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VALORIZATION OF BY-PRODUCTS FROM OLIVE OIL INDUSTRY AND THEIR UTILIZATION FOR INNOVATIVE FOOD FORMULATION

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ABSTRACT

The purpose of this thesis work was the valorization of the main by-products obtained from olive oil production chain (wastewater and pomace) and their utilization in innovative food formulation. In the first part of the thesis, an olive mill wastewater extract rich in phenols were used in the formulation of 3 innovative meat products: beef hamburgers, cooked ham and würstels. These studies confirms that olive mill wastewaters extract rich in phenols could be an alternative for the reduction/total replacement of additives (i.e., nitrites) in ground and cooked meat preparations, which would promote the formulation of healthier clean label products and improve the sustainability of the olive oil industry with a circular economy approach, by further valorizing this olive by-product. In the second part of the thesis, the lipid composition and oxidative stability of a spreadable product obtained from a fermented and biologically de-bittered olive pomace, was assessed during a shelf-life study. This study confirmed that olive pomace represents an excellent ingredient for the formulation of functional foodsIn the third and last part of the thesis, carried out at the Universidad de Navarra (Pamplona, Spain), Department of Nutrition, Food Science and Physiology (School of Pharmacy), under the supervision of Prof. Icíar Astiasarán and Prof. Diana Ansorena during a period abroad (3 months) that was financially supported by the Marco Polo program, three extracts obtained from purification of olive mill wastewaters, were subjected to *in-vitro* digestion and characterized. From the analysis of the three phenolic extracts, it emerged that the most promising extract to be used in the food field is the spry-dried one. Thanks to its formulation containing maltodextrins it manages to maintain its antioxidant capacity even after being underwent to *in-vitro* digestion. This thesis work is a part of the PRIN 2015 project (PROT: 20152LFKAT) "Olive phenols as multifunctional bioactives for healthier food: evaluation of simplified formulation to obtain safe meat products and new foods with higher functionality", coordinated by University of Perugia.

SOMMARIO

Lo scopo di questo lavoro di tesi è stato la valorizzazione dei principali sottoprodotti ottenuti dalla filiera di produzione dell'olio d'oliva (acque reflue e sansa) ed il loro utilizzo per la formulazione di alimenti innovativi. Nella prima parte della tesi, un estratto di acque reflue di frantoio ricco di fenoli è stato utilizzato nella formulazione di 3 prodotti innovativi a base di carne: hamburger di manzo, prosciutto cotto e würstel. Questi studi confermano che l'estratto di acque reflue di frantoio ricco di fenoli potrebbe essere un'alternativa per la riduzione/sostituzione totale degli additivi sintetici (ad es. nitriti) nelle preparazioni a base di carne macinata e nei prodotti carnei cotti, che promuoverebbe la formulazione di prodotti clean label più sani e migliorerebbe la sostenibilità della filiera di produzione dell'olio d'oliva con un approccio di economia circolare, valorizzando ulteriormente questo sottoprodotto. Nella seconda parte della tesi è stata valutata durante uno studio di shelf-life la composizione lipidica e la stabilità ossidativa di un prodotto spalmabile ottenuto da sansa di oliva fermentata e biologicamente deamarizzata. Questo studio ha confermato che la sansa di oliva rappresenta un ottimo ingrediente per la formulazione di alimenti funzionali. Nella terza e ultima parte della tesi, svolta presso l'Universidad de Navarra (Pamplona, Spagna), Dipartimento di Nutrizione, Scienze e Fisiologia degli Alimenti (Scuola di Farmacia), sotto la supervisione della Prof.ssa Icíar Astiasarán e della Prof.ssa Diana Ansorena durante un periodo all'estero (3 mesi) sostenuto finanziariamente dal programma Marco Polo, tre estratti ottenuti dalla depurazione delle acque reflue di frantoio, sono stati sottoposti a digestione *in vitro* e caratterizzati. Dall'analisi dei tre estratti fenolici è emerso che l'estratto più promettente da utilizzare in campo alimentare è quello spray-dry. Grazie alla sua formulazione contenente maltodestrine, infatti, riesce a mantenere la sua capacità antiossidante anche dopo essere stato sottoposto a digestione *in vitro*. Questo lavoro di tesi fa parte del progetto PRIN 2015 (PROT: 20152LFKAT) "*Olive phenols as multifunctional bioactives for healthier food: evaluation of simplified formulation to obtain safe meat products and new foods with higher functionality*", coordinato dall'Università di Perugia.

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Chapter 1 Aim of the thesis

Currently, olive oil is mainly produced in the Mediterranean area and its consumption is increasing globally. During the various stages of olive oil production, however, large quantities of by-products are produced, which have a significant environmental impact. In fact, the production of about 200 kg of virgin olive oil also results in about 800 kg of pomace and 200 kg of wastewater.

Pomace and wastewaters retain remarkable nutritional properties mainly due to the presence of bioactive compounds, including fatty acids, phytosterols, tocopherols and phenolic compounds. The latter include secoiridoids such as oleuropein and phenolic alcohols such as tyrosol (*p*-HPEA) and hydroxytyrosol (3,4-DHPEA), for which antioxidant, anti-inflammatory, antitumor and antidiabetic properties have been demonstrated. Considering the environmental impact of the virgin olive oil production chain, as well as the growing need and interest in more sustainable food production systems, the reuse of bioactive substances naturally present in olive pomace and wastewaters is a valid option for the enhancement of these by-products.

Wastewater contains about 98% of the phenolic compounds present in the olive. These compounds, suitably extracted and purified by membrane technology, can be used for the formulation of innovative food products. Thanks to their high antioxidant potential due to their capacity to act as radical scavengers, they can be used in formulations for the reduction of synthetic antioxidants, such as nitrates and nitrites. In 2015, the International Agency for Research on Cancer (IARC) classified processed meat added with nitrate/nitrite salts as carcinogenic to humans (Group 1), as they can give rise to a series of compounds (N-nitrous compounds (NOCs), nitrosamines and oxidation products), during cooking, subsequent ingestion and digestion that seem to contribute to the onset of colorectal cancer. Independently from the technological advantages, a reduction in the use of nitrates and nitrites has become a matter of primary importance for both industries and consumers. However, their complete or partial replacement with a single natural compound is a challenge, due to the multifunctional characteristics of nitrates and nitrites.

Olive pomace, instead, seems to be an excellent substrate for making functional foods by extracting bioactive compounds from it (such as polyphenols), which can be added to foods. However, the extraction process involves the use of solvents which does not contribute to improve the sustainability of this food chain; a valid alternative could be the use of pomace as such after been suitably treated (for example pitted or dehydrated). The use of pomace, however, significantly changes the consistency of some foods and determines the presence of a strong bitter taste, due to

the phenols present in this by-product. Therefore, it is necessary to de-bitter (chemically and/or biologically) olive pomace, so that it can be used in the formulation of new foods.

The purpose of this thesis work, therefore, was the valorization of the main by-products obtained from olive oil production chain (wastewater and pomace) and their utilization in innovative food formulation. In the first part of the thesis, an olive mill wastewater extract rich in phenols were used in the formulation of 3 innovative meat products: beef hamburgers, cooked ham and würstels.

For beef hamburgers, two experiments were conducted; for the first, the main objectives were: i) to verify the effectiveness of the powder formulation of a phenolic extract from olive mill wastewaters (PE) as preserving agent for the extension of the shelf-life and the oxidative stability on the hamburgers; ii) to define the sensory profile of these new phenol-enriched meat products; and iii) to monitor the perception of sensory characteristics and the presence of unacceptable sensory attributes and/or off-flavors during storage. The aim of second experiment was to evaluate the ability of the powder formulation of the same PE to counteract the formation of cholesterol oxidation products (COPs) and heterocyclic aromatic amines (HCAs) in grilled beef burgers subjected to a shelf-life study, as well as than to evaluate its impact on their mutagenicity and cytotoxicity.

For cooked ham, the study aimed at evaluating the effect of the addition of the PE on the oxidative stability of this cooked meat products, for a partial/total nitrite replacement in cooked meat products.

Finally, for würstels, the study aimed at evaluating the effect of the addition of the PE on the stability and sensory characteristics of steam cooked and grilled würstels. Specifically, to verify the effectiveness of the phenolic extract as preservative agent for the extension of the shelf-life and the oxidative stability on the würstels and to monitor the presence of unacceptable sensory attributes and/or off-flavors during storage.

In the second part of the thesis, the lipid composition and oxidative stability of a spreadable product obtained from a fermented and biologically de-bittered olive pomace, was assessed during a shelf-life study. Two different product formulations were prepared using probiotics (*Lpb. Plantarum*) of human origin and isolated from fermented foods. To ascertain the palatability of the olive cream, a descriptive sensory test (Flash Profile) was performed with untrained consumers.

In the third and last part of the thesis, three extracts obtained from purification of olive mill wastewaters, were subjected to *in-vitro* digestion and characterized. The content and composition of phenols, condensed tannins and antioxidant activity was determined before and after *in-vitro* digestion.

This thesis work is a part of the PRIN 2015 project (PROT: 20152LFKAT) "Olive phenols as multifunctional bioactives for healthier food: evaluation of simplified formulation to obtain safe meat products and new foods with higher functionality", coordinated by University of Perugia.

Chapter 2 Introduction

2.1 Environmental impact of olive oil production

The olive oil industry is considered a very important sector in the Mediterranean Agro-food chain since olive oil is a fundamental part of the various local diets as it has peculiar organoleptic and health characteristics that have caused an increase in consumption and production. Olive oil has excellent nutritional properties, and its consumption is increasing globally, pushing countries such as Argentina, Australia, the United States to emerge as producers by promoting olive cultivation (Maffia et al., 2020). As regards the production of olive oil, according to the latest data released by the International Olive Council (COI) for the 2020/2021 campaign, it is estimated a world production of olive oil of 3.3 million tons (3% more than the 'last year). The main world leader is Spain (1.6 million tons) followed by Greece (265 thousand tons), Italy (255 thousand tons, 30% less than the previous year's campaign) and Tunisia (120 thousand tons) (ISMEA 2020). The increasing production can be explained by the success of the Mediterranean diet, which is associated with a lower incidence of atherosclerosis, some cancers, and cardiovascular and neurodegenerative diseases (Banias et al., 2017). With the world population increasing, the demand for greater food production is also growing and this constitutes a challenge for agriculture, aiming for greater sustainability of the agri-food supply chains. To be sustainable, the olive oil supply chain requires a process of continuous improvement, in which the most impacting techniques, those harmful to the environment, are progressively replaced with more sustainable ones (Maffia et al., 2020). The production of olive oil, however, involves the consumption of large quantities of resources and the generation of emissions into the air that significantly downgrade the natural environment. For example, the use of chemicals (pesticides, fertilizers) causes significant damage to the environment by polluting especially groundwater; in addition, the use of various machinery on farms is responsible for fuel and energy consumption and therefore affects the increase in emissions into the environment. It is in the oil extraction phase, however, that the dominant environmental problem is included, considering that, for example, the water used for washing the olives further increases the production of liquid effluents in the mill. Overall, the main environmental impacts during the production of olive oil (release of CO_2, energy, wastewater, fuel consumption, use of pesticides and fertilizers, emissions) are recorded during the production and distribution chain (**Table 2.1**).

Table 2.1 Recording of the impacts resulting from each phase of olive oil production (Adapted from Banias et al., 2017)

	Farming	Manufacturing	Packaging	Transportation	Warehousing	Reverse logistics
CO_2	✓	✓	✓	✓		
Energy	✓	✓	✓	✓		
Pesticides	✓					
Fertilizers	✓					
Emissions	✓	✓	✓	✓		✓
H_2O	✓	✓				✓
Waste	✓	✓	✓		✓	
Fuel	✓	✓		✓		
Soil	✓					✓

The production of olive oil also generates various waste (wood, branches, leaves) and by-products (olive pomace, vegetation water, olive stones) in large quantities (Donner & Radić, 2021).

Pursuant to Decision 2000/532 of the European Union Commission and the Eurostat database classification, waste from agricultural activities (e.g., corn, wheat, fruit, vegetables, rice, pomace, olive waste) are included in the category of agricultural livestock waste (ALW) (Abbattista et al., 2021). Among the three different extraction processes (traditional hydraulic pressure, two and three-phase centrifugation processes) there is a truly remarkable amount of by-products production: in the traditional process, from a ton of olives used there is a production of 200 kg of olive oil with a waste of 200-400 kg of pomace and 400-600 kg of vegetation water. In the three-phase production process, on the other hand, for the same quantity of olive oil produced, 500-600 kg of pomace and 1000-1200 kg of vegetable water are obtained (Donner & Radić, 2021). These by-products, in addition to representing an economic problem for producers, pose serious environmental concerns; their partial reuse, like that of all agronomic production residues, represents, to date, one of the objectives to be pursued (Abbattista et al., 2021). The enhancement of the by-products of the olive industry must be a general commitment to the environment but not only, as the reuse of waste constitutes a resource in many fields. In fact, if until now the waste and by-products of olives have been more than anything else converted into products with low added value (bioenergy or fertilizers), there are several initiatives that already successfully market products with high added value (e.g., extracts obtained from waste for their use in the cosmetic field, to produce handicraft products, etc.) (Donner & Radić, 2021). This must go hand in hand with the updating and strengthening of technological processes such as, for example, the implementation of biorefineries (Donner & Radić, 2021).

2.2 Olive oil extraction processes

The operations underlying the mechanical extraction process of olive oil generate interactions between water, oil, constituent parts of the pulp and almond and minor components of the oil such as sterols, terpenes, polyphenols, volatile substances of enzymatic derivation, which have important effects on the oil quality. In some mills, especially in the small ones, the practice of storing the olives before processing is widespread. In this phase, the temperature, time and method of conservation of the olives are very important since, for example, excessively long storage periods lead to an increase in the activity of lipases and therefore to an increase in free acidity and a modification the aroma of the oil; in fact, to date, it is concluded that the conservation of the olives should be avoided and that the processing of the olives should be carried out within 24 hours after harvesting (Servili et al., 2012). Then, we move on to the processing of the olives which can be divided into 5 fundamental phases (**Figure 2.1**):

- Washing (with eventual previous defoliation)
- Crushing
- Kneading
- Extraction
- Storage

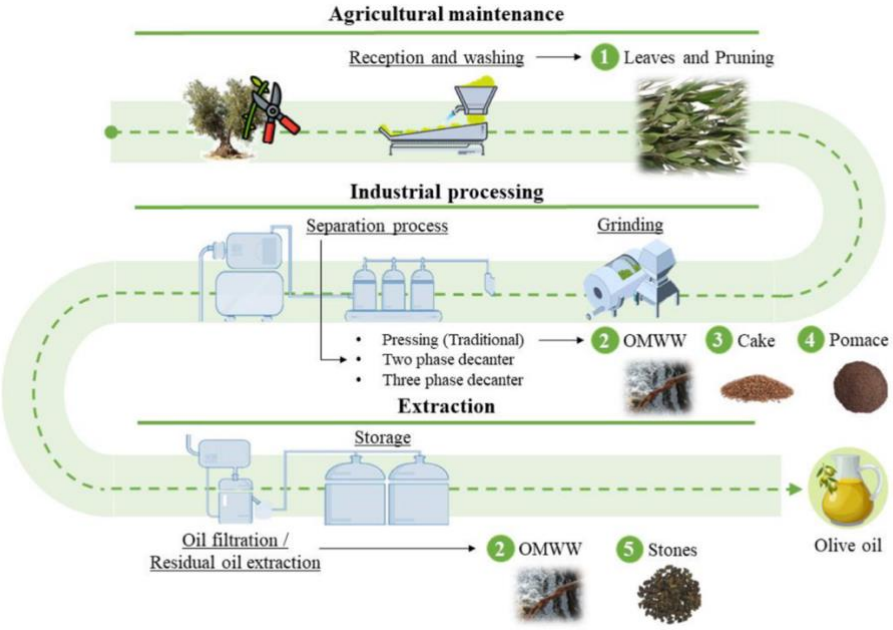

Figure 2.1 Olive oil processing and by-products of the olive oil industry from agricultural maintenance (leaves and pruning biomass) and industrial process (aqueous olive mill wastewater, cake, pomace and stones) (Modified from Otero et al., 2021).

2.2.1 Washing and defoliation

Defoliation is an additional operation but recommended since the presence of leaves does not bring positive effects to the production of the oils but can change the taste and aroma in a negative way, giving hints of bitterness due to the presence of polyphenols in these scraps; for washing (**Figure 2.2)**, continuous washing machines are normally used in which the olives are unloaded, washed by immersion and here undergoes continuous mixing. In this way, the olives freed from impurities pass to the second step, that is the pressing. A critical component of this first process lies in the characteristics of the washing water which should be replaced frequently based on the level of contamination of foreign bodies (e.g., earth) of the olives to be processed: in fact, a water too rich in earthy particles it can give the oil negative sensations, such as the “earth” defect (Servili et al., 2012).

Figure 2.2 Olives defoliation and washing

2.2.2 Crushing

This second phase consists in crushing the entire structure of the drupe, even the stone, giving rise to the so-called "olive paste", to allow the extraction of oils from it. This is a critical phase both from a biochemical and mechanical point of view since:

- endogenous enzymes are activated (with glycosidase and lipoxygenase activity) which allow the formation of volatile substances, subsequently leading to the constitution of aldehydes, saturated and unsaturated alcohols at C5 and C6 which are responsible for the fruity herbaceous aroma of the oil;

- other enzymes are activated such as polyphenol oxidase (PPO) and peroxidase (POD) which begin to degrade phenolic substances only later, during malaxing;
- some phenolic compounds begin to be transferred into the oil, thanks to the mechanical intervention exerted on the cellular structures of the fruit and the activation of endogenous enzymatic complexes that modify the molecular structure of these substances, increasing their liposolubility.

Precisely due to the mechanical intervention, an emulsion process begins between the oil present in the vacuole ("vacuole oil") and colloidal substances which makes it difficult to recover oil, for this reason it is necessary to move on to the third step, or kneading, to allow the re-compacting of the oil drops. During the pressing phase, the olives can be de-stoned which allows, by eliminating the almond, to reduce the peroxidase activity of the enzymes and to preserve the phenolic characteristics of the oil (Servili et al., 2012). Technological innovations have ensured that, for crushing, we pass from a system of mullers to a system of continuous crushing: crushers are machines characterized by the continuity of the crushing work. The objective of this phase is that the process of degradation of the fruit tissues is more marked towards the epicarp and the mesocarp (to favor the release of the oil and the pigments of the peel) rather than intervening on the almond, where the impact must necessarily be more limited (Servili et al., 2012).

2.2.3 Malaxing

Olive oil is present in the vacuoles of the mesocarp cells of the olive fruit and in limited quantities even in small drops that are found in the colloidal system of the cytoplasm, in the epicarp and in the endosperm. The malaxing plays a very important role; the malaxing machine consists of a tank in which warm water circulates and blades rotate inside which allow the mixing of the dough obtained from the pressing which is kept at a controlled temperature (**Figure 2.3**). This operation causes the breakage of the water-oil emulsion caused by pressing, allowing the small oily drops to gather in larger drops (coalescence). The kneading operation involves very important modifications to the minor compounds and the result is strictly connected to the management of three variables at the same time (to be defined and controlled based on the characteristics of the olives to be processed): temperature, duration of the operation and conditions of aeration of the dough in the kneading machine. During the malaxing (in open malaxers, in contact with the air) an important process takes place, the oxidation of phenolic substances. In fact, the concentration of these substances in the olive pastes decreases both in the oils and in the by-products. This phenomenon is less in closed malaxers because they limit the exchange of oxygen with the outside and, in some cases, allow you

to work under nitrogen. In this additional phase, endogenous oxide-reductive enzymes such as PPO and POD act which can, in fact, promote the oxidation of polyphenols during the process (Servili et al., 2012).

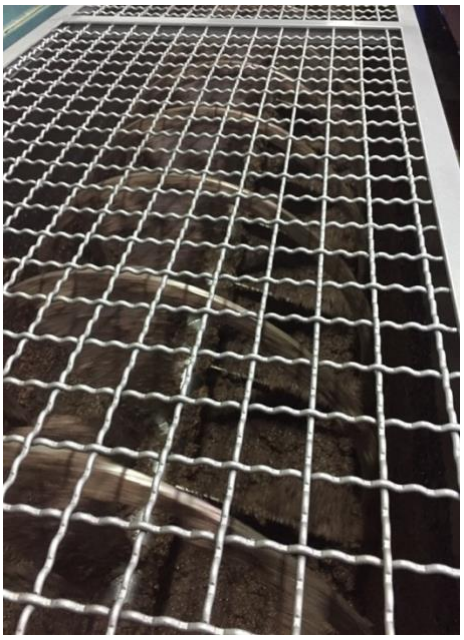

Figure 2.3 Malaxing

2.2.4 Extraction

To obtain the separation of the oil or oily must from the pastes obtained from kneading, several principles must be considered: pressure, centrifugation, selective filtration, or surface tension. The most significant variables mainly concern the amount of water used in the system and there are currently some innovations in the systems used for continuous centrifugation (Servili et al., 2012). To date, the most used process to separate the olive oil from the olive paste is horizontal centrifugation, in turn divided between:

- Two-phase method;
- three-phase method.

