2017) (Figure 5).

Perhydoxy and hydroxyl radicals can be easily formed in the gastric milieu in the presence of dissolved oxygen and ferrous iron (Oueslati et al., 2016). Ferrous iron is endogenously present in
meat and can be released from meat itself after cooking and mastication (Kanner & Lapidot, 2001). Oxygen can be already present in a low amount in the gastric fluid and can be released from meat following mastication (Kanner & Lapidot, 2001). Ferrous iron can generate \( \text{O}_2^- \) from dissolved oxygen (Figure 5). At low pH such as found in the gastric medium \( \text{O}_2^- \) forms \( \text{HOO}^- \), which can initiate lipid peroxidation. Indeed, in acidic medium, an important fraction of \( \text{O}_2^- \) can disproportionate into hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) and oxygen (Oueslati et al., 2016). The formation of \( \text{HO}^- \) is possible by \( \text{H}_2\text{O}_2 \) decomposition, catalysed by ferrous iron (Fenton reactions), or by \( \text{H}_2\text{O}_2 \) reaction with \( \text{O}_2^- \) (Haber–Weiss reaction) (Papuc et al., 2017). Harel & Kanner (1985) estimated the production of \( \text{H}_2\text{O}_2 \) in ground turkey muscle at 0.045 mM/h at 37°C and at pH 5.6. Oueslati et al. (2016) demonstrated that under gastric conditions and in presence of oxidants (a mixture of ferrous iron and \( \text{H}_2\text{O}_2 \)), \( \text{O}_2^-/\text{HOO}^- \) were detected in higher quantity than \( \text{HO}^- \). Increasing the pH from 3.5 to 6.5 hardly affected the kinetics of free radical production. Tagliazucchi et al. (2010) found that the complete chelation of ferrous iron by EDTA decreased the lipid peroxidation during gastric digestion of turkey breast meat by about 70% suggesting that Fenton chemistry is the most important factor in initiating turkey breast meat lipid peroxidation during gastric digestion. The lower contribution of perferrylmioglobin-mediated peroxidation in turkey breast meat can be due to the low heme-iron content of turkey breast meat (Lombardi-Boccia et al., 2002). In other meat-types, the relative contribution of free iron and heme-iron in initiating lipid peroxidation can be different depending on their concentration.

In our gastro-intestinal system, the level of lipid hydroperoxides increased during gastric digestion of turkey breast meat by 3.7-fold. According to the kinetics of formation of \( \text{O}_2^-/\text{HOO}^- \) and \( \text{HO}^- \) under gastric conditions (Oueslati et al. 2016), the greatest increase was found in the first 30 minutes of digestion (3-fold increase respect to time zero). The increase in lipid hydroperoxides during gastric digestion was followed by an increase of 3.2-fold (respect to time zero) in the amount of TBA-RS. The transition in the intestinal fluids caused an immediate 5.2-fold increase in the level of lipid hydroperoxides respect to the end of the gastric digestion. This increase could be a
consequence of the emulsification and micelization of meat fatty acids by bile salts. Previous work showed that lipid oxidation occurs faster in water-in-oil emulsion than in bulk oil or dispersion without emulsifier (Berton-Carabin, Ropers, & Genot, 2014). The causes can be related to different aspects. Firstly, the creation of interfacial area between the fat and the aqueous phase may favour the contacts between oxidants and oxygen, dissolved in the aqueous phase, and fatty acids (Berton-Carabin et al., 2014). Another cause can be attributed to the solubilisation of already formed lipid hydroperoxides in the micelles, which in turn may promote the oxidative reaction in the micelles itself (Donnelly, Decker, & McClements, 1998). In addition, it has been proved that hydrophobic bile acids, in presence of iron, enhanced lipid peroxidation of arachidonic acid (Sreejayan & von Ritter, 1998). Further incubation in simulated intestinal fluid resulted in the disappearance of lipid hydroperoxides. Rodríguez-Malaver, Leake, & Rice-Evans (1997) found that at pH 7.4 (such as found in the intestinal fluid) copper-induced formation of lipid hydroperoxides in LDL was more rapid respect to pH 5 and that lipid hydroperoxides gradually declined towards zero. Therefore, neutral or slightly alkaline pH values seem to enhance the decomposition of lipid hydroperoxides. The increased production of lipid hydroperoxides in the intestinal fluid and their rapid decomposition did not result in an increased production of TBA-RS.