In general, the compounds on which the extraction process intervenes are the phenolic fraction and the volatile component, causing in any case a loss of the former and a modification of the latter. Furthermore, depending on the type of horizontal centrifugation used, different by-products are obtained (**Figure 2.4**).

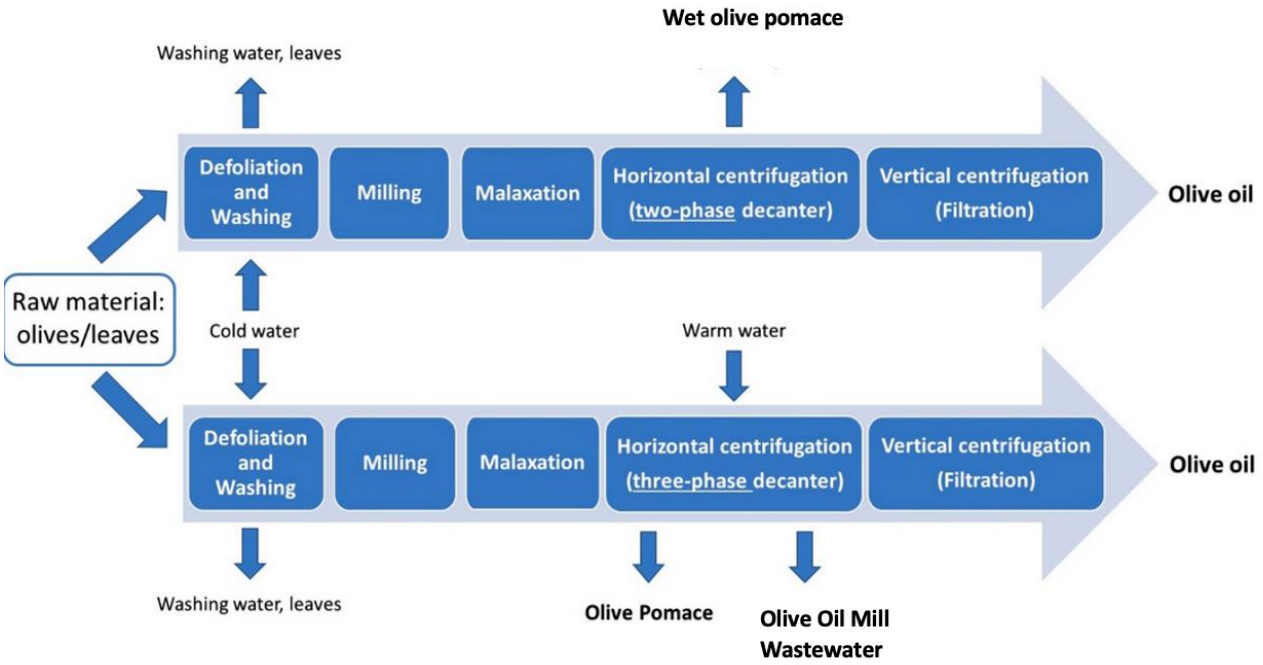

Figure 2.4 Layout of the main steps of olive oil production involving two- or three-phase horizontal centrifugation and the main by-products obtained (Modified from Abbattista et al., 2021).

In the two-phase method for horizontal centrifugation, no water is added to the olive paste, which is obtained from kneading and, generally, a higher level of phenolic compounds is recorded (Servili et al., 2012), and they are also obtained as by-products:

- Wet olive pomace: that is a moist semi-solid, made up of olive peel, crushed olive stones and aqueous solution (Dermeche et al., 2013).

In the three-phase method, however, large quantities of water are used to improve the efficiency of oil extraction and two types of by-products are obtained (Abbattista et al., 2021):

- Olive pomace
- Olive mill wastewater (OMWW)

The most modern method of two-phase centrifugal extraction, named "ecological" due to the reduction of water consumption, returns a single by-product, the wastewater from the mill that groups the pomace with the vegetation waters (oil wastewater) (Abbattista et al., 2021).

2.2.5 Storage

The storage phase represents a further critical point for the quality of the oil in production. To delay the alteration processes such as souring and oxidation, the storage temperatures of the oils must be kept between 12°C and 15°C (Servili et al., 2012). Furthermore, as regards the evolution of phenolic substances in the preservation process, there may be enzymatic activities capable of hydrolyzing oleuropein derivatives and other compounds, resulting in an increase in the

concentration of hydroxytyrosol (3, 4-DHPEA) and free tyrosol (*p*-HPEA). This causes an alteration in the stability and sensory characteristics of the oil. These enzymes being in the colloidal fraction can be removed by filtration (Servili et al., 2012).

2.3 Olive processing by-products

The production of high-quality olive oils involves the generation of large quantities of solid residues and/or wastewater which can have a great impact on the terrestrial and aquatic environment due to their high phytotoxicity (Roig et al., 2006). Among the by-products already mentioned, wastewaters are the most difficult to manage since they can have negative effects on the microbial populations of the soil (Paredes et al., 1987): they have antimicrobial activity, high acidity and phytotoxicity, which make its biological degradation and its disposal for the main olive oil producing countries (also considering the considerable quantities of wastewaters that are generated every year). Lately, attempts have been made to enhance the wastewaters in various ways by trying, for example, to use them as a source of energy, as fertilizers or additives in animal feed (Abbattista et al., 2021). However, a large amount of these by-products remains unused, and this constitutes a problem as well as a challenge for the supply chain, since these compounds, having a high biological value, could be better exploited. These wastes, in fact, keep within them a heterogeneous mixture of many chemical components, such as metal ions, carbohydrates and polyphenols with antioxidant activity (Dermeche et al., 2013). To date, however, still most of the phenolic compounds of olives are mostly retained in the corresponding waste and only a small percentage (approx. 2%) is transferred to the olive oils during production. In recent years, however, scientific research has tried to find alternative solutions to the disposal of these by-products (Abbattista et al., 2021), which go in the direction of extracting bioactive compounds and therefore promoting circular economy and industrial symbiosis paths, with the final goal of improving the sustainability of the supply chain.

2.3.1 Olive mill wastewater

Wastewater was the most abundant by-product generated during the production of the olive oil and is the one that generates the most environmental problems. The annual world production of wastewater is estimated at between 7 and over 30 million m^3 (Abbattista et al., 2021). Wastewater was a slightly acidic liquid (pH from 2 to 6), of a color ranging from red to black, with high conductivity (Abbattista et al., 2021) (**Figure 2.5**).

Figure 2.5 Olive mill wastewater

Its composition varies both qualitatively and quantitatively depending on the variety of olives, climatic conditions, cultivation practices, the storage time of the olives and the oil extraction process. In addition to water (83-92%), the main components of these are phenolic compounds (0.6-2.0%), sugars (1.5-12.2%) and 3-15% of organic material (including carbohydrates, lipids, pectin, organic acids, polysaccharides, phenols, tannins and lignin) (Abbattista et al., 2021). Furthermore, among the minerals the potassium ion content is quite high (Abbattista et al., 2021). For many years this waste has been dumped in the soil and rivers, and it is precisely the long-chain fatty acids present in wastewater that represent a problem for the environment as they are toxic to microorganisms inside plants and soil.

2.3.2 Olive pomace

The virgin pomace constitutes the solid by-product of olive processing, formed by the fibrous part of the fruit, from the stone, of approx. 5% of residual oil and a quantity of water that can vary according to the type of centrifugation used. The pomace, in fact, can appear from humid to semi-solid (**Figure 2.6**): when using two-phase centrifugation plants, the water content is between 50% and 70%, while the water content is reduced with the three-phase centrifugation plants (35-40%) and with the traditional discontinuous pressurized ones (with 20-25% water) (Abbattista et al., 2021).

Figure 2.6 Olive pomace

The approximate composition of the pomace can be summarized as follows:

- water (60-70%),
- lignin (13-15%),
- cellulose and hemicellulose (18-20%),
- oil retained in the pulp (2.5-3%).

While the main organic compounds are sugars (3%), volatile fatty acids (C2-C7) (1%), polyalcohols (0.2%), proteins (1.5%), polyphenols (0.2%), pigments (0.5%), vitamin E (α-tocopherol, β-tocopherol, α-tocotrienol and γ-tocopherol) present however mainly in the form of α-tocopherol (> 2.6 mg/100 g), in the lipid fraction is oleic acid content (approx. 75%), followed by palmitic, linoleic and stearic acids (Abbattista et al., 2021).

Finally, the phenolic content of virgin pomace depends on the oil extraction system used; the virgin pomace obtained from three-phase extraction plants have approx. 48% of phenolic compounds, while in the pomace obtained from the two-phase system there is a higher content of phenolic compounds since the fraction of vegetation water is also present. It is possible to have two types of virgin pomace: one is the pomace that is obtained from post-extraction olive de-stoning, the second is a pomace obtained from pre-de-stoned olives, obtained from oils extracted from de-stoned pastes. These two products differ in their oil content, in the level of fiber and lignin, and above all in the content of antioxidants, which is higher in the pomace obtained from pre-de-stoned olives.

2.4 Phenolic composition of olive processing by-products

About 98% of the phenols contained in the olive, however, are not found in extra virgin olive oil, but in by-products generated during olive oil production (Caporaso et al., 2018). The most present phenolic compounds are hydroxyphenylethanol (or tyrosol, *p*-HPEA), dihydroxyphenylethanol (or hydroxytyrosol, 3,4-DHPEA) the aglyconic forms of oleoeuropein, demethyloleuropein and

ligostroside such as 3,4-DHPEA- EA (or 3,4-DHPEA-elenolic acid mono-aldehyde or oleuropein-aglycone mono-aldehyde), 3,4-DHPEA-EDA (or 3,4-DHPEA-elenolic acid di-aldehyde, or oleuropein-aglycone of -aldehyde), *p*-HPEA-EDA (*p*-HPEA-elenolic acid di-aldehyde or ligstroside-aglycone di-aldehyde), in addition to verbascoside (Proietti et al., 2012) (**Figure 2.7**).

The phenolic substances contained in wastewater belong to different classes of compounds such as phenolic acids and flavonoids, but the phenolic compounds most present are the secoiridoids (Abbattista et al., 2021).

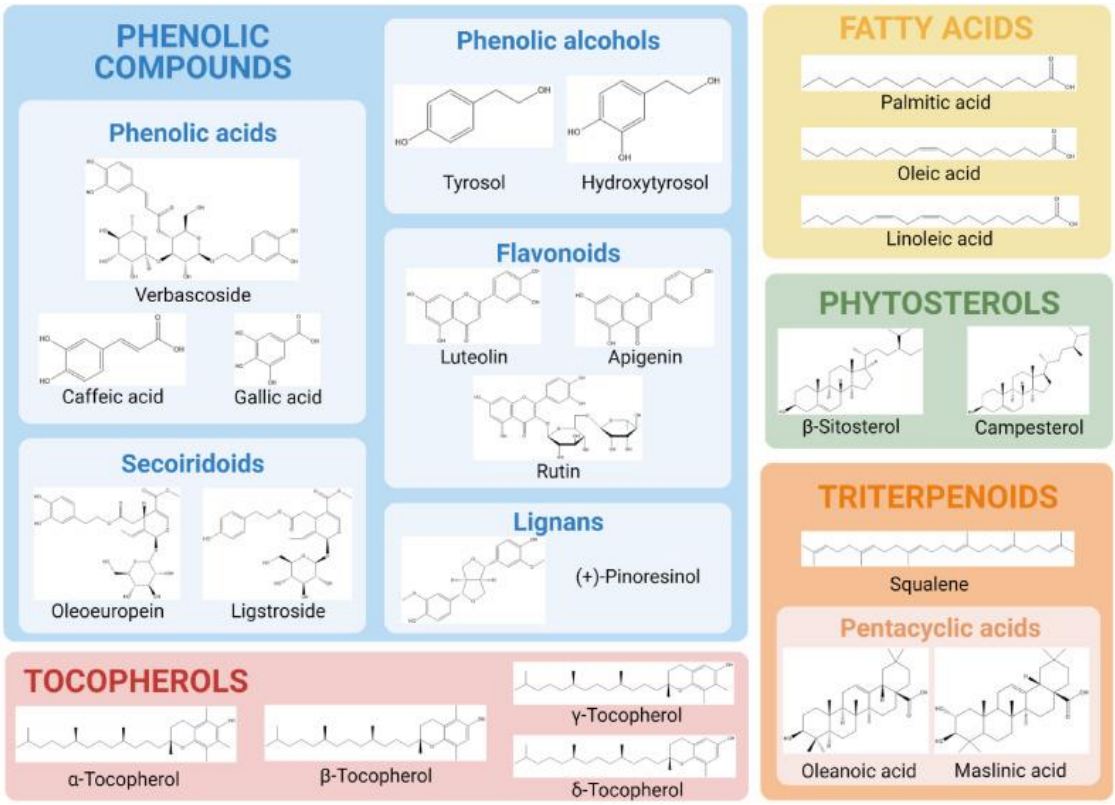

Figure 2.7 Chemical structure of the main bioactive compounds present in olive oil and in the by-products of olive oil industry (Modified from Otero et al., 2021).

1.5 Phenolic compounds properties

Many biological properties have been attributed to the phenolic compounds found in by-products of olive oil industry (**Figure 2.8**) including:

- Antioxidants
- Anti-inflammatory
- Antitumorals
- Antiobesity and antidiabetics
- Cardioprotective

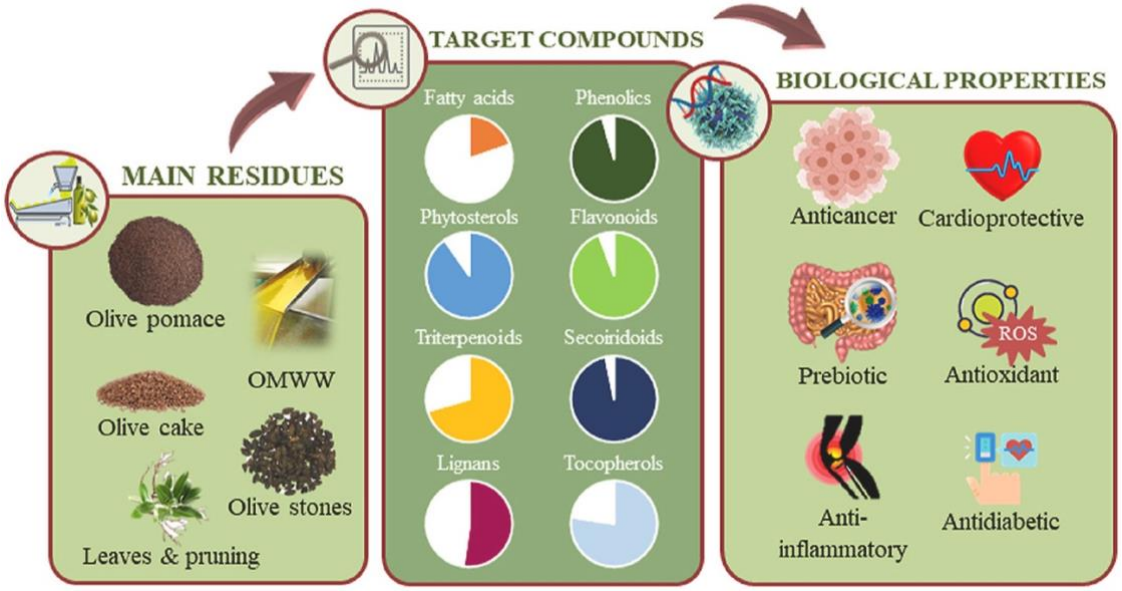

Figure 2.8 Possible biological effects of the main compounds present in olive industry by-products (Modified from Otero et al., 2021).

The antioxidant characteristics found in the by-products of olive oil industry are mainly due to phenolic compounds such as 3,4-DHPEA and derivatives which, having an *o*-diphenolic structure, able to eliminate free radicals and chelate metals (Araújo et al., 2015). Antioxidant effects have not only been reported by DPPH and ABTS assays (Kouka et al., 2017; Pannucci et al., 2019), but also in cell cultures and in *in vivo* studies (Crupi et al., 2020). Similar effects have been also observed with hydroxytyrosol derivatives found in by-products, such as 3,4-DHPEA oleate (Benincasa et al., 2019) and homovanillic alcohol (Ricelli et al., 2020). Several mechanisms of action have been proposed for 3,4-DHPEA, including activation of nuclear factor Nrf2 (nuclear erythroid factor-2), a transcription factor that plays a crucial regulatory role on ARE (antioxidant response element), or it intervenes by activating the NK-p62/SQSTM1 pathway (Nuclear Factor-p62/Sequestosome-1). Both pathways are involved in the cellular response against oxidative stress (Kouka et al., 2017). Furthermore, 3,4-DHPEA appears to stimulate mitochondrial biogenesis (Granados-Principal et al., 2014). Oleoeuropein also has an *o*-diphenolic structure responsible for its antioxidant activity, managing to eliminate reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Czerwin et al., 2012). Furthermore, oleoeuropein and its derivatives have been shown to reduce the production of ROS, RNS and oxidative markers, inhibit lipid peroxidation and improve antioxidant defense systems, increasing the activity of antioxidant enzymes in both *in vivo* and *in vitro* model (Czerwin et al., 2012; Janahmadi et al., 2017; Jemai et al., 2008). As expected, oleoeuropein derivatives present in olive and in olive oil industry by-products, like oleoeuropein-aglycone or oleacin have shown analogous effects (Czerwin et al.,2012; Nardi et al., 2017). **Table 1.2** shows the main biological properties attributable to the main phenolic compounds present in olive industry by-products.

Table 1.2 Biological properties of compounds present in olive oil industry by-products (Modified from Otero et al., 2021).

Activity	Compounds	Main Mechanisms	References
Antioxidant	**Hydroxytyrosol** and its **derivates**	- Ability to scavenge free radicals and chelate metals. - Reduction of lipid peroxidation. - Reduction of mitochondrial dysfunction. - Activation of Nrf2 and upregulation of antioxidant genes.	(Araújo et al., 2015; Karković Kouka et al., 2017; Marković et al., 2019; Robles-Almazan et al., 2018)
	Oleoeuropein and its **derivates**	- Ability to scavenge ROS and RNS. - Reduction of lipid peroxidation. - Activation of Nrf2 and upregulation of antioxidant genes.	(Czerwin et al., 2012; Janahmadi et al., 2017; Jemai et al., 2008; Sherif, 2018; Yin et al., 2019)
Anti-inflammatory	**Hydroxytyrosol** and its **derivates**	- Inhibition of pro-inflammatory molecules (NO, PGE2, TNF-α, NF-κB…).	(Aparicio-Soto et al., 2017; Bigagli et al., 2017; Fki et al., 2020; Plastina et al., 2019; Robles-Almazan et al., 2018)
	Oleoeuropein and its **derivates**	- Activation of Nfr2 related pathways and downregulation of inflammation related genes (COX-2, iNOS…).	(Aparicio-Soto et al., 2017; Feng et al., 2017; Hassen et al., 2015; Janahmadi et al., 2017; Sherif, 2018; Yin et al., 2019)
Antitumor	**Hydroxytyrosol**	- Inhibition of proliferation, induction of cell cycle arrest. - Induction of pro-apoptotic pathways (PI3K/Akt/FOXO3a, PI3K/Akt/mTOR, caspasecascade…).	(Calahorra et al., 2020; Goldsmith et al., 2018; Imran et al., 2018; Karković Marković et al., 2019; Robles-Almazan et al., 2018)
	Oleoeuropein	- Alteration of pro/anti-apoptotic Bcl-2 family proteins ratio. - Downregulation of anti-apoptotic factors, oxidative stress and inflammation.	(Asgharzade et al., 2020; Boss et al., 2016; Goldsmith et al., 2018; Imran et al., 2018; Shamshoum et al., 2017)
Anti-obesity & anti-diabetic	**Oleoeuropein** and its **derivates**	- Enhancement of GPBAR1. - Better insulin secretion. - Reduction of glycaemia. - Activation of ERK/MAPK signaling pathway.	(Sato et al., 2007; Ling Wu et al., 2017)

Cardioprotective effec	**Hydroxytyrosol** and **tyrosol; Oleoeuropein** and its **derivates**	- Reduction of systolic blood pressure, cardiac hypertrophy, cholesterol and angiotensin II plasma levels. - Downregulation of oxidative stress and inflammation	(Bendini et al., 2007; Covas et al., 2006; Gómez-Caravaca et al., 2015; Janahmadi et al., 2015; Soler-Rivas et al., 2000; Tuck & Hayball, 2002; Vazquez et al., 2019; Lixing Wu et al., 2018)

Abbreviations: Nrf2, nuclear factor (erythroid-derived 2)-like 2; ROS, reactive oxygen species; RNS, reactive nitrogen species, HO-1, heme oxygenase 1; NO, nitric oxide; PGE2, prostaglandin E2; TNF-α, tumor necrosis factor-alpha; NF-κB, nuclear factor kappa B; iNOS, inducible nitric oxide synthase; COX-2, cyclo-oxygenase-2.

Olive and olive oil by-products also exert anti-inflammatory effects, although they were reported by less studies compared with those focused on antioxidant activity. Recently, phenolic extracts from olive mill wastewater and olive pomace have been shown to reduce NO production in lipo-polysaccharide (LPS)-stimulated RAW-264.7 macrophages (Plastina et al., 2019) and inhibit the production of the interleukin-8 (IL-8) pro-inflammatory cytokine in human colorectal adenocarcinoma Caco-2 cells (Di Nunzio et al., 2018), respectively. Anti-inflammatory effects have been also observed in *in vivo* studies: in rats, for example olive oil industry by-products promoted an inflammation reduction associated with gastrointestinal disorders (Parisio et al., 2020). In general, these properties of by-products have been attributed to the presence of compounds such as hydroxytyrosol, oleoeuropein and their derivatives, as anti-inflammatory activity is usually linked to antioxidant activity. 3,4-DHPEA and derivatives, such as 3,4-DHPEA oleate or 3,4-DHPEA stearate, have shown anti-inflammatory properties in both *in vitro* and *in vivo* studies. Several cellular studies have reported that these compounds inhibited NO, prostaglandin E2 (PGE2) and pro-inflammatory cytokines production, tumor necrosis factor-alpha (TNF-α) secretion and expression and repressed inflammatory-related genes expression (Bigagli et al., 2017; Plastina et al., 2019; Robles-Almazan et al., 2018). These effects and mechanisms have been also observed in various animal models (Aparicio-Soto et al., 2017; Fki et al., 2020). Regarding oleoeuropein and derivatives, such as oleacin or oleocanthal, similar anti-inflammatory effects and mechanisms have been observed in cell cultures, including the inhibition of the nuclear factor κ-B (NF-κB) and its translocation into the nucleus and the production of pro-inflammatory cytokines and also the downregulation of genes like COX-2 and iNOS (Aparicio-Soto et al., 2017). It has been reported that OLE also inhibits the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways, being both important

regulators of the inflammatory process (Feng et al., 2017). This anti-inflammatory activity has been also confirmed on animal models, where oleoeuropein reduced the levels and the expression of pro-inflammatory mediators and genes, which has been attributed to the activation of Nrf2/heme-oxygenase-1 (HO-1) signaling pathway (Sherif, 2018; Yin et al., 2019).

The antitumor properties of olive by-products have been also described in the literature. To cite some of the most recent studies, phenolic extracts from olive leaf and olive pomace exerted inhibitory effects against mouse sarcoma S180, HeLa, Caco-2 and HCT116 cell lines (Lanza et al., 2020; Wang et al., 2019) and the antitumor properties have been mainly correlated to hydroxytyrosol and oleoeuropein (Imran et al., 2018; Robles-Almazan et al., 2018). Regarding hydroxytyrosol, it has been demonstrated to inhibit the proliferation and growth and induce apoptosis in different cancer cell lines and *in vivo* models, by promoting cell cycle arrest in the G0/G1 phase and by modulating the expression of different pathways and genes involved in tumor progression (Karkovi'c Markovi'c et al., 2019; Robles-Almazan et al., 2018).

Oleoeuropein has shown also antitumor effects in different cancers, reducing viability, and inducing cell cycle arrest and apoptosis (Boss et al., 2016; Imran et al., 2018). Like 3,4-DHPEA, it has been observed that oleoeuropein exerts antitumor properties affecting many different pathways, such as caspase pathway, PI3K/Akt/mTOR pathway, or extracellular signal-regulated protein kinases 1 and 2 (ERK-1 and ERK-2), and downregulating inflammatory and oxidative factors (Shamshoum et al., 2017). In the study of Goldsmith et al (2018), oleoeuropein induced apoptosis in MIA PaCa-2 cells trough activation of caspase 3/7, increased pro/anti-apoptotic Bcl2 proteins ratio and augmented the expression of c-Jun and c-Fos. Other compounds to which anti- tumor properties are attributed are luteolin or apigenin, due to their ability to reduce oxidative damage and modulate the inflammatory response mediated by NF-κB, and tumor progression-related pathways (Boss et al., 2016).

Obesity is known to be associated with a series of metabolic diseases, including insulin resistance, which can lead to type II diabetes (Rabe et al., 2008). Phenolic acids, flavonoids and their derivatives seem to be responsible for the greatest antidiabetic activities of olive and its by-products, since phenolic compounds are inhibitors of α-glucosidase and α-amylase enzymes, which are therapeutic targets of anti-diabetic drugs (Kamiyama et al., 2010; Vlavcheski et al., 2019). Among the by-products of the food industry, olive leaves, skin and pomace are the main components with antidiabetic and anti-obesity properties as evaluated in several works (Abunab et al., 2017; Guex et al., 2019). These biological activities are mainly attributed to phenolic compounds, in particular to

oleoeuropein and some derivatives; it has been reported, in fact, that oleoeuropein and oleanolic acid enhance the role of G-protein-coupled bile acid receptor 1 agonists, improving metabolic disorders with greater peripheral use of glucose and better insulin secretion (Sato et al., 2007). More recently, oleoeuropein has shown to reduce glycemia and enhance glucose tolerance in several animal models. This compound stimulates the insulin secretion promoted by glucose in pancreatic β-cells with a dose-dependent effect, activating the ERK/MAPK signaling pathway (Ling et al., 2017). Finally, there are many studies that support the cardioprotective properties of olive by-products, such as antiarrhythmic and vasodilator effects (Covas et al., 2006), linked to their antioxidant and anti-inflammatory properties, being 3,4-DHPEA, *p*-HPEA, oleoeuropein, and their derivatives the major responsible of the cardioprotective effect (Bendini et al., 2007; Gomez-Caravaca et al., 2015). To cite some examples evaluating by-products and bioactive compounds, a study assessed the effect of extra virgin olive oil (EVOO) enriched with phenolic compounds obtained from its by-products in rats. The results showed that the group supplied with enriched EVOO presented decreased systolic blood pressure, cardiac hypertrophy, and reduced plasma levels of total cholesterol and angiotensin II (Vazquez et al., 2019). In other study, oleoeuropein displayed cardioprotective effects in rats with acute myocardial infarction because it prevents cardiac deterioration by reducing oxidative stress and decreasing the release of pro-inflammatory cytokines (Janahmadi et al., 2015). Regarding the antihypertensive effect, a study reported that the consumption of 500 mg twice a day of olive leaf extract for 8 weeks was able to lower blood pressure in a similar extent than the group treated with captopril, and also the olive leaf extract was able to lower triglyceride levels (Susalit et al., 2011).

2.6 Recovery of phenolic compounds from olive mill wastewater

As already seen above most of the phenolic substances contained in the fresh olive mill wastewaters are the same molecules present in virgin olive oil. In fact, in olive mill wastewaters other than small percentages of 3,4-DHPEA and *p*-HPEA, the dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA or oleacein), is usually the prevalent phenolic species, such as in virgin olive oil where, due to their high hydrophilicity, no more than 2% of the total phenolic compounds contained in the olive fruits is transferred during the mechanical separation. Additionally, verbascoside is also generally abundant in olive mill wastewaters, on a cultivar-dependent basis (Veneziani et al., 2017). In virgin olive oil producer countries, the volume of resulting olive mill wastewaters is very large (especially in Italy, where the separation is mainly based on the high olive mill wastewaters producing three-phases centrifugation system), determining the need to implement disposal practices. The growing interest in the phenolic compounds of olives and

their abundance in olive mill wastewaters, up to 25 g/L, have led to the change of the traditional approach from that of the problem of waste disposal to that of waste valorization by recovering the bioactive phenolic fraction (Veneziani et al., 2017).

Membrane filtration method, combining ultrafiltration and reverse osmosis, has demonstrated very satisfactory selectivity and concentration capability in recovering and concentrating the olive mill wastewaters phenolic fraction, giving a crude phenolic extract that can be further purified and processed for subsequent uses. Nevertheless, the multi-step treatment by membrane filtration is able to break down the polluting load, giving a permeate with a very low BOD (biochemical oxygen demand) and COD (chemical oxygen demand). This technological approach, processing fresh and previously untreated olive mill wastewaters, appears very promising in comparison to other proposed methods in terms of environmental impact. Since no solvents are required for the recovery of phenolic compounds, in terms of yield, reaching the crude extract a concentration up to four times of the original olive mill wastewaters, and in terms of keeping the compositional profile of the phenolic species occurring in fresh olive mill wastewaters, since no spontaneous or induced hydrolyses takes place (Ianni et al., 2021).

2.6.1 Application of phenolic extract from olive mill wastewater in food systems

In the last few years, several different uses have been proposed and tested for the incorporation of phenolic extract from olive mill wastewater in various food systems. For fresh and processed food products (meat and non-meat ones), extracts rich in phenols can be used for the complete or partial replacement of chemical additives, such as nitrates and nitrites. In 2015, the IARC has classified processed meat added with nitrate/nitrite salts as carcinogenic to humans (Group 1), as they can give rise to a series of compounds (N-nitrous compounds (NOCs), nitrosamines and oxidation products), during cooking, subsequent ingestion and digestion that seem to contribute to the onset of colorectal cancer (Bouvard et al., 2015). Independently from the technological advantages, a reduction in the use of nitrates and nitrites has become a matter of primary importance for both industries and consumers. However, their complete or partial replacement with a single natural compound is a challenge, due to their multifunctional characteristics. Successful results on oxidative stability and sensory characteristics were obtained by Balzan et al. (2017) by using a phenolic extract from olive mill wastewater, rich in 3,4-DHPEA, *p*-HPEA, verbascoside and 3,4-DHPEA-EDA, in raw and cooked pork sausages formulated without chemical additives and subjected to a shelf-life study. The phenolic extract concentrations used modified the sensory perception of the samples, but they were never considered unpleasant by the panelists. Effectively counteracting the oxidative process,

with TBARS values lower than 1 mg/kg and COPS oxidation ratios consistently low. The phenolic extract concentration of 0.075 g/100 g is effective to maintain oxidative stability, but lower levels may well be equally efficient. Again, regarding meat products, the study of Barbieri et al. (2021) demonstrated the efficacy of a powder formulation of a phenolic extract from olive vegetation water at improving the overall oxidative stability and sensory quality of raw and grilled beef hamburgers, which had been previously subjected to cold storage for 9 days. Both phenolic extract concentrations used (87.5 and 175 mg of phenols/kg meat), proved to effectively reduce primary and secondary lipid oxidation, as well as cholesterol oxides, during the burgers' shelf-life study and after cooking. Sensory analysis also confirmed the effectiveness of PE addition in beef hamburgers, having a positive effect especially on the intensity of the red color (raw product) as it resulted in a reduction of browning during storage. Furthermore, the presence of phenols was not perceived by panelists, so they did not negatively influence the organoleptic characteristics of the products. However, the discriminant test evidenced a qualitative decay of all products during storage, which was more relevant in the control and the phenol-enriched burgers at the highest PE dose.

Miraglia et al. (2021) evaluated quality traits and sensory profile of cooked pink shrimps treated with an olive wastewater phenolic extract. The results provide evidence on the effectiveness of the phenolic extract both from a hygienic point of view and in terms of shrimp quality in general. The bactericidal and antioxidant activity of the phenolic compounds was proportional to their concentration, as were the pleasant sensory attributes. In this regard, the phenolic fractions found in the shrimps after cooking, albeit at different percentages of reduction, enriched the nutraceutical value of the rose shrimps, which in addition to being safer with longer-lasting shrimp sensory characteristics, were also without or with less use of additives. Furthermore, at the concentrations used by the authors (2 and 1 g of phenols/L), the phenolic extract did not modify either the sensory characteristics of the fresh shrimp or during the storage period. Finally, sulphites combined with the extract demonstrated a less effective antibacterial action, although they appear to have protected the phenolic compounds from the oxidative processes of cooking and storage.

Taticchi et al. (2017) tested the protective effect of a phenolic extract from olive wastewater on carotenoids and other phytonutrients during a home-cooking procedure to prepare tomato sauce. To specifically investigate on the complex mechanisms involved in the thermal degradation and solubilization of carotenoids, the olive phenols were added to a refined olive oil, chosen as control fat, to distinguish the effect of the simple oily matrix from that of the phenols occurring in virgin olive oils. During cooking steps, the added olive phenolic extract is effective in the improvement of

retention of most of the quantitatively important phenolic constituents of the vegetable mix and of tomato (flavonoids and α-tocopherols), for most of them according to the level of addition (the dose of 60 mg/100 g of oil is more efficacious than 40 mg/100 g). The antioxidant activity exerted by the phenolic extract was able as well to preserve a significantly higher content of carotenoids (α− and β-carotene and *E*+*Z* –lycopene), guaranteeing in the final tomato sauce more abundant bioactive components available for ameliorating the health promoting properties of this traditional dish. Nevertheless, after cooking, the sauces still contain appreciable quantities of the phenolic species from olives claimed to reduce blood lipid oxidation.

2.7 Re-utilization of olive pomace

As for the olive pomace, its composition makes this waste of considerable importance and in recent years research has worked a lot, with different technological approaches, for the recovery of bioactive molecules from this waste, such as polyphenols, as well as developing innovative treatments to try to make pomace an ingredient that can be used as it is for the formulation of innovative and functional foods. Vegetable oils, for example, especially those with a high amount of unsaturated fatty acids, are more susceptible to oxidation and therefore difficult to store. Several studies have been advanced on the possibility of improving the qualitative and oxidative parameters of these oils through the addition of phenolic extracts obtained from pomace, also automatically obtaining an enrichment in polyphenols (Difonzo et al., 2019). With the same idea, a fermented milk was formulated that was richer in polyphenols and with a greater antioxidant activity (Aliakbarian et al., 2015). The extraction of polyphenols from pomace inevitably leads to the use of chemical solvents, as well as the production of an extraction residue. To avoid this, the use of pomace as it is added directly to food, properly treated (destoned or dehydrated), could represent a very valid alternative (Difonzo et al., 2019). In fact, several researchers have used destoned and dehydrated olive pomace in foods such as pasta and bread: for example, functional spaghetti were formulated with 10-15% of olive paste flour added and these showed a higher content of flavonoids, fibers, tocopherols and carotenoids (Padalino et al., 2018). Bread fortified with olive pomace, present in the dough at 10%, was richer in phenolic compounds (Cedola et al., 2019). These studies, however, have shown that it is necessary to keep attention to the quantity of pomace that is added to these foods as it strongly tends to modify the texture and to highlight some sensory defects: in fact, pasta added with 15% of olive paste flour turned out to have a dark green color, once cooked it showed little elasticity, and when tasted it resulted in a very intense taste and smell due to the bitter and spicy notes provided by the polyphenols (Padalino et al., 2018; Difonzo et al., 2021). To resolve this

type of alteration, it may be advisable to carry out a preliminary treatment of the pomace. Durante et al. (2019) subjected pomace to fermentation before it could be incorporated into the taralli dough. In the study, enriched taralli were shown to have a higher content of polyphenols (mainly 3,4-DHPEA), triterpene acids and carotenoids, a lower saturated fatty acid content, as well as an improved shelf-life compared to controls (traditional taralli) (Durante et al., 2019). In a more recent study, an olive patè was extracted thanks to the inclusion of a novelty in the two-phase extraction system, a multiphase decanter which does not require any addition of water during the process. From this innovative process were obtained a dried olive paste (similar to that of the three-phase decanter) and a by-product called "paté" consisting of pulp and wastewater, without a trace of stone (it is very similar to the pomace obtained from de stoned olives). This has been shown to have a high content of polyphenols, a fatty acid composition similar to extra virgin olive oil and a high humidity (between 75 and 90%). It has therefore resulted in a product with a high added value and can be reused in various fields. In fact, this by-product as such is would be suitable for human consumption but, also, due to its high content in polyphenols, which gives it a bitter taste, it is not edible without any treatment; then, it underwent to several transformation like sequential filtration and fermentations (Lanza et al., 2020).

Always in food sector, use of pomace is having good results in packaging and animal feed. To meet environmental sustainability, several scientific research have been focused on the creation of sustainable food packaging, using a dehydrated pomace powder or in the form of microparticles in chitosan-based films. In addition, animal nutrition is an important aspect to consider in order to obtain products that are, in turn, qualitatively better for human consumption: in this sense, attempts have been made to improve the diet of rabbits by adding olive pomace; as a result, an improvement in the fatty acid profile was obtained with an increase in monounsaturated fatty acids in the meat and a higher content in polyphenols (Difonzo et al., 2021).

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Chapter 3. Improved oxidative stability and sensory quality of beef hamburgers enriched with a phenolic extract from olive vegetation water

3.1 DETAIL OF THE PUBLICATION

Title: Improved oxidative stability and sensory quality of beef hamburgers enriched with a phenolic extract from olive vegetation water

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Abstract:

This study aims at evaluating the effect of a phenol-rich extract obtained from the concentration and purification of olive mill wastewaters (added at a ratio of 87.5 and 175 mg of phenols/kg meat) on the stability and sensory quality of beef hamburgers packed under modified atmosphere and stored under alternating exposure to fluorescent light at 4±2°C for 9 days. Hamburgers were sampled at different times (0, 6 and 9 days) and grilled at 200°C. After 9 days, more than 56% of the added phenols in the raw burgers and more than 20% the grilled ones, were retained. The results show that both concentrations of phenolic extract proved to effectively reduce primary and secondary lipid oxidation, as well as cholesterol oxidation products (COPs), during the shelf-life of raw hamburgers. Peroxide value, thiobarbituric acid reactive substances and total COPs were up to 1.4-, 4.5- and 8.8-fold lower in phenol-enriched raw hamburgers, respectively, than in control samples; a similar trend was noted also in phenol-enriched cooked hamburgers (1.3-, 5.7- and 4-fold lower). Sensory analysis also confirmed the effectiveness of the addition of phenolic extract, having a positive effect on the red color intensity (raw product) and thus reducing browning during storage.

Keywords: Beef hamburger; phenolic extract; olive vegetation water; olive by-product; clean label; lipid oxidation; cholesterol oxidation; sensory analysis.

3.2. INTRODUCTION

The consumption of fresh ground meat preparations, like hamburgers, is wide-spread due to their pleasant taste and ease of cooking. The increasing demand for healthy and high-quality ground meat-based products has pushed the food industry towards the search for suitable strategies to render these products more stable during their shelf-life and thus limit changes of both color and flavor, two important factors that directly affect consumer's acceptance (Wang et al., 2021). In fact, one of the main problems that fresh ground meat preparations face during processing and storage, is lipid oxidation, due to the cell membrane disruption during grinding and to their large surface/mass ratio. This is particularly true for ground red meat products, which have a high concentration of pigments (around 12%) that act as photosensitizers, as well as other oxidation catalyzing agents like cytochromes, non-heme iron, and other heavy transition metals (Delgado et al., 2021); moreover, the ferritin fraction can significantly promote lipid oxidation in heated meat systems (Karwowska et al., 2020). Considering the high susceptibility of red meat to lipid oxidation, the International Agency for Research on Cancer (IARC) (IARC, 2018) has classified it in Group 2A (probably carcinogenic to humans), as it can give rise to a series of compounds (i.e. 4-hydroxy-nonenal, malondialdehyde) that seem to contribute to adenocarcinoma formation and to the onset of colorectal cancer (Bouvard et al., 2015). In addition, meat cholesterol can also undergo oxidation and generate cholesterol oxidation products (COPs), which have been associated with the development of several human diseases (i.e., cancer, cardiovascular, neurodegenerative or autoimmune ones) (Testa et al., 2018; Duc et al., 2019; Zmysłowski et al., 2019). To control lipid oxidation in fresh ground meat preparations and extend their shelf-life, food additives with antioxidant properties are usually employed. In a clean label perspective of meat product formulation, synthetic food antioxidants could be replaced by natural extracts obtained from agri-food by-products and waste, which are rich in bioactive compounds (such as carotenoids, phenolic compounds, essential oils or β-glucans) (Carpentieri et al., 2021; Ramìres-Pulido et al., 2021) that display different health properties, antioxidant and antimicrobial activities. In this regard, one of the most interesting food by-products is olive mill wastewater (OMWW), which is generated during olive processing for the production of olive oils and is characterized by a high content of organic compounds (sugars, tannins, pectins and phenolic substances) and mineral salts (Carrara et al., 2021). This particular composition, together with its high generation rate, makes OMWW a highly polluting by-product whose uncontrolled disposal such be avoided; in particular, large quantities of polyphenols can exhibit marked antimicrobial, phytotoxic and anti-nutritional properties and

resistance to degradation, thus leading to negative effects on ecosystems (Carrara et al., 2021). However, OMWW could be considered an exploitable source of hydrophilic phenols (mainly secoiridoids, which are found exclusively in the *Oleaceae* family) with antioxidant, antimicrobial and anti-inflammatory activity (Servili et al., 2014; Veneziani et al., 2017). Thus, phenols can be recovered from OMWW by using suitable membrane technology (Servili et al., 2011), for further applications in the food, pharmaceutical or cosmetic industries (Veneziani et al., 2017; Galanakis et al., 2018; Caporaso et al., 2018). Nevertheless, adding phenols from OMWW to extend ground meat products' shelf-life requires careful sensory evaluation of the newly formulated food product before a possible market launch. In fact, phenolic substances affect bitter taste and pungent perception (Cui et al., 2021), which may be negative drivers of liking (Barbieri et al., 2015). Regarding the use of phenols from OMWW in meat products, they may have a negative impact sensory quality and consumers acceptability. In fact, if not correctly treated and dosed, phenolic OMWW extracts could result in a bitter taste and astringency sensation (De Toffoli et al., 2019). Therefore, considering the need of prolonging the maintenance of quality during shelf-life in fresh ground meat preparations in a formulation perspective of clean label food products, this study aimed at evaluating the effect of the addition of OMWW extracts rich in phenols on the stability and sensory characteristics of raw and grilled beef hamburgers. Specifically, the main objectives were to verify the effectiveness of a powder formulation of the phenolic extract as preservative agent for the extension of the shelf-life and the oxidative stability on the hamburgers, to define the sensory profile of these new phenol-enriched meat products and to monitor the perception of sensory characteristics and the presence of unacceptable sensory attributes and/or off-flavors during storage.