Meals supplemented with EVOO may equivocally affect lipid peroxidation during gastro-intestinal digestion, as found in this study. At low realistic concentration of 2.5% of EVOO, respect to meat (w/w), a significant inhibition of lipid oxidation was observed, whereas lipid peroxidation was greatly enhanced when the EVOO amount was increased in the gastro-intestinal system. *In vitro* digestion of turkey breast meat in presence of EVOO-phenolic rich fraction at the same concentration as found in 2.5% EVOO resulted in a decrease in turkey breast meat lipid peroxidation similar to that observed during co-digestion of turkey breast meat and 2.5% EVOO (Figures 2 and 3). This effect was due to the antioxidant properties of EVOO phenolic compounds. EVOO used in this study contained 127.8 mg/100g of total phenolic compounds. Wide ranges (from 10 to 140 mg/100g) have been previously reported for the concentration of total phenolic
compounds in EVOO (Del Carlo et al., 2004; Samaniego Sánchez et al., 2007). Furthermore, we demonstrated that EVOO phenolic compounds were efficient scavengers of free radicals. Our findings suggest that EVOO phenolic compounds may act at different levels (Figure 5). In the first step of the reaction, they may scavenge superoxide anion (O$_2$•⁻/HOO•) and hydroxyl radical (HO•) preventing the initiation step of the reaction. Indeed, EVOO phenolic compounds may act as radical scavengers by reacting directly with hydroperoxyl (LOO•) or alkoxy (LO•) radical, preventing the propagation step of the reaction. EVOO phenolic compounds could act by donating a hydrogen atom to the LOO• or LO• radicals as suggested by other authors (Saija et al., 1998). There was no evidence of chelation of Fe$^{2+}$ by EVOO phenolic-rich extract, suggesting that iron sequestering is not a mechanism of lipid peroxidation prevention by EVOO phenolic compounds. EVOO phenolic compounds exerted an ambiguous pro- or antioxidant effect, depending on their concentration. In fact, in vitro co-digestion of meat and EVOO phenolic-rich fraction at 5% and 10% showed an increased lipid hydroperoxide production (Figure 3). A hypothesized reaction mechanism explaining this pro-oxidant outcome of the EVOO phenolic compounds is proposed in Figure 5. This study provides evidence that phenolic compounds are able to reduce Fe$^{3+}$ to Fe$^{2+}$, thereby stimulating the Fenton and Haber–Weiss reactions and the formation of O$_2$•⁻, HOO•, H$_2$O$_2$ and HO•. Indeed, phenolic compounds containing a catechol moiety such as hydroxytyrosol and derivative (oleuropein aglycone) may generate both O$_2$•⁻ and H$_2$O$_2$ in presence of Fe$^{3+}$ and dissolved oxygen(Eghbaliferiz & Iranshashi, 2016; Fabiani, Fuccelli, Pieravanti, De Bartolomeo, & Morozzi, 2009) (Figure 6). It can be speculated that, at high EVOO phenolic compounds concentration, the oxidative stress induced by iron re-cycling and O$_2$•⁻ and H$_2$O$_2$ produced by phenolic compounds exceed their O$_2$•⁻/HOO• and HO• scavenging properties resulting in an increased production of lipid hydroperoxides. It is however not clear why this enhanced formation was not observed for TBA-RS during the turkey breast meat digestion in presence of high EVOO phenolic compounds concentration. It is possible that, at high concentration, EVOO phenolic compounds may still
effectively scavenge LO• and LO• radicals preventing the decomposition of lipid hydroperoxides in aldehydic TBA reactive compounds.

Our results differ from Kuffa, Piesbe, Krueger, Reed, & Richards (2009) who found that the addition of low concentrations of grape seed extract during simulated gastric digestion of high-fat turkey meat had a pro-oxidant effect, while higher concentrations exerted an antioxidant effect. Similarly, Van Hecke et al. (2016) found that, during digestion of high-fat beef, phenolic acids displayed either pro-oxidant or antioxidant behaviour at lower and higher doses, respectively; whereas ascorbic acid was pro-oxidant at all doses. However, previous studies found that increasing concentrations of caffeic and chlorogenic acids as well as rutin and quercetin stimulated the formation of HO• and O₂•⁻/HOO• in a reaction mixture containing H₂O₂ and Fe³⁺ (Oueslati et al., 2016). Indeed, Tirosh et al. (2015) established that vitamin E behaved as an antioxidant or pro-oxidant depending on the concentration of un-saturated fatty acids. Probably, the balance between the pro-oxidative and antioxidative activity of phenolic compounds on lipid oxidation during gastrointestinal digestion is dependent on the type and concentration of fatty acids, the type of iron catalyzers, and type and amounts of phenolic compounds.

Since the pro-oxidant effect of polyphenols only partially explained the enhanced formation of lipid hydroperoxides during the digestion of turkey breast meat in presence of EVOO, we decided to study the involvement of EVOO fatty acids. In vitro digestion of 10% EVOO without meat did not result in the production of lipid hydroperoxides. On the contrary, as shown in Figure 4, when the fat fraction of EVOO was digested with turkey breast meat, at the same concentration as found in 10% EVOO, a significant increase in the amount of lipid hydroperoxides and TBA-RS was observed respect to the turkey meat sample. These observations indicated that, the presence of meat catalyzers promoted the peroxidation of EVOO lipids that, otherwise, did not occur during in vitro digestion of EVOO alone.