3.3. MATERIALS AND METHODS

3.3.1 OMWW phenol extract

A crude phenolic extract (PE) was obtained from fresh OMWWs of olives harvested in Umbria (Central Italy) from trees of Moraiolo cultivar, by a 3-step membrane filtration of fresh OMWW as previously reported (Ianni et al., 2021). The PE was added with maltodextrin (1:1 d.w.), as a support, and then spray-dried to get a powder formulation of the PE.

3.3.2 Preparation of phenol-enriched burger samples

For the preparation of burgers, adult bovine meat (beef rump and shoulder muscle trimming) was obtained from animal reared and slaughtered in Spain, while the carcasses were sectioned in Italy according to the Regulation EC/853/2004. The meat was trimmed and minced at a 6-mm

diameter with a professional meat mincer (TCS32, Cavalli Meat Processing Machinery, Felino, Italy). The minced meat was mixed with salt (0.8 g/100 g) and starter cultures SafePro® (B-SF-43, *Leuconostoc carnosum*) and Bactoferm® (S-B-61, *Staphylococcus carnosus*) (Chr. Hansen GmbH, Germany) using a two-paddle mixer (IMP50-Bipala, Cavalli Meat Processing Machinery, Felino, Italy). The protective culture was added with the primary aim to control the spoilage bacteria (mainly *Enterobacteriaceae*) that can contaminate the beef meat; in this way, the unwanted variability factors that are not included in the experimental design (such as spoilage bacteria), are restrained. The dough was then divided into three batches: i) Control, meat dough plus maltodextrin (0.35 g/100 g, Maltodextrin Glucidex 19, Roquette, France); ii) L1, meat dough plus PE equivalent to 87.5 mg phenols/kg of meat; iii) L2, meat dough plus PE equivalent to 175 mg phenols/kg of meat. Each batch was further mixed for 1 min and the burgers were then molded (about 80 g each pattie), packed 2 per tray under modified atmosphere Alipak 333 mixture (50% nitrogen, 20% oxygen, 30% carbon dioxide) and wrapped with a film made of polyethylene terephthalate (PET) + polyethylene (PE)/ethylene vinyl alcohol (EVOH)/PE (12+50 µm thickness, anti-fog and anti-UV; Guillin 5025N, Usmate-Velate, Italy). The trays containing the hamburgers were randomly divided and placed in a display refrigerator at 4 ± 2°C for 9 days, under alternating exposure to fluorescent light (12 h light/12 h darkness) to simulate retail storage conditions. Burgers were sampled at fixed time periods (just after produced, T0; 6 days of storage, T6; 9 days of storage, T9) and frozen at -80°C. At the same sampling times, the same number of hamburgers from each batch were grilled in an electrical grilling plate (Fimar FRY1L230M, Rimini, Italy) at 200°C for 4 min per side until the core temperature reached 70°C. For chemical analyses after cooking, burgers were cooled down at room temperature for 5 min, placed in a blast chiller (TBF051B, Moduline, Treviso, Italy) at -40°C for 15 min, packed in a plastic bag under vacuum and stored at -80°C until analysis. Two independent batches of burgers' preparation were run.

3.3.3 Proximate composition

Moisture, crude protein and ash of raw samples were measured according to the AOAC Official methods no. 950.46.B, 981.10 and 920.15 (AOAC, 1990). Crude fat was calculated by difference, the carbohydrate fraction was not taken into account. Measurements were carried out in duplicate.

3.3.4 Phenols analysis

In the PE. Fifty mg of spray-dried PE were solubilized in 10 mL of a methanol:water mixture (80:20, v/v), filtered with a 0.2 µm polyvinylidene fluoride (PVDF) syringe filter (Agilent Captiva, Agilent

Technologies, Santa Clara, CA, USA) and injected into a high-performance liquid chromatograph coupled to a diode array detector (HPLC-DAD Agilent Technologies system Mod. 1100). The HPLC equipment and analytical conditions were those described by Selvaggini et al. (2014). Each measurement was done in duplicate.

In the hamburgers. Five grams of hamburgers were mixed with 100 mL of methanol:water (80:20, v/v) containing 20 mg/L of butylated hydroxytoluene (BHT) + 0.2% trichloroacetic acid 1 M. The operations of homogenization, recovery, concentration until a final volume of 40 mL of extract and purification by solid-phase extraction (SPE) from 10 mL of this aqueous extract were carried out as previously described (Miraglia et al., 2020). The purified extract was then subjected to HPLC-DAD analysis using the same equipment and conditions of the PE analysis (Selvaggini et al.,2014). Each measurement was done in duplicate.

3.3.5 Chemical analysis

3.3.5.1 Lipid extraction

Lipids were extracted according to Boselli et al. (2011). The extraction was performed on 5 g of hamburgers, which were added with 5α-cholestane (internal standard for the quantification of main lipid classes, see paragraph 2.5.2) (Sigma Chemical, St. Louis, USA). The fat content was determined gravimetrically and expressed as percentage. Three independent replicates were run per sample.

3.3.5.2 Determination of main lipid classes

The qualitative-quantitative profile of the main lipid classes (free fatty acids, FFA; monoacylglycerols, MAG; free sterols, STE; diacylglycerols, DAG; esterified sterols, E-STE; triacylglycerols, TAG) was determined by gas chromatography-flame ionization detection (GC-FID), as reported by Gallina Toschi et al. (2014) and Luise et al. (2018). An aliquot of 20 mg of the lipid extract dissolved in 1 mL of *n*-hexane, was used for this analysis. The internal standard method, with the response factor of each main lipid class (estimated with commercial standards), was used to determine the amount of each lipid class (expressed as g/100 g of lipids). Three independent replicates were run per sample.

3.3.5.3 Determination of total FA

The composition of total fatty acids was determined on 20 mg of lipid extract by GC-FID (Cardenia et al., 2015), after previous methylation and transmethylation. FAME quantification was performed according to the internal standard method (using tridecanoic acid methyl ester) and expressed as a

proportion of the identified total FAME (g/100 g). Three independent replicates were run per sample.

Based on the total FA composition, the atherogenic index (AI) and thrombogenic index (TI) were also determined (Ulbricht & Soutage, 1991).

3.3.5.4 Determination of peroxide value (PV)

PV were determined in the lipid fraction extracted from hamburgers, using 10 g of meat and 50 mL of Folch solution (Kim et al., 2013). After extraction, 10 mL of the recovered mixture were evaporated under nitrogen and used for PV determination by titration, according to Kim et al. [30]. Results were expressed as meq of active O_2/kg meat.

3.3.5.5 Determination of thiobarbituric acid reactive substances (TBARs)

Secondary lipid oxidation was assessed as TBARs on raw and grilled meat burgers samples (Tarladgis et al., 1960). Two g of each sample were used for this spectrophotometric determination and the absorbance was measured at 530 nm. A 1,1,3,3-tetramethoxypropane standard calibration curve was used for the quantification of TBARs (concentration range of 0.045–0.113 µg/mL; $y=0.0077x+0.0072$, $r^2=0.9998$) and the values were expressed as mg MDA/kg meat. Three independent replicates were made per sample.

3.3.5.6 Determination of cholesterol and oxysterols (COPs)

Cholesterol and COPs were extracted by cold saponification of 200 mg of lipid extract, followed by purification with aminopropyl SPE (Cardenia et al., 2015). Silylated cholesterol and COPs were analyzed by Fast GC/MS (Cardenia et al., 2012), using betulinol (Sigma Chemical, St. Louis, USA) and 19-hydroxycholesterol (Steraloids, Newport, Rhode Island, USA) as internal standards, respectively. Mass spectra were acquired in full scan mode (total ion current, TIC), while they were integrated with single ion monitoring (SIM) mode using the characteristic ions with a high abundance (Cardenia et al., 2012); quantification was carried out by means of calibration curves built for each compound. Cholesterol and total COPs were expressed as mg/kg of meat. Three independent replicates were run per sample. The rate of total cholesterol oxidation (%OR) was also estimated as reported by Cardenia et al. (2015).

3.3.7 Physical analysis

3.3.7.1. Image analysis

The instrumental measurement of appearance was carried out by an “electronic eye” (visual analyzer VA400 IRIS, Alpha MOS, France), a high-resolution CCD (charge-coupled device) camera (resolution 2592x1944p) combined with powerful data processing software as described by Barbieri et al. (2016). The software application allows to discriminate samples according to their different shape, size, color intensity and color uniformity and, after building a data library, to process the data using multivariate statistical techniques. The image analysis using the electronic eye was performed on hamburgers before cooking as common purchase conditions.

3.3.8 Sensory analysis

3.3.8.1 Descriptive analysis

The sensory quality of all samples (C, L1, L2) at the 3 different storage times (T0, T6 and T9) was evaluated by a panel of twelve fully trained judges of both genders, aged between 20 and 65 years and recruited on the basis of their previous experience in the field of sensory analysis and/or knowledge of product (staff and PhD students at the Department of Agricultural and Food Sciences, *Alma Mater Studiorum*-University of Bologna, Italy). A conventional profiling method was applied (Meligaard et al., 2007; Heymann et al., 2014). Sensory attributes were evaluated on a linear scale of 100 mm anchored at their extremes (0: absence of sensation; 100: maximum of sensation intensity); the results for five replicates and average values were calculated. Regarding the sample’s preparation method, for the olfactory-retrolfactory, gustatory and texture attributes, burgers were evaluated after cooking (200°C for a total of 8 min, 4 min for each side); for the visual attributes, the sensory analysis was performed on whole and raw samples, in order to mimic as much as possible the normal conditions of product purchase. Moreover, to avoid judges influences by appearance and/or color of samples, the evaluation of the visual attributes was carried out at the end of the sensory evaluation. Finally, to standardize the tasting conditions and reduce bias, panelists evaluated visual attributes by observing the same sample inside a plate, whereas evaluation of other attributes (smell, taste, and texture) was performed by providing minced samples placed in plastic cups.

Samples were coded with three-random numbers and were presented to the assessors in randomized blocks with a break between samples.

3.3.8.2 Discriminant test

In this study, the triangle test was applied to identify sensory differences between the control sample (C) and the treated samples (L1 and L2) right after being produced (T0) and to investigate

the effect of different storage times, i.e. 0, 6 and 9 days (CT0-CT6-CT9; L1T0-L1T6-L1T9, L2T0-L2T6-L2T9). Having three samples at three different storage times to be compared, the test was conducted in several successive sessions (two different days) to perform all possible combinations. The sessions were held in the tasting room of the Food Science Campus in Cesena (Department of Agricultural and Food Sciences, *Alma Mater Studiorum*-University of Bologna), involving 30 (first day) and 28 (second day) untrained judges, aged between 20 and 65 years; the test were carried out according to the procedures described by ISO 4120:2007. For the samples' preparation, the same indications followed during the descriptive analysis were applied, except for the visual attributes which, in this test, were evaluated on the cooked product. In each session, 6 triad of samples were evaluated. Data processing was performed by comparing the number of correct answers with values reported in a double entry probability table, indicating the minimum number of correct answers corresponding to the number of judges involved in the test or the number of judgments (number of judges for the number of replicas) for the different levels of significance (ISO 4120:2007).

3.3.9 Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, France) was used to elaborate chemical, sensory and physical data.

The chemical data are reported as mean values of independent replicates of each analytical determination. First, normal distribution of data was tested (p<0.05) with Shapiro-Wilk method. Chemical data were analyzed using two-way or three-way analysis of variance (ANOVA), including formulation (Form), storage time (St), grilling (Gr) and their interaction (Form*St; Form*Gr; St*Gr; Form*St*Gr) as factors. Tukey's honest significance test was performed at a 95% confidence level (p≤0.05), to separate means of statistically different parameters. A principal component analysis (PCA), with a Varimax rotation, was also carried out.

For sensory data, significant differences between samples by one-way and two-way ANOVA (multiple comparison test, Fisher LDS with p <0.05), were evaluated. Moreover, to investigate the relationships between instrumental data (physical analysis) and the color of samples (sensory analysis), principal component analysis (PCA) and multiple factor analysis (MFA) was applied.

3.4. RESULTS AND DISCUSSION

3.4.1 Proximate composition

The proximate composition of the hamburger (with a moisture of 70%, a crude protein of 22% and fat around 5%) is roughly comparable to that of fresh lean meat (**Table 3.1**). The concentration of ash close to 2% is due to the salt addition during dough mixing. No differences were detected among the different experimental batches, as well as between the beginning (0 days) and the end of the storage time (9 days). Such behavior confirms that the film barrier prevented evaporation losses, which would have led to an increase in dry matter after 9 days of storage.

Table 3.1. Proximate composition of raw hamburger samples after 0 and 9 days of storage. C, Control (minced beef meat + maltodextrin + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat; L2, minced beef meat + starter cultures + 175 mg phenols/kg of meat.

		Protein	Ash	Moisture	Fat
			(%)		
Raw samples			0 days		
	C	22.16±1.14	1.74±0.14	70.23±0.65	5.87±0.94
	L1	21.86±1.27	1.68±0.29	70.74±0.80	5.73±0.81
	L2	23.29±1.15	1.85±0.14	69.87±0.60	4.99±1.06
			9 days		
	C	22.65±1.10	1.74±0.06	70.16±0.43	5.44±0.79
	L1	22.46±0.91	1.83±0.05	70.42±0.27	5.29±0.92
	L2	22.19±1.09	1.84±0.12	70.31±0.34	5.66±0.99
	Factor		F value		
	Form	0.82 *ns*	1.64 *ns*	2.64 *ns*	0.37 *ns*
	St	0.00 *ns*	0.91 *ns*	0.01 *ns*	0.04 *ns*
	Form*St	2.17 *ns*	1.06 *ns*	1.49 *ns*	1.42 *ns*

Results as reported as mean±s.d of 2 independent replicates. no significant differences (Tukey's test; $p \leq 0.05$) between the same sample during the shelf-life, no significant differences (Tukey's test; $p \leq 0.05$) between treatments. Form, formulation; St, storage.

3.4.2 Evolution of phenolic compounds

The PE used for the preparation of the burgers had a total phenol content of 25.7 mg/g of dried product, of which 61.5% was 3,4-DHPEA-EDA (oleacein), 20.6% 3,4-DHPEA (hydroxytyrosol), 13.2% verbascoside and 4.7% *p*-HPEA (tyrosol).

As shown in **Table 3.2**, part of the added phenols was lost during storage and cooking. In terms of total phenols, there was a loss of 34% and 43% of phenolic compounds in L1 samples after 6 and 9 days of storage, respectively, whereas a minor loss was detected in L2 burgers (16.2 and 36.5%, respectively). In particular, the highest variation was observed for 3,4-DHPEA-EDA, which decreased by 84% and 93% in L1 sample after 6 and 9 days, respectively, while in L2 its decrease was limited to 31% and 70%, respectively. Conversely, a significant increase in 3,4-DHPEA was detected (24.5% and 5.6% in L1 and L2, respectively) after 6 days; however, after 9 days, this compound was partly lost in L1, while it displayed a further increase in L2 burgers, reaching a total positive variation of 19%.

Table 3.2. Evolution of phenolic compounds of raw and grilled hamburger samples after 0, 6 and 9 days of storage. C, Control (minced beef meat + maltodextrine + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat; L2, minced beef meat + starter cultures + 175 mg phenols/kg of meat.

		3,4-DHPEA	*p*-HPEA	VB	3,4-DHPEA-EDA	Total phenols
		(mg/Kg)				
Raw samples		0 days				
	C	-	-	-	-	-
	L1	$29.37\pm1.15^{B,X}$	$4.46\pm0.46^{B,X}$	$10.30\pm0.82^{B,X}$	$40.83\pm1,93^{a,B,X}$	$84.96\pm4.37^{a,B,X}$
	L2	$40.98\pm0.20^{b,A,X}$	$9.23\pm0.04^{A,X}$	$22.80\pm0.21^{a,A,X}$	$95.29\pm1.61^{a,A,X}$	$168.29\pm2.06^{a,A,X}$
		6 days				
	C	-	-	-	-	-
	L1	$36.53\pm1.92^{B,X}$	$3.81\pm0.10^{B,X}$	$9.12\pm0.53^{B,X}$	$6.60\pm0.16^{b,B,X}$	$56.05\pm2.71^{b,B,X}$
	L2	$43.28\pm0.56^{b,A,X}$	$9.12\pm0.02^{A,X}$	$22.76\pm0.10^{a,A,X}$	$65.91\pm3.00^{b,A,X}$	$141.07\pm3.69^{b,A,X}$
		9 days				
	C	-	-	-	-	-
	L1	$32.08\pm2.23^{B,X}$	$3.32\pm0.03^{B,X}$	$9.79\pm0.75^{B,X}$	$2.82\pm0.58^{b,B,X}$	$48.01\pm3.58^{b,B,X}$
	L2	$48.87\pm1.82^{a,A,X}$	$8.82\pm0.29^{A,X}$	$20.80\pm0.53^{b,A,X}$	$28.37\pm1.43^{c,A,X}$	$106.86\pm4.07^{c,A,X}$
Grilled samples		0 days				
	C	-	-	-	-	-
	L1	$11.48\pm0.87^{a,B,Y}$	$3.35\pm0.40^{a,B,Y}$	$5.46\pm0.49^{B,Y}$	nd^{Y}	$20.29\pm1.75^{a,B,Y}$
	L2	$19.31\pm0.90^{a,A,Y}$	$8.14\pm0.27^{a,A,Y}$	$21.96\pm0.65^{a,A,Y}$	nd^{Y}	$49.42\pm1.82^{a,A,Y}$
		6 days				
	C	-	-	-	-	-
	L1	$4.56\pm0.40^{b,B,Y}$	$2.46\pm0.28^{ab,B,Y}$	$4.46\pm0.17^{B,Y}$	nd^{Y}	$11.48\pm0.85^{b,B,Y}$
	L2	$8.92\pm1.29^{b,A,Y}$	$7.27\pm0.06^{a,A,Y}$	$21.95\pm0.87^{a,A,Y}$	nd^{Y}	$38.14\pm2.23^{b,A,Y}$
		9 days				
	C	-	-	-	-	-
	L1	$nd^{c,B,Y}$	$1.38\pm0.28^{b,B,Y}$	$4.57\pm0.17^{B,Y}$	nd^{Y}	$5.95\pm0.45^{c,B,Y}$
	L2	$1.31\pm0.30^{c,A,Y}$	$4.66\pm0.37^{b,A,Y}$	$15.68\pm0.68^{b,A,Y}$	nd^{Y}	$21.65\pm1.34^{c,A,Y}$
	Factor	F value				
	Form	2449.72^{***}	4043.62^{***}	6493.57^{***}	3262.06^{***}	4755.42^{***}
	St	31.62^{***}	89.59^{***}	40.84^{***}	944.93^{***}	338.66^{***}
	Gr	4031.08^{***}	314.37^{***}	244.21^{***}	6484.39^{***}	4794.17^{***}
	Form*St	13.18^{***}	24.79^{***}	36.62^{***}	320.75^{***}	108.76^{***}
	Form*Gr	1048.99^{***}	91.57^{***}	86.15^{***}	3262.06^{***}	1613.62^{***}
	St*Gr	146.39^{***}	29.44^{***}	12.08^{***}	944.93^{***}	55.62^{***}
	Form*St*Gr	50.22^{***}	14.75^{***}	8.84^{***}	320.75^{***}	17.07^{***}

Results as reported as means±s.d of 2 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) within the same sample during the shelf-life. A-B indicate significant differences (Tukey's test; $p \leq 0.05$) among treatments, X-Y indicate significant differences (Tukey's test; $p \leq 0.05$) between raw and grilled samples. *** $p < 0.001$. 3,4-DHPEA, hydroxytyrosol; Form, formulation; Gr, grilling; *p*-HPEA, tyrosol; St, storage; VB, verbascoside; 3,4-DHPEA-EDA, oleacein; *nd*: not detected

Since the increase of 3,4-DHPEA, in presence of 3,4-DHPEA-EDA, has already been observed in different food matrices and at different temperatures (Servili et al., 2011; Esposto et al., 2015; Taticchi et al., 2017; Balzan et al., 2017), the phenols evolution in L1 and L2 hamburgers strengthens the hypothesis of its hydrolytic origin from the degradation of oleuropein derivatives during storage (Brenes et al., 2001). Nevertheless, the oxidative degradation of these two phenols has been appointed as the main cause of their decrease (Obied et al., 2008; Di Maio et al., 2011). For hydroxytyrosol, therefore, two contemporary phenomena would be at the basis of its particular evolution over time: the first is the hydrolysis of 3,4-DHPEA-EDA after which this phenolic alcohol is released in free form, while the second one is the oxidative degradation which leads to its decrement. In the case of samples L1 and L2, in the early stages of storage, hydroxytyrosol is

limitedly involved in oxidation reactions and, therefore, the resultant balance between the decrease on the one hand and the increase on the other, is an increase of the concentration of hydroxytyrosol. In the more advanced phases, the oxidative degradation prevails in L1 hamburgers, due to the decrease in the concentration of the other more reactive phenols (such as 3,4-DHPEA-EDA); in the L2 samples, on the contrary, being the concentration of 3,4-DHPEA-EDA still high until the ninth days of storage, the balance is evidently still in favor of the increase of 3,4-DHPEA by hydrolysis rather than its decrease by oxidation.

During storage, the concentration of *p*-HPEA and verbascoside did not significantly vary, as already found in other shelf-life studies for food matrices (Menchetti et al., 2020). Despite the significant decrease during storage, in terms of total polyphenols, the percentage retained was 56.5% and 63.5% of the initial amount added to L1 and L2, respectively.

Regarding the behavior of phenolic compounds during cooking, the loss was greater than those found during storage. In all grilled samples, 3,4-DHPEA-EDA completely disappeared, confirming its high susceptibility to high cooking temperatures (Lonzano-Castellón et al., 2020), while a severe loss of hydroxytyrosol (ranging between 53 and 100% from 0 to 9 days) was observed as well. Differently from the evolution noticed during storage, *p*-HPEA and verbascoside also showed a consistent decrease with grilling. However, even after cooking, the amount of the retained phenols ranged between 12.4% (L1 T9) and 29.4% (L2 T0).

3.4.3 Chemical analysis

3.4.3.1 Lipid content and main lipid classes

As reported in **Table 3.3**, the lipid content ranged from 4.9 to 6.3% and from 6.1 to 7.5% in raw and grilled burgers, respectively. Formulation and storage did not significantly affect the lipid content of both raw and grilled samples. However, it is possible to note a significant increase in the lipid amount of the grilled samples, which could be attributed to the free water loss during grilling, with consequent dehydration of the meat product (Gruffat et al., 2021).

Regarding main lipid classes (**Table 3.3**), the most abundant class was TAG, followed by FFA, DAG, E-STE, STE and MAG in both raw and grilled burgers. While shelf-life did not significantly affect the percentage distribution of the single lipid classes, formulation apparently had an effect on FFA, MAG, STE, E-STE and TAG, but without displaying a clear trend. These slight differences could be partly due to the non-homogeneous nature of hamburgers as they were prepared with minced beef meat from different cuts. After cooking, some significant differences were detected in the total lipid

profile (in particular MAG, DAG, and STE), which may have been influenced by a combined effect of lipolysis and a partial/selective loss of the lipid components during grilling, due to their different melting points.

Table 3.3. Lipid content and main lipid classes profile of raw and grilled hamburger samples after 0, 6 and 9 days of storage. C, Control (minced beef meat + maltodextrine + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat; L2, minced beef meat + starter cultures + 175 mg phenols/kg of meat.

		Lipid content	FFA	MAG	STE	DAG	E-STE	TAG
		(%)	(% of total lipids)					
Raw samples				0 days				
	C	4.93±0.60^{Y}	5.87±0.21^{X}	0.26±0.01^{X}	1.87±0.44	4.26±0.79	2.01±0.05	85.62±1.18
	L1	6.33±0.53	4.15±0.70	0.13±0.01	1.10±0.12	3.50±0.50^{Y}	1.18±0.64^{Y}	89.87±1.01^{X}
	L2	5.29±0.45^{Y}	3.51±0.45	0.10±0.02	1.42±0.38	4.20±0.59	2.12±0.07	88.58±1.25
				6 days				
	C	4.98±0.30^{Y}	6.12±0.54	0.24±0.03^{X}	1.88±0.45	4.18±1.01	1.68±0.57	85.80±1.45
	L1	5.13±1.02^{Y}	5.50±1.28	0.16±0.06	1.28±0.36	4.49±0.76	2.24±0.12	86.26±1.91
	L2	5.39±0.62^{Y}	3.10±0.21^{Y}	0.08±0.02	1.35±0.15	4.51±0.54	2.41±0.26	88.49±0.46^{X}
				9 days				
	C	5.04±0.35^{Y}	5.55±0.12	0.18±0.01	1.70±0.06	3.83±0.12	2.00±0.02	86.68±0.17
	L1	5.29±0.45^{Y}	4.36±0.74	0.11±0.00	1.14±0.16	4.34±0.66	2.19±0.02	87.79±0.07
	L2	6.06±0.18^{Y}	5.13±0.56^{X}	0.15±0.01^{X}	1.13±0.15	4.64±0.24	2.28±0.01^{X}	86.61±0.76
Grilled samples				0 days				
	C	6.12±0.47^{X}	5.38±0.21^{Y}	0.19±0.02^{Y}	1.97±0.22	4.81±0.63	2.00±0.07	85.57±0.91
	L1	6.15±0.22	3.56±0.55	0.10±0.03	1.66±0.27	4.84±0.33^{X}	2.20±0.19^{X}	87.57±0.57^{Y}
	L2	6.21±0.10^{X}	4.21±0.39	0.13±0.02	1.56±0.10	5.97±0.26	2.11±0.23	86.86±0.63
				6 days				
	C	7.09±0.35^{X}	6.06±0.37	0.18±0.01^{Y}	1.65±0.10	4.29±0.20	1.95±0.31	85.79±0.28
	L1	6.47±0.90^{X}	4.98±0.32	0.10±0.04	1.66±0.38	5.98±0.88	2.13±0.12	87.08±1.42
	L2	6.66±1.15^{X}	4.07±0.24^{X}	0.11±0.01	1.69±0.22	5.24±0.51	2.18±0.08	86.75±0.88^{Y}
				9 days				
	C	7.49±1.02^{X}	5.24±1.03	0.17±0.04	1.72±0.60	4.37±0.99	1.79±0.57	86.65±2.00
	L1	6.47±0.74^{X}	3.76±0.32	0.10±0.01	1.67±0.33	4.87±0.46	2.06±0.08	87.42±0.51
	L2	6.89±1.22^{X}	3.85±0.24^{Y}	0.10±0.01^{Y}	1.44±0.33	4.45±0.52	2.09±0.06^{Y}	88.02±1.16
	Factor				F value			
	Form	0.30 *ns*	46.62***	58.68***	8.30**	1.32 *ns*	3.66*	11.52***
	St	1.05 *ns*	2.34 *ns*	1.15 *ns*	0.82 *ns*	0.52 *ns*	0.53 *ns*	1.81 *ns*
	Gr	35.04***	3.59 *ns*	11.10**	5.90*	6.89*	0.01 *ns*	1.35 *ns*
	Form*St	0.72 *ns*	4.03**	3.21*	0.35 *ns*	0.74 *ns*	1.56 *ns*	1.78 *ns*
	Form*Gr	2.03 *ns*	3.07 *ns*	4.01*	3.68*	0.77 *ns*	1.74 *ns*	0.84 *ns*
	St*Gr	2.35 *ns*	2.05 *ns*	0.24 *ns*	0.21 *ns*	0.33 *ns*	2.14 *ns*	1.78 *ns*
	Form*St*Gr	0.34 *ns*	3.45*	4.58**	0.08 *ns*	0.84 *ns*	1.91 *ns*	2.52 *ns*

Results as reported as means±s.d of 3 independent replicates. X-Y indicate significant differences (Tukey's test; $p \leq 0.05$) between raw and grilled samples. *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$. DAG, diacylglycerols; E-STE, esterified sterols; FFA, free fatty acids; Form, formulation; Gr, grilling; MAG, monoacylglycerols; STE, sterols; St, storage; TAG, triacylglycerols.

3.4.3.2 Total fatty acid profile

About the total FA composition (**Table 3.4**), the most represented FA class was monounsaturated fatty acids (MUFA, 55-58%), followed by saturated fatty acids (SFA, 36-42%) and polyunsaturated fatty acids (PUFA, 2-4%). This FA profile agrees with that reported by Gruffat et al. (2021) for beef meat.

Table 3.4. Fatty acid classes (expressed as % of total fatty acids) of raw and grilled hamburgers after 0, 6 and 9 days of storage. C, Control (minced beef meat + maltodextrine + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat; L2, minced beef meat + starter cultures + 175 mg phenols/kg of meat.

		SFA	MUFA	PUFA	*n*-3	*n*-6
		(% total fatty acids)				
Raw samples		0 days				
	C	38.95±1.22	57.40±1.13	3.65±0.14	0.68±0.10	2.39±0.21
	L1	40.13±0.56	55.81±0.50	4.06±0.55^{a}	0.56±0.18	2.80±0.46^{a}
	L2	38.42±1.42	57.56±0.39	4.01±1.26	0.71±0.16	2.50±0.90
		6 days				
	C	36.90±3.79^{B}	58.94±3.09	4.17±0.95	0.65±0.15	2.89±0.82^{A}
	L1	40.59±1.59^{A}	57.58±1.29	4.82±0.52^{a}	0.73±0.06	2.99±0.48^{a,B}
	L2	40.01±3.16^{B}	57.71±2.60	4.27±0.58	0.58±0.03	2.43±0.40^{AB,Y}
		9 days				
	C	39.35±1.36	56.67±1.64	3.98±0.37	0.61±0.07	2.66±0.44
	L1	41.67±0.67	56.84±0.85	3.50±0.19^{b,Y}	0.65±0.17	2.77±0.30^{a,Y}
	L2	38.30±0.85^{Y}	58.64±1.49^{Y}	3.07±1.49	0.53±0.01^{X}	2.01±1.10
Grilled samples		0 days				
	C	39.70±1.32	56.37±0.99	3.93±0.95	0.64±0.16	2.81±0.77
	L1	42.49±3.78	55.12±1.78	2.27±2.32	0.51±0.26	2.70±1.77
	L2	39.26±0.43^{b}	57.44±0.91	2.82±0.49	0.52±0.08	2.12±0.49
		6 days				
	C	39.55±2.51	56.87±1.97	3.58±0.86	0.48±0.05	2.78±0.75
	L1	40.10±1.92	55.10±3.34	3.12±0.95	0.62±0.21	2.19±0.62
	L2	39.73±0.53^{b}	57.33±0.63	2.94±0.14	0.43±0.11	2.32±0.23^{X}
		9 days				
	C	37.80±4.39	57.83±3.67	4.00±0.16^{A}	0.59±0.18	2.99±0.28
	L1	39.70±2.54	56.99±2.09	3.31±0.55^{AB,X}	0.57±0.23	2.40±0.15^{X}
	L2	41.18±0.21^{a,X}	56.14±0.27^{X}	2.69 ±0.38^{B}	0.41±0.04^{Y}	2.17±0.42
	Factor	F value				
	Form	2.96 *ns*	1.19 *ns*	10.08***	1.70 *ns*	10.16***
	St	0.04 *ns*	0.40 *ns*	1.88 *ns*	0.07 *ns*	1.28 *ns*
	Gr	0.39 *ns*	2.37 *ns*	0.35 *ns*	7.20*	4.94*
	Form*St	0.20 *ns*	0.18 *ns*	0.50 *ns*	2.16 *ns*	0.64 *ns*
	Form*Gr	0.29 *ns*	0.10 *ns*	0.36 *ns*	0.93 *ns*	0.21 *ns*
	St*Gr	0.24 *ns*	0.56 *ns*	1.80 *ns*	0.20 *ns*	1.89 *ns*
	Form*St*Gr	1.61 *ns*	0.88 *ns*	4.48**	1.09 *ns*	3.32*

Results as reported as means±s.d. of 3 independent replicates. a–b indicate significant differences (Tukey's test; $p \leq 0.05$) between the same sample during the shelf-life, A-B indicate significant differences (Tukey's test; $p \leq 0.05$) between treatments, X-Y indicate significant differences (Tukey's test; $p \leq 0.05$) between raw and grilled samples. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Form, formulation; Gr, grilling; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; St, storage; UFA, unsaturated fatty acids.

No significant effect of preservation time on the FA profile of hamburgers, was observed (**Table 3.4**). As expected, PE addition significantly stabilized PUFA in raw hamburgers, in particular PUFA *n-6*, which could be mainly ascribed to the antioxidant activity of the remaining 3,4-DHPEA and oleacein (≈ 73% of total remaining phenols; **Table 3.2**). However, grilling significantly affected the single PUFA classes, but no defined trend was observed. In addition, a significant interaction among the product formulation, storage time and grilling was noted only for PUFA and PUFA *n-6*. In this study, grilling may have induced modifications in the FA composition of hamburgers by oxidation of unsaturated FA and/or selective melting of lipids (Simopoulos, 2006); these two effects add up to the selective

degradation of phenols in the grilled PE-enriched products (**Table 3.2**), as oleacein completely disappeared after cooking.

Table 3.5. Ratios of fatty acid classes, atherogenic index (AI) and thrombogenic index (TI) of raw and grilled hamburgers after 0, 6 and 9 days of storage. C, Control (minced beef meat + maltodextrine + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat; L2, minced beef meat + starter cultures + 175 mg phenols/kg of meat.

		n-6/*n*-3	UFA/SFA	PUFA/SFA	AI	TI
Raw samples				**0 days**		
	C	3.59±0.80	1.57±0.08	0.09±0.01	0.58±0.01	1.17±0.06
	L1	5.45±2.00^{a}	1.49±0.03	0.10±0.01^{a}	0.60±0.01	1.24±0.04
	L2	3.69±1.80	1.60±0.09	0.11±0.04	0.56±0.03	1.14±0.07
				6 days		
	C	4.59±1.44^{A}	1.73±0.29	0.12±0.04^{A}	0.53±0.08	1.08±0.18
	L1	4.09±0.81^{b,B}	1.47±0.09	0.05±0.01^{b,B}	0.63±0.04	1.22±0.05
	L2	4.18±0.68^{AB,Y}	1.51±0.19	0.06±0.02^{AB}	0.62±0.06	1.22±0.15
				9 days		
	C	4.42±1.17	1.54±0.09	0.10±0.01	0.58±0.02	1.19±0.07
	L1	4.26±0.85^{b,Y}	1.40±0.04	0.04±0.00^{b}	0.66±0.01	1.27±0.06
	L2	3.77±2.04	1.61±0.06^{X}	0.08±0.04	0.57±0.02^{Y}	1.15±0.04^{Y}
Grilled samples				**0 days**		
	C	4.58±1.66^{B}	1.52±0.08	0.10±0.03	0.61±0.04	1.21±0.08
	L1	5.29±1.92^{A}	1.36±0.21	0.06±0.06	0.69±0.08	1.41±0.23
	L2	4.14±1.42^{B}	1.53±0.03^{a}	0.07±0.01	0.59±0.03	1.20±0.02^{b}
				6 days		
	C	5.85±1.84	1.54±0.17	0.09±0.03	0.60±0.06	1.21±0.13
	L1	3.99±0.28	1.46±0.17	0.08±0.03	0.58±0.05	1.23±0.11
	L2	5.71±1.73^{X}	1.52±0.03^{a}	0.07±0.00	0.59±0.02	1.22±0.22^{b}
				9 days		
	C	5.42±1.80	1.66±0.31	0.11±0.02^{A}	0.56±0.07	1.13±0.20
	L1	4.85±2.38^{X}	1.53±0.16	0.08±0.02^{AB}	0.61±0.06	1.22±0.15
	L2	5.42±1.40	1.43±0.01^{b,Y}	0.07±0.01^{B}	0.63±0.03^{X}	1.30±0.01^{a,X}
	Factor			**F value**		
	Form	1.37 *ns*	3.23 *ns*	9.46***	4.01*	2.30 *ns*
	St	0.98 *ns*	0.03 *ns*	1.31 *ns*	0.18 *ns*	0.11 *ns*
	Gr	14.99***	0.57 *ns*	0.14 *ns*	0.14 *ns*	1.42 *ns*
	Form*St	2.68*	0.24 *ns*	0.56 *ns*	0.69 *ns*	0.20 *ns*
	Form*Gr	0.93 *ns*	0.26 *ns*	0.45 *ns*	0.31 *ns*	0.18 *ns*
	St*Gr	0.48 *ns*	0.39 *ns*	1.36 *ns*	0.48 *ns*	0.18 *ns*
	Form*St*Gr	0.32 *ns*	1.47 *ns*	3.92*	2.73*	1.43 *ns*

Results as reported as means± s.d of 3 independent replicates. a–b indicate significant differences (Tukey's test; $p \leq 0.05$) between the same sample during the shelf-life, A-B indicate significant differences (Tukey's test; $p \leq 0.05$) between treatments, X-Y indicate significant differences (Tukey's test; $p \leq 0.05$) between raw and grilled samples. *$p < 0.05$, ***$p < 0.001$. AI, Atherogenic Index; Form, formulation; Gr, grilling PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids, St, storage; TI, thrombogenic index; UFA, unsaturated fatty acids.

Regarding FA classes ratios (**Tables 3.5**), the PUFA *n*-6/PUFA *n*-3 ratio varied between 3.59 and 5.45 in raw hamburgers, while it ranged from 3.99 to 5.85 in grilled ones; these values agree with those found by Gruffat et al. (2021) for raw and grilled beef meat. The PUFA *n*-6/PUFA *n*-3 ratio is a suitable index to compare the nutritional value of food. According to Simopoulos (2006), a low PUFA *n*-6/PUFA *n*-3 ratio (< 4) is desirable for a healthy human diet. Grilling significantly impacted this ratio as a consequence of its effect on the single PUFA classes, but no common trend was observed

among the different types of samples. A significant interaction between the product formulation and storage time was detected for this ratio.

The UFA/SFA ratio is useful for observing the oxidative stability of fatty acids in food, as it decreases when UFA oxidize. The UFA/SFA ratio ranged from 1.40 to 1.73 in raw burgers, while it varied from 1.36 to 1.66 in grilled samples, respectively. These values agree with those reported by Gruffat et al. (2021) for raw and grilled beef meat. None of the factors here tested (product formulation, storage time and grilling) significantly influenced this ratio.

The PUFA/SFA ratio is also used for evaluating the nutritional quality of foods lipids and it has been suggested by nutritional guidelines that it should be around 0.4. In our case, this ratio ranged between 0.05 and 0.12 in raw hamburgers, whereas it varied from 0.06 to 0.30 in grilled ones. The results of the present study agree with the PUFA/SFA ratio reported by Gruffat et al. (2021) in raw and after grilling beef meat. This ratio reflected the behavior of the PUFA class, as it was also significantly influenced by formulation and showed a significant interaction among the product formulation, storage time and grilling.

Based on FA composition, AI and TI were also calculated, which are useful indices for understanding the role of FA composition on both atherogenic and thrombogenic risks. The indices ranged from 0.53 to 0.69 and from 1.08 to 1.41 for AI and TI, respectively, which agree with those reported by Gruffat et al. (2021) for beef meat cooked with diverse techniques. While TI values were not significantly different, AI was significantly affected by formulation, confirming the protective effect of PE on unsaturated FA, mainly observed in raw hamburgers. A significant interaction among the three factors (product formulation, storage time and grilling) was also detected in AI.