Based on our results, we believe that the pro-oxidant effect of EVOO at high concentration was a consequence of the interaction between the different variables in the system. EVOO phenolic
compounds behaved as pro-oxidants increasing the generation of lipid hydroperoxides from meat (Figure 3). At the same time, the presence in the digestive system of catalyzers from meat induced the peroxidation of EVOO fatty acids, which was further intensified by the pro-oxidant activity of EVOO phenolic compounds (Figure 4).

In a previous study, Tirosh et al. (2015) found that the addition of olive oil to turkey red meat decreased the entity of meat lipid peroxidation in a concentration-dependent manner. At the same concentration that enhanced lipid peroxidation in our system (i.e. 5% and 10% EVOO), olive oil exhibited inhibitory effect on lipid peroxidation in Tirosh et al. (2015). The different results could arise from differences in the type of meat (red vs white) used in the studies which reflected different mechanisms of initiation of lipid peroxidation. In turkey breast (white) meat, Fenton and Haber-Weiss chemistry represented the major initiators of lipid peroxidation during gastro-intestinal digestion, whereas in turkey red meat met-myoglobin had a predominant role in lipid peroxidation initiation (Kanner & Lapidot, 2001; Tagliazucchi et al., 2010). Indeed, also the phenolic composition of EVOO may have influenced the results. Our EVOO was rich in hydroxytyrosol derivative (such as oleuropein aglycone, which represented more than 60% of EVOO phenolics, Table 1) which are prone to exert pro-oxidant effects. Vice versa, tyrosol and its derivative (such as ligstroside aglycone) are not pro-oxidant (Fabiani et al., 2009). It is possible that EVOO rich in tyrosol-derivative and poor in hydroxytyrosol-derivative do not exert pro-oxidant effect during meat lipid oxidation. Thus, the specific composition of different EVOO preparations and meat but also the different heating treatments of the meat might contribute to explaining some contrasting literature findings.
5. Conclusions

This study provided evidence of a possible paradoxical effect of EVOO on lipid peroxidation during digestion of turkey breast meat. At the lowest tested concentration of 2.5%, EVOO addition to turkey breast meat resulted in an inhibition of the generation of lipid hydroperoxides and TBA-RS during gastro-intestinal digestion. This effect was ascribed to the phenolic content of EVOO. However, at higher concentration EVOO enhanced the formation of lipid hydroperoxides (but not of TBA-RS) during co-digestion with turkey breast meat. This effect was attributed to the ability of meat catalyzers to promote the peroxidation of the EVOO fat fraction and to the pro-oxidant behaviour of EVOO phenolic compounds. The balance between the enhancing effect of EVOO fat and the inhibitory effect of EVOO phenolic compounds on TBA-RS production may help to explain the results obtained with the TBA-RS assay when turkey breast meat was co-digested with EVOO. The EVOO tested in this study was particularly rich in hydroxytyrosol-derivative that are able to increase the oxidative stress during in vitro gastro-intestinal digestion as a consequence of the presence of a catechol group. Since the phenolic composition of EVOO is greatly variable depending on the cultivar and agro-climatic factors (such as growing, harvesting time, seasonal variability), it is plausible that different EVOO with different phenolic composition (i.e. high in tyrosol-derivative and low in hydroxytyrosol-derivative) may have a different impact on oxidative phenomena on lipids. Therefore, it is of paramount importance to study the phenolic composition of antioxidant-rich foods used in this type of study to better understand their impact on lipid peroxidation during the digestion of meat. Based on our findings, we therefore recommend that future studies investigate the phenolic profile of the tested antioxidant-rich foods, since some pro-oxidant effects were observed which depend on the amount and type of phenolic compounds. Indeed, our study underlined the importance of the timing and amount of consumption of EVOO in limiting the peroxidative phenomena on meat lipids, which have been partially associated with the potential adverse effects of meat consumption on human health.
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References


Figure captions

Figure 1. Turkey breast meat lipid peroxidation as affected by extra-virgin olive oil during *in vitro* gastro-intestinal digestion. (A) Changes in lipid hydroperoxides concentration. (B) Changes in advanced lipoxidation end-products measured as TBA-RS and expressed as nmol malondialdehyde (MDA)/g of meat. ( ) Meat alone, ( ) meat with 2.5% extra-virgin olive oil (w/w), ( ) meat with 5% extra-virgin olive oil (w/w), ( ) meat with 10% extra-virgin olive oil (w/w).