Regarding single FA, the most abundant FA in both raw and grilled samples was oleic acid (C18:1 n-9), followed by palmitic acid (C16:0) and stearic acid (C18:0). No significant differences were found in single FA between control and PE-enriched samples during shelf-life. Grilling significantly decreased the content of palmitoleic acid and its *trans* isomer (C16:1 *n7* and C16:1 *t n7),* as reported by Gruffat et al. (2021).

3.4.3.3 Lipid oxidation

Primary lipid oxidation products were monitored by PV, while TBARs and total COPs were determined as secondary oxidation products (**Table 3.6**).

PV ranged from 0.84-5.63 and 5.65-17.74 meq O_2/kg of fat in raw and grilled samples, respectively. Product formulation, storage time and grilling significantly influenced this oxidative parameter; a significant interaction between storage and grilling was also noticed. All this resulted in PV data that

were up to 1.4- and 1.3-fold lower in phenol-enriched raw and cooked hamburgers, respectively, than in control samples. Significantly lower PV levels (0.84-3.58 and 5.65-13.12 meq O_2/kg of fat in raw and grilled samples, respectively) were found in the L2 samples, added with 175 mg phenols/kg of meat; in fact, a PE concentration-dependent effect was observed in grilled hamburgers that had been previously stored for 6 and 9 days. Preliminary lipid oxidation may have already initiated during meat mincing, as grinding was carried out in presence of air and oxygen availability is known to be particularly critical for highly pigmented meats as beef; the salt addition during dough mixing might have also exerted a pro-oxidant effect (Ortuño et al., 2021). Despite being packed under modified atmosphere, the latter contained 20% oxygen, which might have contributed to further formation and accumulation of hydroperoxides during storage. However, due to their high instability, hydroperoxides greatly decomposed when hamburgers were grilled, giving rise to free radical's species that led to propagation of lipid oxidation. In any case, due to their free radical scavenging action (Miraglia et al., 2020), added phenols demonstrated to efficiently contrast the formation of lipid hydroperoxides during storage and after grilling, despite the selective degradation of phenols in the grilled PE-enriched products (**Table 3.2**).

When hydroperoxides decompose, they give rise to alkoxyl and hydroxyl radicals, which can evolve into secondary oxidation products such as aldehydes and ketones, whose presence can be determined as TBARs. In the present study, TBARs ranged from 0.48-4.86 and 0.52-4.07 mg MDA/kg in raw and grilled burgers, respectively, and were up to 4.5- and 5.7-fold lower in phenol-enriched raw and cooked hamburgers, respectively, than in control samples. Product formulation and storage time significantly affected TBARs, and the interaction between these two factors was significant as well. A PE concentration-dependent effect was observed in both raw and grilled samples. As observed for PV, PE was also able to limit the formation of secondary oxidation products during storage and after grilling, in spite of cooking-induced pro-oxidant conditions that arise from iron release from denaturated heme pigments (chiefly myoglobin) (Rodriguez-Carpena et al., 2012). In our study, the PE added to the hamburgers did not lose its effectiveness after grilling thanks to its formulation with the addition of maltodextrins during spray-drying, which exerted a protective role. In fact, TBARs values of phenol-enriched raw and grilled samples, were below or very close to 1 mg MDA/kg, value above which rancid flavor begins to develop in meat (Gray & Pearson, 1987). Our TBARs results are similar to those reported by Aouidi et al. (2017) and Shalaby et al. (2018) in beef burgers added with a phenol rich extract derived from olive leaf in a ratio of 105 mg of PE/100 g of minced beef. In the study of Aouidi et al. (2017), the antioxidant activity of olive leaves extract

against lipid oxidation was lower in cooked meat than in raw meat, which was attributed to the inhibition of the antioxidant activity of olive leaves extract by substances produced during cooking. On the other hand, cholesterol is an important constituent of cell membranes and, as FA, it is also susceptible to oxidation. The total content of cholesterol (**Table 3.6**) ranged from 538.3 to 926.1 mg/kg in raw hamburgers and from 1140.1 to 1225.1 mg/kg in grilled ones. These data agree with those reported by Barriuso et al. (2015) for beef hamburger. Cholesterol content was not significantly affected by the addition of the phenolic extract, nor by the storage time. However, grilling significantly increased the cholesterol content of the hamburgers, which could be ascribed to the water loss and consequent dehydration of the product during grilling (Gruffat et al., 2021).

Regarding total COPs, they varied from 0.90 to 6.32 mg/kg in raw hamburgers and 2.9 to 17.0 mg/kg in cooked ones (**Table 3.6**). The oxysterols profile and amount detected in the present study is similar to the one reported by Barriuso et al. (2015) for raw and cooked beef burgers, where oxidation products deriving from monomolecular reaction pathway (i.e. 7-derivatives) were more abundant than those generated by bimolecular ones (i.e. epoxy and triol derivatives); in particular, triol was present at very low levels (< 0.3 mg/kg) in grilled samples, whose formation is known to be favored by water in acid conditions. In the present study, product formulation, storage time and grilling significantly influenced total COPs, and all the 2-factor and 3-factor interactions were significant as well. COPs displayed a similar behavior to PV and TBARs, as total COPs were up to 8.8- and 4-fold lower in PE raw and PE cooked hamburgers, respectively, than in control samples, thus confirming a PE concentration-dependent effect related to their chain-breaking antioxidant activity.

Table 3.6 PV, TBARs, cholesterol, total COPs and COR of raw and grilled hamburgers after 0, 6 and 9 days of storage. C, Control (minced beef meat + maltodextrine + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat; L2, minced beef meat + starter cultures + 175 mg phenols/kg of meat.

		PV (meq O_2/kg fat)	TBARs (mg MDA/kg burger)	Cholesterol (mg/kg burger)	Total COPs	COR (%)
Raw samples				0 days		
	C	0.91±0.08^{c,Y}	0.81±0.16^{c,A}	538.30±20.32^{Y}	1.59±0.30^{b,Y}	0.29±0.05^{b,A}
	L1	0.89±0.09^{c,Y}	0.53±0.04^{b,AB}	743.04±66.67	1.28±0.14^{Y}	0.17±0.04^{B,Y}
	L2	0.84±0.06^{b,Y}	0.48±0.06^{b,}	728.32±17.02^{Y}	0.90±0.25^{Y}	0.12±0.03^{B,Y}
				6 days		
	C	3.57±0.17^{b,Y}	4.19±0.20^{b,A,X}	560.44±22.92^{Y}	5.38±0.07^{a,A,Y}	0.97±0.17^{a,A}
	L1	3.01±0.20^{b,Y}	1.07±0.31^{ab,B}	773.74±8.23^{Y}	2.03±0.35^{B,Y}	0.26±0.04^{B,Y}
	L2	2.88±0.15^{a,Y}	0.55±0.09^{C}	827.74±79.71^{Y}	1.17±0.36^{B,Y}	0.14±0.06^{B,Y}
				9 days		
	C	5.63±0.20^{a,A,Y}	4.86±0.57^{a,A}	587.53±63.62^{Y}	6.32±0.71^{a,A,Y}	1.08±0.01^{a,A}
	L1	4.05±0.31^{a,B,Y}	1.22±0.25^{a,B}	778.46±25.49^{Y}	2.31±0.48^{B}	0.30±0.04^{B}
	L2	3.58±0.38^{a,B,Y}	0.51±0.03^{C}	926.07±51.88^{Y}	1.19±0.28^{B,Y}	0.13±0.04^{B,Y}
Grilled sample				0 days		
	C	8.04±0.68^{c,X}	0.93±0.14^{c,A}	1040.97±113.46^{X}	2.88±0.42^{c,X}	0.30±0.07^{c}
	L1	6.14±0.55^{b,X}	0.63±0.09^{B}	1145.94±166.70	3.98±0.21^{X}	0.35±0.06^{X}
	L2	5.65±0.66^{c,X}	0.52±0.05^{b,C}	1097.02±59.73^{X}	2.85±0.24^{X}	0.26±0.04^{X}
				6 days		
	C	13.62±0.92^{b,A,X}	3.17±0.47b^{A,Y}	1146.78±124.46^{X}	11.88±0.03^{b,A,X}	1.07±0.30^{b,A}
	L1	11.44±0.86^{a,A,X}	0.97±0.02^{B}	1022.39±9.76^{X}	4.96±1.01^{B,X}	0.49±0.10^{B,X}
	L2	9.15±0.72^{b,B,X}	0.64±0.04^{a,B}	1041.24±10.81^{X}	3.04±0.51^{B,X}	0.29±0.05^{B,X}
				9 days		
	C	17.74±1.01^{a,A,X}	4.07±0.20^{a,A}	1126.62±100.13^{X}	17.00±1.47^{a,A,X}	1.51±0.01^{a,A,X}
	L1	14.87±1.09^{a,AB,X}	1.02±0.16^{B}	1225.14±100.72^{X}	3.70±0.30^{B}	0.31±0.10^{B}
	L2	13.12±0.99^{a,B,X}	0.72±0.03^{a,B}	1104.97±114.31^{X}	3.22±0.76^{B,X}	0.30±0.10^{B,X}
	Factor			**F value**		
	Form	28.13***	90.43***	2.44 *ns*	705.66***	284.00***
	St	127.38***	35.94***	0.88 *ns*	238.65***	85.21***
	Gr	769.06***	4.26 *ns*	105.30***	742.57***	49.33***
	Form*St	0.86 *ns*	17.17***	0.50 *ns*	175.40***	60.26***
	Form*Gr	5.05 *ns*	1.10 *ns*	3.50 *ns*	110.80***	0.87 *ns*
	St*Gr	29.68***	1.26 *ns*	0.01 *ns*	42.23***	0.74 *ns*
	Form*St*Gr	0.73 *ns*	0.94 *ns*	1.72 *ns*	58.73***	5.78**

Results as reported as means± s.d of 3 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) between the same sample during the shelf-life, A-C indicate significant differences (Tukey's test; $p \leq 0.05$) between treatments, X-Y indicate significant differences (Tukey's test; $p \leq 0.05$) between raw and grilled samples. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. COR, cholesterol oxidation ratio; COPs, cholesterol oxidation products; Form, formulation; Gr, grilling PV, peroxide value; St, storage; TBARs, thiobarbituric acid reactive substances.

The cholesterol oxidation ratio (COR) ranged from 0.12% to 1.51% and from 0.12% to 1.08% in raw and grilled samples (**Table 3.6**), respectively, being significantly higher in control samples than in phenol-enriched ones. Similarly to total COPs, product formulation, storage time and grilling significantly influenced this ratio, but only Form * St and the 3-factor interaction were significant. While both control and L1 showed a constant increase of COR during storage, L2 exhibited a steady level of COR. In addition, COR of most cooked PE hamburgers was about 2 times higher than their corresponding raw samples.

As emerged from the study, the use of phenol extracts from agricultural by-products allows to limit lipid oxidation and the formation of potentially harmful compounds such as cholesterol oxidation products (Testa et al., 2018; Duc et al., 2019; Zmysłowski et al., 2019). Successful results were obtained by using a phenolic extract rich in 3,4-DHPEA, *p*-HPEA, verbascoside and 3,4-DHPEA-EDA, in raw and cooked pork sausages (Balzan et al., 2017).

3.4.4 Principal Component Analysis (PCA) of chemical data

To better understand which parameters were the most relevant for assessing the effects of phenolic enrichment, cooking treatment and storage on the hamburgers, the chemical composition and phenolic composition data were subjected to principal component analysis (PCA) for raw and grilled hamburgers (**Figure 3.1**).

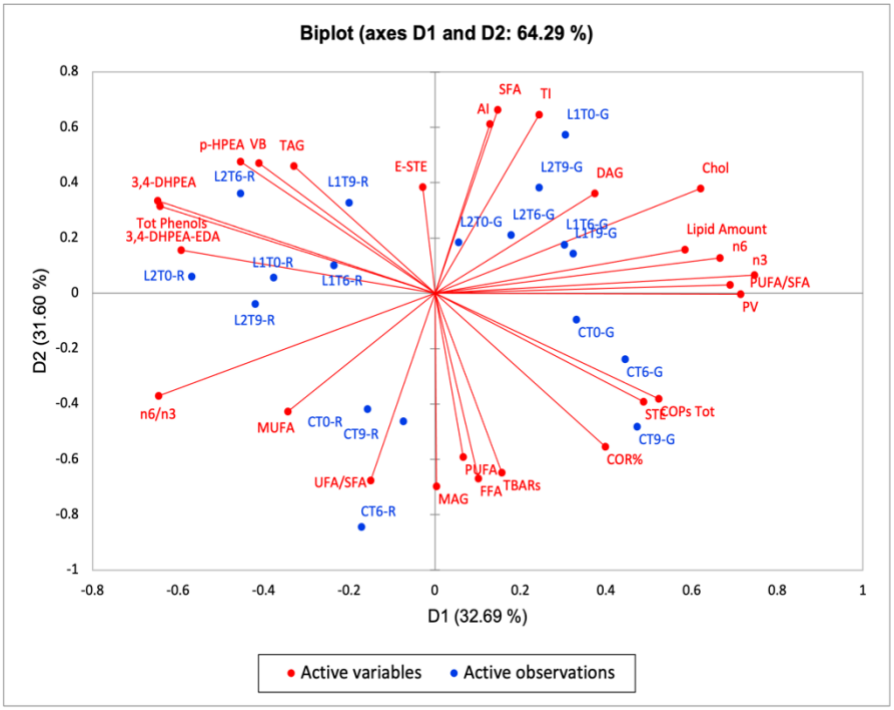

Figure 3.1. Biplot of raw and grilled hamburgers. 3,4-DHPEA, Hydroxytyrosol; 3,4-DHPEA-EDA, decarboxymethyl oleuropein aglycone; AI, Atherogenic Index; COPs, cholesterol oxidation products; COR, cholesterol oxidation ratio; DAG, diacylglycerols; E-STE, esterified sterols; FFA, free fatty acids; MAG, monoacylglycerols; MUFA, monounsaturated fatty acids; *p*-HPEA, tyrosol; PV, peroxide value; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; STE, sterols; TAG, triacylglycerols; TBARs, thiobarbituric acid reactive substances; TI, thrombogenic index; UFA, unsaturated fatty acids; VB, verbascoside.

The first two components explained 64.29% of the total variance (32.69% for PC1 and 31.60% for PC2). All oxidative parameters (COPs, TBARs, COR, PV) are in the opposite quadrant (3) with respect to total phenols and the single phenolic compounds (3,4-DHPEA, 3,4-DHPEA-EDA, *p*-HPEA and VB; quadrant 1).

Grilled samples are well separated from raw ones and are located in different quadrants. Control grilled samples are more correlated to COPs tot and COR, while L1 and L2 grilled samples are more related to DAG. This correlation could be explained by the fact that the phenolic extract present in L1 and L2 was able to limit cholesterol oxidation, but not TAG hydrolysis. Control raw samples are more correlated with UFA/SFA ratio, while L1 and L2 raw samples are more related to total and single phenols. This could be due to the susceptibility of phenols to high temperatures, which led to a greater loss of phenols in L1 and L2 grilled samples compared to the corresponding raw samples over the shelf-life (Table 2). In all grilled samples, in fact, 3,4-DHPEA-EDA completely disappeared. These results confirm that the addition of PE can limit oxidative phenomena in both raw and grilled hamburgers. Although grilling tends to decrease the phenol content, these are still able to exert their antioxidant activity. In fact, as described in paragraph 3.2, even after cooking, the amount of phenols retained by hamburgers ranged between 12.4% and 29.4%.

3.4.5 Physical analysis

3.4.5.1 Image analysis

The electronic eye was applied to support the development of the sensory profile of hamburgers; it performs the analysis of the appearance of the product by acquisition of images in extremely short times, subsequently processed. The processing of the images of the samples (C, L1, L2) stored at 3 times (0, 6, 9 days) acquired by the instrument were processed using the Alphasoft software (version 14.0).

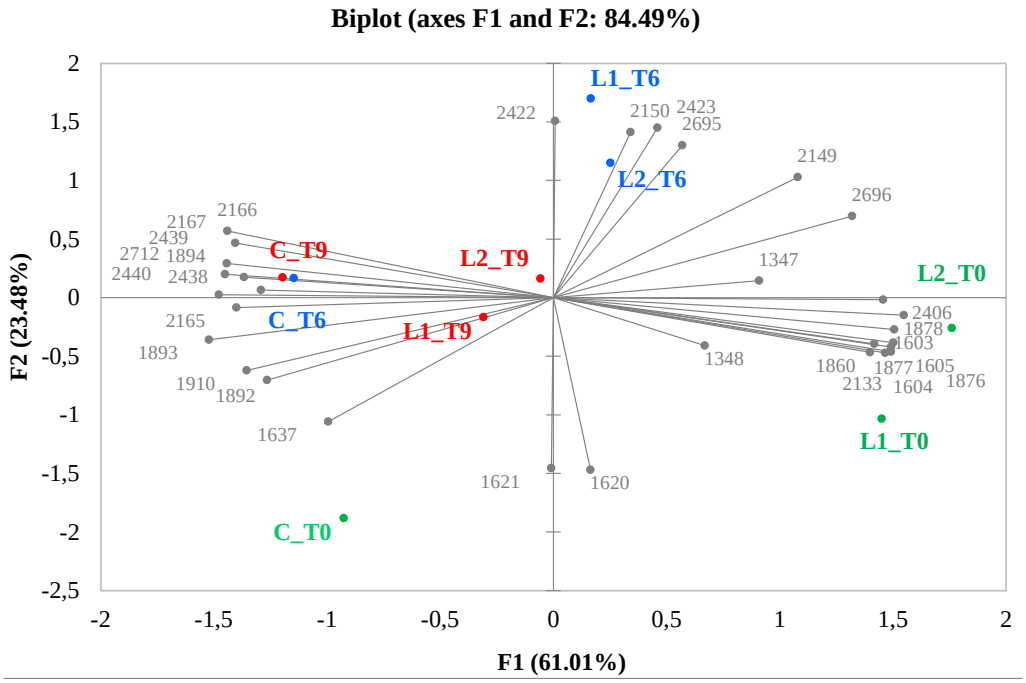

Figure 3.2. Representation of the cases and variables obtained from the PCA related to the results of the image analysis (electronic eye) for the 3 samples under examination (C, Control (minced beef meat + maltodextrine + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat; L2, minced beef meat + starter cultures + 175 phenols/kg of meat) evaluated at all the storage times (T0, T6 and T9).

Samples and instrumental (electronic eye) data were subjected to PCA (**Figure 3.2**) and projected into a two-dimensional plane composed of four quadrants to highlight possible correlations. The first two components explained 84.49% of the total variance (61.01% for F1 and 23.48% for F2). The different direction of vectors (loading PCA) showed the variables (colors) involved in the variations of appearance between the samples. By analyzing the different positions of the samples on the surface (PCA score), it is possible to observe that the control samples (CT0, CT6 and CT9) were more characterized by the variables present between the second and third quadrant that describe the greatest intensity of color and influence the position of the samples with a brown/gray color. This would therefore indicate that sample C is more oxidized; the brown color, in fact, could be due to a prevalence of metmyoglobin, the oxidized form of myoglobin. On the contrary, the phenol-enriched samples at time 0, were close to the variables that describe a low color intensity (fourth quadrant), that is a color that tends more to red. In this case, however, the addition of PE in the samples tends to create a reducing environment, with a consequent prevalence of oxymyoglobin, responsible for the bright red color of the sample. On the other hand, an intermediate color characterized the phenol-added samples stored at 6 and 9: they tended to be lighter for L1T6 and L2T6 (first quadrant) and darker for L1T9 and L2T9 (second and third quadrant). It can therefore be deduced that the presence of phenolic extracts and bioprotective cultures contributed to improving the visual quality of the burgers at both time 0 and during storage.

3.4.6 Sensory analysis

3.4.6.1 Descriptive analysis

The sensory profile of the phenol-enriched burgers was obtained by applying the QDA® method. The final list of descriptors included: i) 2 relative to appearance (presence of fat/connective tissue (total amount of fat/connective tissue inside the whole hamburger), color intensity (from red to brown/gray)); ii) 2 perceived by orthonasal and retronasal routes (beef flavor (flavor associated with cooked beef), bloody (flavor associated with blood or raw beef)); iii) 1 gustatory (salty- basic taste); iv) 2 relative to the texture: (tenderness (how easily it is chewed or cut), juiciness (amount of juice released from the product during mastication)).

When the training was completed, the sensory evaluation was carried out by the panel in five replicates for each type of sample at 3 different storage times (frozen just produced, T0; frozen after

6 days of storage, T6; frozen after 9 days of storage, T9). The average values for each attribute assessed are reported in **Table 3.7.**

Table 3.7. Mean values (five replicates) of the intensity of the attributes evaluated by quantitative-descriptive analysis relative to the samples (C, Control (minced beef meat + maltodextrine + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat; L2, minced beef meat + starter cultures + 175 phenols/kg of meat) at all the storage times (T0, T6 and T9), expressed on a scale from 0 to 100 (0 indicates the absence of perception of the attribute, 100 the maximum perception of the attribute) * extremes of the scale.

	Sensory descriptors							
Sample codes	**Beef flavor**	**Bloody**	**Salty**	**Juiciness**	**Granularity**	**Tenderness**	**Fat/connective tissue**	**Color**
	*(low; high)**	*(low; high)**	*(low; high)**	*(low; high)**	*(low; high)**	*(low; high)**	*(low; high)**	*(red; brown)**
CT0	47.2^{ab}	26.5^{ab}	38.2^{b}	55.3^{a}	36.7^{a}	55.6^{a}	42.3^{a}	67.2^{bc}
CT6	39.1^{bc}	28.7^{a}	39.9^{ab}	52.3^{a}	32.9^{a}	54.8^{a}	43.1^{a}	77.9^{ab}
CT9	37.7^{c}	24.5^{a-c}	38.9^{b}	37.6^{d}	38.6^{a}	45.3^{ab}	49.6^{a}	78.6^{a}
L1T0	51.9^{a}	19.0^{bc}	41.5 ^{ab}	51.3^{ab}	29.8^{a}	55.7^{a}	39.3^{a}	42.0^{ef}
L1T6	50.2^{a}	19.3^{bc}	41.4 ^{ab}	51.3^{ab}	38.3^{a}	51.1^{ab}	40.8^{a}	47.3^{d-f}
L1T9	50.8^{a}	24.1^{a-c}	42.0 ^{ab}	39.1^{cd}	38.2^{a}	41.6^{b}	40.2^{a}	49.9^{de}
L2T0	52.0^{a}	22.6^{a-c}	40.4 ^{ab}	51.3^{ab}	34.6^{a}	50.6^{ab}	43.6^{a}	38.0^{f}
L2T6	54.9^{a}	20.1^{bc}	42.6 ^{ab}	49.8^{a-c}	33.9^{a}	54.2^{a}	48.7^{a}	39.7^{ef}
L2T9	48.6^{a}	17.5^{c}	45.9^{a}	40.0^{b-d}	36.7^{a}	39.5^{b}	48.4^{a}	56.4^{cd}

Different letters (a- f) indicate significantly different values from each other (multiple comparison test, Fisher LDS with p <0.05)
*sensation intensity of attributes.

Concerning the control samples (C), it is possible to notice that there were no statistically significant differences in the attributes assessed by the panel during product storage except for the juiciness and color intensity. In particular, the juiciness decreased passing from 6 to 9 days of storage, while between T0 and T6, no differences were perceived for this texture attribute. The intensity of the color (from red to brown/gray), instead, showed the opposite trend, increasing significantly after 6 days of storage and thus confirming that myoglobin oxidation is already detectable after this time. The PE addition in sample L1 (87.5 mg phenols/kg of meat) did not affect most of the sensory attributes during storage; the only changes were related to the juiciness and tenderness texture attributes: in both cases, a decrease was observed over time. Unlike the control sample, L1 did not show any differences in color intensity. Having a higher concentration of phenolic extract (175 mg/kg of phenols), L2 displayed statistically significant differences only for the color intensity attribute, which significantly increased after 9 days of storage.

Considering the comparison among samples at the diverse storage times, it is possible to note that, for samples just produced (T0), the addition of the phenolic extract results in a color variation that shows lower values in both samples L1 and L2. In fact, the panel did not highlight any changes in the other attributes evaluated in the olfactory-retro-olfactory, gustatory and texture phases.

After 6 and 9 days of storage, the effect of the addition of phenolic extract on the color of the meat was confirmed: for both storage times a decrease in color intensity (0: red; 100: brown/gray) was found in the 2 phenol-enriched samples compared to the control one. Furthermore, the intensity of the beef flavor was higher in L1 and L2 compared to C, confirming the positive effect on the product performance during storage.

Samples and sensory attributes (most significant according to ANOVA) evaluated by the panel, were projected into a two-dimensional plane composed of four quadrants to highlight possible correlations by PCA (**Figure 3.3**).

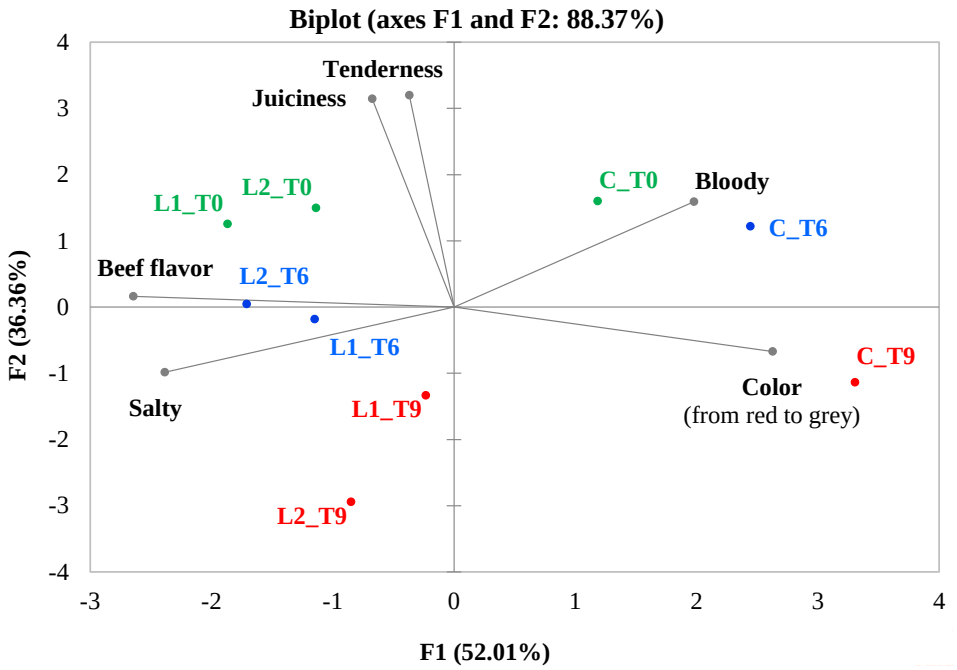

Figure 3.3. Representation of the cases and variables obtained from PCA related to the results of the quantitative-descriptive analysis for the 3 samples under examination (C, Control (minced beef meat + maltodextrine + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat; L2, minced beef meat + starter cultures + 175 phenols/kg of meat) evaluated at all the storage times (T0, T6 and T9).

The first two components explained 88.37% of the total variance (52.01% for PC1 and 36.36% for PC2). Control samples at 0 and 6 days of conservation (CT0 and CT6) were located in the first quadrant and characterized by a strong blood aroma and high tenderness. The fresh phenol-enriched samples (L1T0, L2T0) were placed in the second quadrant, whose position was influenced by the high intensity of juiciness and beef perceived by the panel. Samples at 6 days of storage (L1T6, L2T6) with similar characteristics were between the second and the third quadrant, being characterized by a low color intensity (0: red; 100: brown/gray). After 9 days of storage, L1 and L2 samples positioned in the third quadrant: although these samples were characterized by a high

intensity of color, compared to their performance at time 0 and 6, they showed a reduction of juiciness, tenderness and beef flavor, as well as an increase in salty. Finally, in the fourth quadrant is located the control sample at time 9, which was very similar to the C at 0 and 6 days, but less tender and juicy and with a dark color (high color intensity).

In addition to the evaluation of the descriptors by linear scale, assessors were asked to indicate the presence of off-flavors and negative sensations. Elaborating these comments, the presence of olfactory-gustatory anomalies (fermented, oxidized, rancid) emerged for the control sample at time 6 and 9, while for both L1 and L2, these anomalies were not significant.

3.4.6.2 Discriminant test

The triangle test was carried out to verify the existence of significant differences between the two phenol-enriched hamburgers (L1, L2) and the control sample (C) and between each type of sample stored at different times (0, 6 , 9 days), to provide useful information to evaluate the shelf-life of the new formulated products. The test was organized in several sessions in which all possible combinations of the samples to be compared were presented to the involved subjects (**Table 3.7**).

Table 3.7. Sessions, samples, number of subjects involved, number of correct given answers and significance level of the triangle test conducted on hamburger samples (C, Control (minced beef meat + maltodextrine + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat; L2, minced beef meat + starter cultures + 175 phenols/kg of meat) at all the storage times (T0, T6 and T9).

Session n.	Compared samples	Judges n.	Correct answers	Significance
1	CT0 vs. L1T0	30	15	0.05
2	CT0 vs. L2T0	30	17	0.01
3	L1T0 vs. L2T0	30	14	0.1
4	CT0 vs. CT6	30	16	0.05-0.01
5	CT0 vs. CT9	30	13	0.2
6	CT6 vs. CT9	30	9	*n.s.*
7	L1T0 vs. L1T6	28	10	*n.s.*
8	L1T0 vs. L1T9	28	13	0.2
9	L1T6 vs. L1T9	28	16	0.01
10	L2T0 vs. L2T6	28	12	0.3
11	L2T0 vs. L2T9	28	13	0.2
12	L2T6 vs. L2T9	28	10	*n.s.*

The significance is expressed in terms of α-risk level. *n.s.* indicates no significant perceptible difference between samples was found.

The sessions 1-3 aimed to compare all samples (C, L1, L2) at time 0 and the results, obtained from the 30 subjects, showed significant differences. In particular, the L1T0 sample was recognized as different from CT0 because it was characterized by a more intense beef flavor and different texture (session 1), while the CT0 sample resulted different from L2T0 for the less intense meat flavor and juiciness (session 2). The L2T0 sample was discriminated from L1T0 for the lower intensity of beef

flavor, tenderness and juiciness (session 3). These results were in agreement with those of Balzan et al. (2017): the subjects involved could discriminate between the three formulations (C, L1, L2) and the color was never mentioned as a discriminating factor even if, as evidenced by physical and sensory results previously discussed, the color variations were significant especially on the raw product. On the other hand, the hamburgers with added phenolic extract (L1, L2) were perceived as significantly different. In sessions 4-6, a comparison between the control samples at the 3 storage times, was carried out. Only the session in which CT0 was compared with CT6 and CT9 evidenced significant differences, while no differences between CT6 and CT9 were detected (session 6). When CT0 and CT6 were compared (session 4), people indicated that differences were related to a lower intensity of beef flavor (sometimes considered unpleasant and/or anomalous), tenderness and juiciness of the sample stored for 6 days. In the comparison between CT0 and CT9 (session 5), judges correctly identified the different sample (CT9) because it was more salty, acid, hard, dry and characterized by unpleasant taste (sometimes subject indicated rancid and/or “expired”). Further sessions were conducted for the evaluation of the phenol-enriched samples at the 3 storage times. Interviewees (n=28) found statistically significant differences between L1T0 and L1T9 (session 8) due to lower overall taste (tasteless) of sample L1 at time 0, whereas the differences between L1T6 and L1T9 (session 9) were attributed to a higher overall taste (more acid) of L1T9. No significant differences in L1 at 0 and 6 days of storage, were perceived (session 7). Concerning L2 sample, significant differences were detected when comparing the sample just produced (T0) with those at 6 (session 10) and 9 days of storage (session 11), while no differences were perceived when comparing L2T6 and L2T9 (session 12). In both significant sessions, a higher overall taste (more acid and sometimes unpleasant) of stored samples (L2T6, L2T9), was perceived.

3.4.6.3 Correlation between sensory (QDA®) and instrumental (electronic eye) data

The data obtained from both sensory and instrumental approaches were statistically processed through multivariate factor analysis (MFA). Results showed that the sensory evaluation of color intensity (QDA) was correlated with the instrumental one (electronic eye). In particular, positive correlations were obtained between the sensory parameter and the instrumental variables (colors) that characterized the samples with darker coloring (Pearson's correlation coefficients: 0.95 (variable 2165), 0.91 (variable 2712); p <0.05), whereas negative correlations were found between the sensory parameter and the instrumental variables (colors) that describe a lighter staining of the samples (Pearson correlation coefficients: 0.87 (variable 1878), 0.80 (variable 2696); p <0.05).

3.5. CONCLUSIONS

This study demonstrated the efficacy of a powder formulation of a phenolic extract from olive vegetation water at improving the overall oxidative stability and sensory quality of raw and grilled beef hamburgers, which had been previously subjected to cold storage for 9 days. The added phenolic compounds underwent a progressive decrease during the shelf-life period, but more than 55% and 63% of the added phenols were still retained (for L1 and L2, respectively) after 9 days of storage. On the other hand, cooking caused a more drastic reduction of phenolic compounds, leaving only 29.4% of the amount added in L1 at the beginning of storage. Both PE concentrations (87.5 and 175 mg of phenols/kg meat) proved to effectively reduce primary and secondary lipid oxidation, as well as cholesterol oxides, during the burgers' shelf-life study and after cooking. In particular, PV, TBARs and total COPs were up to 1.4-, 4.5- and 8.8-fold lower in PE raw hamburgers, respectively, than in control samples; a similar trend was also noted in cooked hamburgers (1,3-, 5.7- and 4-fold lower). Moreover, COR in PE hamburgers was about half as much that of control samples and never exceeded 0.5%. Sensory analysis also confirmed the effectiveness of PE addition in beef hamburgers, having a positive effect especially on the intensity of the red color (raw product) as it resulted in a reduction of browning during storage. Furthermore, the presence of phenols was not perceived by panelists, so they did not negatively influence the organoleptic characteristics of the products. However, the discriminant test evidenced a qualitative decay of all products during storage, which was more relevant in the control and the phenol-enriched burgers at the highest PE dose. In conclusion, this study confirms that OMWW extracts rich in phenols could be an alternative for the reduction of synthetic additives in ground meat preparations, which would promote the formulation of healthier clean label products and improve the sustainability of the olive oil industry with a circular economy approach, by further valorizing this olive by-product.

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Chapter 4. Effects of phenols extracted from olive vegetation water on COPs, HCAs, mutagenicity and genotoxicity of cooked beef hamburgers

ABSTRACT

IARC has classified the consumption of red meat as probable human carcinogen (Group 2A). These unhealthy properties have been correlated to the presence in red meat of carcinogenic chemicals compounds that are formed during it processing and cooking. Most of these compounds appear to derive from the oxidation of lipidic and proteic meat fraction and seem to contribute to the development of various chronic diseases (i.e., cardiovascular disease, diabetes, cancer, etc.). The aim of this study was to evaluate the effect of an extract rich in phenols obtained from olive vegetation water on COPs and HCAs formation in grilled beef hamburgers during storage, as well as on their mutagenicity and genotoxicity. The results show that the phenolic extract at both concentrations proved to effectively reduce COPs formation during shelf-life. Moreover, the extract of cooked hamburgers proved to be genotoxic on PBMCs (Primary Peripheral Blood Mononuclear Cells) humans' cells, while they were not mutagenic. Nevertheless, the genotoxicity was reduced by presence of the phenolic extract.

In conclusion, the phenolic extract from olive oil wastewater proved to be an effective antioxidant and to reduce the production of genotoxic compounds (responsible for carcinogenicity of red meats, according to IARC), thus confirming to be a promising ingredient for meat products.

KEY WORDS: beef hamburgers, COPs, HCAs, phenolic extract; wastewater, mutagenicity, citoxicity

4.1 INTRODUCTION

Nutrition is widely recognized as one of the main determinants of the onset of various chronic degenerative diseases like cardiovascular diseases, diabetes and cancer (Guardiola et al., 1996; Guardiola et al., 2002; Sottero et al., 2009). In the case of cancer, a conspicuous percentage of cancer cases could be prevented by changes in dietary habits. Different foods can act both as risk factors and preventive factors towards carcinogenesis. Among the risk factors, IARC has classified the consumption of red meat as probable human carcinogen (Group 2A) and processed meat as carcinogen (Group 1). These unhealthy properties have been correlated to the presence in red meat of carcinogenic chemicals compounds that are formed during it processing and cooking. In these regards, much attention has been given to N-nitroso-compounds (NOC), polycyclic aromatic

hydrocarbons (PAH) and heterocyclic aromatic amines (HCAs). Furthermore, even the compounds that derive from the oxidation of lipids, cholesterol in particular, seem to contribute to the development of various chronic diseases, in addition to those already mentioned above (Guardiola et al., 1996; Guardiola et al., 2002; Sottero et al., 2009), they can contribute to the development of Alzheimer's (Bjorkhem et al., 2006; Cao et al., 2007) and Parkinson's diseases (Bosco et al., 2006; Rantham Prabhakara et al., 2008) and recently they have been associated with human inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease (Biasi et al., 2009; Biasi et al., 2013a). Cholesterol is a monounsaturated constituent of cell membranes and is involved in their permeability and fluidity. Due to the presence of a double bond (carbon 5), a wide range of cholesterol oxidation products (COPs) can be produced endogenously or exogenously through different reaction mechanisms and pathways (chemical, photosensitized and enzymatic oxidation). The faster reaction in the formation of cholesterol oxides is photooxidation that start with the addition of singlet oxygen ($^1O_2$) on the side of double bond present in caron 5, generating 5α-/6α-/6β-hydroperoxysterols, of which the most abundant was 5α-OOH which rearranges in 7α-OOH isomer, the most stable form. Autoxidation, instead, initiates with the abstraction of a reactive allylic hydrogen at C7, giving rise to a radical molecule that reacts with triplet oxygen ($^3O_2$) to form a 7-peroxy radical. The latter neutralizes with a hydrogen radical from other sterol/fatty acid molecule, thus promoting the classical radical-mediated mechanism and generating 7α-/7β-hydroperoxysterols. After dysmutation of 7- hydroperoxysterols, 7α-/7β-hydroxysterols and 7-ketosterol are formed. Other major sterol autooxidation products derive from the bimolecular reaction between a hydroperoxy radical and an unoxidized sterol molecule, leading to the formation of 5α,6α-/5β,6β-epoxysterols, which can undergo an oxirane ring opening in presence of water in acidic conditions and thus convert into sterol triols. Metabolic dysfunctions or the frequent consumption of COP-containing foods can be potentially harmful to health, several studies, in fact, have discussed their major biological effects.

During cooking of red meet, in addition, heterocyclic aromatic amines (HCAs) are produced. These compounds are mutagen and carcinogen and his amount produced depends upon the type of meat, more specifically by the amount of protein contained, cooking method, temperature, and duration of cooking. High temperature cooking method, like pan-frying, barbecuing, or grilling produces the highest amounts of HCAs (Ni et al., 2008). The most common HCAs, called “aminoimidazoarenes” which includes 2-amino-1-methyl-6-phe-nylimidazo[4,5-b] pyridine (PhIP), 2-amino-3-methyl-3H-imidazo[4,5-f] quinoline (IQ), 2-amino-3,8-dimethyl-imidazo[4,5-f] quinoxaline (MeIQx), and 2-

amino-3,4,8-trimethyl-3H-imidazo[4,5-f] quinoxaline (DiMeIQx) (Kizil et al., 2011), are produced by the pyrolysis of amino acids or a complex reactions that involving the product of the heat degradation of amino acids (pyridine or pyrazine) and creatinine (Turesky, 2007).

PhIP is the most abundant HCA in the human diet while IQ, MeIQx and DiMeIQx are among the most potent mutagen compounds ever tested in the Ames/*Salmonella* assay (Sugimura et al., 2004). Several epidemiological studies have indicated that high consumption of well-done meat could increase the risk of cancer in humans, mainly due to the presence of high quantity of HCAs (Khan et al., 2022).

In the last few years, several *in vitro*, animal, and epidemiological studies have demonstrated the importance of healthy nutrition and dietary components on disease prevention. The Mediterranean diet has been considered as an example of a diet capable of limiting the onset of various diseases, in particular cardiovascular diseases, and various types of cancer (Kalkuz & Demircan, 2021). This is thanks, above all, to the high consumption of vegetables and the presence of extra virgin olive oil as the main source of fats (Ly et al., 2021). In addition to the oleic acid, olive oil contains a lot of other compounds possessing distinct biological activity (Servili et al., 2009). Among them great attention has be given to the secoiridoid phenols and their derivatives such as hydroxytyrosol (3,4-DHPEA) and tyrosol (pHPEA) which have been shown to have anti-cancer activities *in vivo* (Fabiani, 2016) and to prevent the DNA damage induced *in vitro* by different compounds in a variety of cellular systems (Fuccelli et al., 2014). About 98% of the phenols contained in the olive, however, are not found in extra virgin olive oil, but in olive mill wastewater (OMWW), a by-product that originates during the pressing of olives and which has multiple activities, including antioxidant and antimicrobial (Caporaso et al., 2018).