Figure 2. Effect of extra-virgin olive oil concentration on the amount of lipid hydroperoxides and advanced lipoxidation end-products measured at the end of the gastro-intestinal digestion of turkey breast meat. Lipid hydroperoxides (■) were expressed as nmol H$_2$O$_2$/g of meat (left y-axis) whereas advanced lipoxidation end-products (●) were measured as TBA-RS and expressed as nmol malondialdehyde (MDA)/g of meat (right y-axis). EVOO: extra-virgin olive oil.

Figure 3. Effect of extra-virgin olive oil phenolic compounds concentration on the amount of lipid hydroperoxides and advanced lipoxidation end-products measured at the end of the gastro-intestinal digestion of turkey breast meat. Lipid hydroperoxides (■) were expressed as nmol H$_2$O$_2$/g of meat (left y-axis) whereas advanced lipoxidation end-products (●) were measured as TBA-RS and expressed as nmol malondialdehyde (MDA)/g of meat (right y-axis). Left y-axis shows that EVOO phenolic-rich fraction at low concentration (2.5% w/w respect to meat) strongly inhibited the formation of lipid hydroperoxide at the end of the digestion, whereas at high concentration (5% and 10% w/w respect to meat) exerted a pro-oxidant effect leading to an increase in lipid hydroperoxide concentration. Differently, the right y-axis shows that EVOO phenolic-rich fraction generally inhibited the production of TBA-RS at each concentration. EVOO: extra-virgin olive oil.

Figure 4. Lipid oxidation during *in vitro* digestion of 10% extra-virgin olive oil alone and during the co-digestion of 10% extra-virgin olive oil fat fraction and meat. (A) Figure 4A describes the variations in the levels of lipid hydroperoxides at the end of the gastro-intestinal
digestion expressed as nmol H$_2$O$_2$/g of meat. (B) Figure 4B describes the variations in the levels of advanced lipoxidation end-products measured as TBA-RS at the end of the gastro-intestinal digestion expressed as nmol malondialdehyde (MDA)/g of meat. ( ) Meat alone, ( ) meat with 10% extra-virgin olive oil (w/w), ( ) 10% extra-virgin olive oil phenolic-rich fraction (w/w). Results showed how the EVOO fat fraction interacted with meat catalysers leading to an increased production of lipid hydroperoxides and TBA-RS. Different letters indicate that the values are significantly different ($P<0.05$).

**Figure 5. Proposed underlying mechanism explaining the anti- or pro-oxidant effect of extra-virgin olive oil phenolic compounds during gastro-intestinal digestion of turkey breast meat.**

At low concentration, extra-virgin olive oil phenolic compounds act as antioxidant inhibiting meat lipid peroxidation (phenolic compounds in light-grey boxes). Extra-virgin olive oil phenolic compounds may act at different levels. In the first step of the reaction, they may scavenge superoxide anion (O$_2^•$/HOO$^•$) and hydroxyl radical (HO$^•$) preventing the initiation step of the reaction. Indeed, extra-virgin olive oil phenolic compounds may act as radical scavengers by reacting directly with hydroperoxyl (LOO$^•$) or alkoxy (LO$^•$) radical, preventing the propagation step of the reaction. At high concentration, extra-virgin olive oil phenolic compounds act as pro-oxidant enhancing meat lipid peroxidation (phenolic compounds in black boxes). Extra-virgin olive oil phenolic compounds are able to reduce Fe$^{3+}$ to Fe$^{2+}$, thereby stimulating the formation of O$_2^•$, HOO$^•$, H$_2$O$_2$ and HO$^•$ and increasing the oxidative stress in the gastro-intestinal milieu. Indeed, phenolic compounds containing a cathecol moiety such as hydroxytyrosol and derivative (oleuropein) may generate, in presence of Fe$^{3+}$ and dissolved oxygen, both O$_2^•$ and H$_2$O$_2$ (see Figure 6). R$^1$ may indicate either a hydrogen (tyrosol) or a hydroxyl group (hydroxytyrosol). R$^2$ may indicate either a hydroxyl group (tyrosol or hydroxytyrosol) or an elenolic acid group (ligstroside aglycone or oleuropein aglycone).
Figure 6. Production of $\text{O}_2^*$ and $\text{H}_2\text{O}_2$ by phenolic compounds containing a catechol moiety in presence of $\text{Fe}^{3+}$. $R^2$ may indicate either a hydroxyl group (hydroxytyrosol) or an elenolic acid group (oleuropein aglycone).
Table 1. Mass spectra and quantitative data for phenolic compounds identified in the extra-virgin olive oil phenolic-rich extract. Values represent means ± standard deviation of triplicate determination.

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<th>Compound</th>
<th>[M-H]$^-$(m/z)</th>
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*Total phenolic compounds* 98.93 ± 1.84

*Quantified as hydroxytyrosol equivalent with the exception of luteolin which was quantified as luteolin equivalent*