The purpose of this work, therefore, was to evaluate the ability of a phenolic extract, obtained from the purification of OMWW, to counteract the formation of COPs and HCAs in grilled beef burgers subjected to a shelf-life study, as well as than to evaluate its impact on their mutagenicity and cytotoxicity.

4.2 MATERIAL AND METHODS

4.2.1 Phenolic extract (PE)

From fresh olive mill wastewater of olives from trees of Moraiolo cultivar, a PE was obtained as previously reported by Ianni et al. (2021), with a 3-step membrane filtration of fresh OMWW. The

extract was added with maltodextrin, in a 1: 1 (d.w.) ratio, as a support, and then spray-dried to get a powder formulation.

4.2.2 Preparation of phenol-enriched hamburger samples

For the preparation of burgers, an adult bovine meat mix specific for hamburgers preparation was bought on the market. The meat was trimmed and minced at a 6-mm diameter with a professional meat mincer (TCS32, Cavalli Meat Processing Machinery, Felino, Italy). The minced meat was mixed with salt (0.8 g/100 g) and starter cultures SafePro® (B-SF-43, *Leuconostoc carnosum*) and Bactoferm® (S-B-61, *Staphylococcus carnosus*) (Chr. Hansen GmbH, Germany) using a two-paddle mixer (IMP50-Bipala, Cavalli Meat Processing Machinery, Felino, Italy). The protective culture was added with the primary aim to control the spoilage bacteria (mainly *Enterobacteriaceae*) that can contaminate the beef meat; in this way, the unwanted variability factors that are not included in the experimental design (such as spoilage bacteria), are restrained. The dough was then divided into three batches:

- Control, meat dough+maltodextrin (0.35 g/100 g, Maltodextrin Glucidex 19, Roquette, France),
- L1, meat dough + PE (87.5 mg phenols/kg of meat),
- L2, meat dough + PE (175 mg phenols/kg of meat).

Each batch was further mixed for 1 min and the burgers were then molded (about 80 g each pattie), packed 2 per tray under modified atmosphere Alipak 333 mixture (50% nitrogen, 20% oxygen, 30% carbon dioxide) and wrapped with a film made of polyethylene terephthalate (PET) + polyethylene (PE)/ethylene vinyl alcohol (EVOH)/PE. The trays containing the hamburgers were randomly divided and placed in a display refrigerator at 4 ± 2°C for 9 days, under alternating exposure to fluorescent light (12 h light/12 h darkness) to simulate retail storage conditions. Burgers were sampled at fixed time periods (just after produced, T0; 6 days of storage, T6; 9 days of storage, T9) and grilled in an electrical grilling plate (Fimar FRY1L230M, Rimini, Italy) at 200°C for 4 min per side until the core temperature reached 70°C. After cooking, burgers were cooled down at room temperature for 5 min, placed in a blast chiller (TBF051B, Moduline, Treviso, Italy) at -40°C for 15 min, packed in a plastic bag under vacuum and stored at -80°C until analysis. Two independent batches of burgers' preparation were run.

4.2.3 Phenols analysis

For the PE analysis was used the method described by Selvaggini et al. (2014). Briefly 50 mg of spray-dried PE were solubilized in 10 mL of a methanol:water mixture (80:20, v/v), filtered with a 0.2 µm

polyvinylidene fluoride (PVDF) syringe filter (Agilent Captiva, Agilent Technologies, Santa Clara, CA, USA) and injected into a high-performance liquid chromatograph coupled to a diode array detector (HPLC-DAD Agilent Technologies system Mod. 1100). Each measurement was done in duplicate.

For the hamburgers, was used the method described by Miraglia et al. (2020). 5 grams of hamburgers were mixed with 100 mL of methanol:water (80:20, v/v) containing 20 mg/L of butylated hydroxytoluene (BHT) + 0.2% trichloroacetic acid 1 M. A homogenization, followed by recovery, concentration until a final volume of 40 mL of extract and purification by solid-phase extraction (SPE) from 10 mL of this aqueous extract were carried out. The purified extract was then subjected to HPLC-DAD analysis using the same equipment and conditions described by Selvaggini et al. (2014). Each measurement was done in duplicate.

4.2.4 Lipid extraction

Lipids were extracted according to a modified version of the Folch method (Boselli et al., 2001). Briefly, lipid fraction of 5 g of cooked burgers was extracted using a chloroform: methanol solution (1:1, v/v) followed by the addition of another aliquot of chloroform. After mixing with 1 M KCl, the organic phase was separated and taken to dryness; the fat content was determined gravimetrically. Three independent replicates for each sample were carried out.

4.2.5 Determination of cholesterol oxidation products (COPs)

Cholesterol and COPs were extracted and purified as described previously (Cardenia et al., 2015). Briefly, the lipid extract containing internal standard (12.5 µg of 19-hydroxycholesterol (Steraloids, Newport, Rhode Island, USA)) were subjected to cold saponification. One-tenth of the unsaponifiable matter was used to determine the sterol composition, while the remaining 9/10 were purified by SPE-NH_2 for COPs quantification. COPs fraction was silylated by adding 1 mL of pyridine:hexamethyldisilazane:trimethylchlorosilane (5:2:1, v/v/v) at 40°C for 20 min, taken to dryness under a nitrogen stream, re-dissolved in n-hexane and injected in Fast GC/MS (Cardenia et al., 2012). The identification and quantification COPs were carried out by comparing their mass spectra and retention times with those of the corresponding chemical standards (Sigma Chemical; Steraloids (Newport, Rhode Island, USA); Avanti Polar Lipids (Alabaster, Alabama, USA)) in the SIM acquisition mode by using calibration curves built for each chemical compound. Amount of single COP was expressed as mg/Kg of lipids. Three independent replicates for each sample were carried out.

The rate of total cholesterol oxidation (%OR) was also determined according to the following formula: %OR = (Total COPs/Total cholesterol) x 100 (Cardenia et al., 2015).

4.2.6 Determination of HCAs

HCAs were determined as reported by Jan et al. (2019).

4.2.7 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from leucocyte-enriched human peripheral blood on a density gradient as previously reported (Fabiani et al., 2007). Briefly, blood samples (2 mL) diluted to 8 mL with RPMI 1640 without serum, were layered over 2 mL of Histopaque 1077 and centrifuged at 1600 rpm for 20 min. The layer containing the mononuclear cells at the interface between the plasma and the Histopaque was recovered and washed twice with RPMI 1640. The viable PBMCs obtained were counted by the trypan-blue exclusion technique and the density was adjusted to 1 × 106 cells/mL with RPMI 1640. The cells were then used for the different experiments.

4.2.8 Treatment of PBMC with meat extracts

The PBMC suspensions were exposed to meat extracts in RPMI 1640 medium supplemented with 10% FCS, 2.0 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (complete RPMI medium). After incubation for 30 min at 37 °C and 5% CO_2, the cell viability and the DNA damage were evaluated by the trypan-blue exclusion technique and the comet assay, respectively. In some experiments the treatment of PBMC with meat extracts was carried out in different experimental conditions regarding the incubation time (15 min - 24 h), medium (simple RPMI 1640 and PBS) and temperature (4 °C), inclusion of DNA repair inhibitors cytosine 1-β-d-arabinofuranoside (AraC) and hydroxyurea (HU), inclusion of external metabolic activation (S9-mix).

4.2.9 Single-cell gel electrophoresis (comet assay)

The single-cell gel electrophoresis assay was performed essentially as previously described (Singh, et al., 1988). After treatment, aliquots of the cell suspension (50–100 µL, 0.5–1.0 ×105 cells) were transferred to 1.5-mL Eppendorf tubes and centrifuged at 1300 rpm for 6 min. The supernatant was discarded and the pellet was mixed with 75 µL of low melting-point agarose (0.7% in PBS), which was then distributed into conventional microscope slides pre-coated with normal melting-point agarose (0.5% in PBS), and dried at 50 °C. After the agarose had solidified (4 °C for 10 min), a third

layer of normal melting-point agarose was applied similarly to the second. The slides were then immersed in the lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, pH 10, containing freshly added 1% Triton X100 and 10% DMSO) for 1 h at 4°C and then placed into a horizontal electrophoresis apparatus filled with freshly made buffer (1 mM Na_2EDTA, 300 mM NaOH). After 20 min of pre-incubation (unwinding of DNA), the electrophoresis was run for 20 min at a fixed voltage of 25 V (0.83 V/cm) and 300 mA, adjusted by raising or lowering the level of the electrophoresis buffer in the tank. At the end of the electrophoresis, the slides were washed three times with neutralisation buffer (0.4 M Tris–HCl, pH 7.5), stained with 50 µL ethidium bromide (20 µg/mL), and kept in a moisture chamber in the dark at 4 °C until analysis. All steps described above were carried out under red light to prevent any additional DNA damage.

4.2.9.1 Comet detection

The cells were analyzed 24h after staining at 400X magnification with a fluorescence microscope (Zeiss, Germany) equipped with a 50-W mercury lamp. The extension of each comet was analyzed by means of a computerized image-analysis system (Comet assay II, Perceptive Instruments, UK) which, amongst several other parameters, gave the "Tail Intensity %", which represents the percentage of fluorescence intensity in the tail relative to the total intensity of the comet (Collins and Duthie, 1995).

4.2.10 Ames test

The *Salmonella typhimurium* His reversion test (Ames test) was carried out according to previous procedures (Wahab et al., 2018).

4.2.11 Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, France) was used to elaborate data. The data are reported as mean values of independent replicates of each analytical determination. First, normal distribution of data was tested (p<0.05) with Shapiro-Wilk method. After, data were analyzed using two-way analysis of variance (ANOVA), including formulation (Form), storage time (St) and their interaction (Form*St) as factor. Tukey's honest significance test was performed at a 95% confidence level (p≤0.05), to separate means of statistically different parameters. For the Comet Assay significant differences of the results of each experiment, repeated at least four times with different PBMC preparations, were assessed using Student's t-test (p<0.05). Finally, a principal component analysis (PCA), with a Varimax rotation, was also carried out.

4.3 RESULTS AND DISCUSSION

4.3.1 Evolution of total phenols during conservation

The PE used for the preparation of the burgers was the same used in the study of Miraglia et al. (2020) and had a total phenol content of 25.7 mg/g of dried product, of which the most abundant was 3,4-DHPEA-EDA (oleacein), followed by 3,4-DHPEA (hydroxytyrosol), verbascoside and *p*-HPEA (tyrosol).

As shown in **Table 4.1**, part of the added phenols was lost during storage. In fact, there was a loss of 34% and 43% of phenolic compounds in L1 at T6 and T9, respectively, whereas a minor loss was detected in L2 burgers (16.2 and 36.5%, respectively). As reported by Obied et al. (2008) and Di Maio et al. (2011), the main cause of phenols decrease was oxidative degradation products, moreover, cooking could also have contributed to the decrease of these compounds. It is in fact known that phenolic compounds are thermolabile molecules (Miraglia et al., 2020).

Table 4.1. Total phenols (mg/kg of meat), single COPs (mg/kg of lipids), cholesterol oxidation ratio (OR %), single and total HCAs (ng/g of meat) of grilled hamburgers after 0, 6 and 9 days of storage. C, Control (minced beef meat + maltodextrins + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat; L2, minced beef meat + starter cultures + 175 mg phenols/kg of meat.

		Storage Time (days)						SEM	*P*		
	Samples	0		6		9			Form	St	Form*St
Total phenols	C	-		-		-		0.52	***	***	***
	L1	20.29	a,B	11.48	b,B	5.95	c,B				
	L2	49.92	a,A	38.14	b,A	21.65	c,A				
7β -HC	C	0.96	b,B	4.03	ab,A	5.36	a,A	0.35	***	***	***
	L1	1.44	A	1.97	B	1.47	B				
	L2	1.17	A	1.35	B	1.36	B				
5β,6β-EC	C	0.65	c	2.15	b,A	3.54	a,A	0.23	***	***	***
	L1	0.85	*NS*	1.06	B	0.72	B				
	L2	0.74	*NS*	0.74	B	0.76	B				
5α,6α-EC	C	0.21	*NS*	1.28	A	0.99	A	0.11	**	*NS*	*NS*
	L1	0.23	*NS*	0.35	B	0.38	B				
	L2	0.11	*NS*	0.15	B	0.17	B				
CT	C	0.11	*NS*	0.31	A	0.26	A	0.02	**	*NS*	*NS*
	L1	0.10	*NS*	0.13	B	0.11	B				
	L2	0.07	*NS*	0.06	B	0.11	B				
7-KC	C	0.94	*NS*	4.03	A	6.84	A	0.48	***	***	***
	L1	1.36	*NS*	1.46	B	1.01	B				
	L2	0.77	*NS*	0.74	B	0.83	B				
OR	C	0.30	c	1.07	b,A	1.51	a,A	0.10	***	***	***
	L1	0.35	*NS*	0.49	B	0.31	B				
	L2	0.26	*NS*	0.29	B	0.30	B				
IQ	C	*n.d.*		*n.d.*		*n.d.*					
	L1	*n.d.*		*n.d.*		*n.d.*					
	L2	*n.d.*		*n.d.*		*n.d.*					
8-MeIQx	C	0.25	*NS*	0.33	*NS*	0.31	*NS*	0.01	*NS*	*NS*	*NS*
	L1	0.29	*NS*	0.31	*NS*	0.33	*NS*				

	L2	0.26	*NS*	0.29	*NS*	0.27	*NS*				
4,8-DiMeIQx	C	0.14	*NS*	0.12	*NS*	0.13	*NS*	0.00	*NS*	*NS*	*NS*
	L1	0.12	*NS*	0.13	*NS*	0.13	*NS*				
	L2	0.12	*NS*	0.14	*NS*	0.14	*NS*				
PhIP	C	*n.d.*		*n.d.*		*n.d.*					
	L1	*n.d.*		*n.d.*		*n.d.*					
	L2	*n.d.*		*n.d.*		*n.d.*					
HCAs Tot	C	0.39	*NS*	0.45	*NS*	0.44	*NS*	0.01	*NS*	*NS*	*NS*
	L1	0.41	*NS*	0.44	*NS*	0.45	*NS*				
	L2	0.38	*NS*	0.42	*NS*	0.40	*NS*				

Results as reported as means±s.d of 2 or 3 independent replicates. a–b indicates significant differences (Tukey's test; $p \leq 0.05$) within the same sample during the shelf-life. A-B indicate significant differences (Tukey's test; $p \leq 0.05$) among different formulations. $^{**}p<0.01$, $^{***}p<0.001$. 4,8-DiMeIQx, 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoxaline; 5α,6α-EC, 5α,6α-Epoxy Cholesterol; 5β,6β-EC, 5β,6β-Epoxy Cholesterol; 7β-HC, 7β-Hydroxy Cholesterol; 7-KC, 7-Keto Cholesterol; 8-MeIQx, 2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline; CT, Cholestane Triol; Form, formulation; HCAs Tot, Heterocyclic Aromatic Amines; IQ, 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline; *nd*, not determined; *NS*, non-significant; OR, Oxidation Ratio; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; St, storage.

4.3.2 Evolution and formation of COPs and HCAs

As reported in **Table 4.1**, COPs profile detected in the present study is similar to the one reported by Barriuso et al. (2015) for cooked beef burgers. The most abundant COPs detected in the samples were 7β-HC and 7-KC, 0.96-5.36 and 0.94-6.84 mg/kg of lipids respectively, that deriving from monomolecular reaction pathway, followed by 5β,6β and 5α,6α-EC, 0.65-3.54 and 0.21-1.28 mg/kg of lipids respectively, those generated by bimolecular ones. CT was present at very low levels (< 0.3 mg/kg lipids) whose formation is known to be favored by water in acid conditions.

Going to analyze the single compounds, it is possible to note how, for all COPs, the highest content was found in C, compared to L1 and L2 samples. Product formulation, storage time and their interaction, significantly influenced the presence of 7β-HC, 7-KC and 5β,6β-EC. On the other hand, for 5α,6α-EC and CT, only product formulation seems to have an impact on their content. Generally, products containing cholesterol, like red meat, are susceptible to oxidation, especially when submitted to high temperatures, as well as those that are cooked in the presence of oxygen (Guardiola et al., 2002; Vejux et al. 2008). Indeed, under these conditions, food cholesterol is exposed to numerous reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), which can contribute to the generation of cholesterol hydroperoxides, hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH). During thermal-induced oxidation, hydroperoxides in C-7 are mainly generated, thus 7-keto is one of the most representative COPs in food systems ranging from 30% to 70% of the total content of COPs. This has suggested that 7-keto can be the most reliable biomarker of cholesterol oxidation due high temperature processing of foods (Rodriguez-Estrada et al., 2014).

Several studies conducted on laboratory animals have shown that oxysterols incorporated through the diet or excreted by the liver, where cholesterol is oxidized by enzymatically and/or non-

enzymatically mediated reaction, can be absorbed in human intestine and distributed in tissues (Otaegui-Arrazola et al., 2010). The absorption of COPs is lower than that of cholesterol, this difference is probably due to the lower solubility of COPs. Each type of COP, in fact, is absorbed to different degrees; 7β-HC, 7-KC and 5α,6α-EC are detected in greater amounts than 5β,6β-EC and 25-HC, which are not quantified in some studies done on laboratory animals and healthy humans (Osada et al., 1994; Linseisen et al., 1998; Vine et al., 1998). On the contrary to 7β-HC, the 7-KC is slightly absorbed and rapidly metabolized by the liver (Schweizer et al. 2004). A correlation exists between serum levels of COPs and their concentration in the diet. The studies carried out by Staprans et al. (2003) in healthy humans have shown that after a diet containing about 400 mg of α-EC, serum α-EC can be detected, in comparison with the control group in which α-EC serum level was not quantified. Linseisen et al. (1998) have found that the intake animal origin food, like cheese and salami that providing, respectively, 0.84 mg and 2.64 mg of COPs, raises plasma COPs levels. Free oxidized cholesterol concentration increased three hours after the meal, but with very high interindividual variation. In contrast, plasma total COP levels were much higher after 6–8 h of intake salami and cheese. It was also found that, COPs concentrations found in plasma and tissues of the subjects under study are higher than those that would be predicted from the concentrations in the test diets (Linseisen et al., 1998). Despite their lower absorption compared to cholesterol, COPs are absorbed more quickly in the intestine, have faster plasma clearance and are quickly collected by tissues. Oxysterols in human plasma or serum may vary from about 1 μM (0.05% of total cholesterol) in healthy subjects to 20-30 μM (0.5-0.75% of total cholesterol) in diseased individuals, but much higher concentrations of plasma oxysterols have also been reported (Schroepfer, 2000).

The cholesterol oxidation ratio (OR) ranged from 0.12 to 1.08% in samples (**Table 4.1**) and being significantly higher in control samples than in phenol-enriched ones. Similarly to 7β-HC, 7-KC and 5β,6β-EC, product formulation and storage time and their interaction significantly influenced this ratio. While both C and L1 showed an increase of OR during storage, L2 exhibited a steady value of OR.

Furthermore, during a high temperature cooking of protein rich foods such as red meat, other particularly harmful compounds, the HCAs, are formed. As said previously, HCAs are potent mutagen and carcinogen compounds (Sugimura et al., 2004). To limit and inhibit the formation of HCAs, natural extracts from leaves, peel and by-products of the agricultural industry are often used which, being rich in phenolic compounds, exert antioxidant function. In fact, they act as a radical

scavenger to trap free radicals created in different pathways of HCAs formation (Gibis and Weiss 2012).

In the present study (**Table 4.1**) the presence of 4 main HCAs was evaluated, IQ, 8-MeIQx, 4,8-DiMeIQx and PhIP. Among these it was found the presence, in the samples under analysis, only of 8-MeIQx and 4,8-DiMeIQx, with values ranging from 0.25-0.33 and 0.12-0.14 ng/g of meat, respectively. HCAs content was not significantly affected by the addition of the phenolic extract, nor by the storage time.

The values found in the present study appear to be very low and there are no statistically significant differences between the C and the L samples. This could probably be due to the temperature, 200 °C, and the relatively short time, 4 minutes per side, to whom the burgers were cooked. In fact, the results obtained by Sepahpour et al. (2018), in which beef samples marinated with different herbs and spices were grilled at 240°C for 10 min, show that the herbs and spices used in this study as well as their combinations positively inhibited HCAs formation in grilled beef. Concentration of HCAs in the control sample were higher than those measured in marinated grilled beef samples with exception of PhIP in grilled beef marinated with curry leaf. The concentrations of MeIQ and MeIQx both in control and marinated samples were lower than the limit of detection (LOD).

The addition of phenol-rich plant extracts in the formulation has proven to be an excellent strategy in preventing the formation and accumulation of COPs and HCAs. As can be seen from this study, in fact, in samples L1 and L2, added with phenolic extract from OMWW, have significantly lower levels of COPs, if compared to sample C, formulated without PE. This trend is due to the PE action related to their chain-breaking antioxidant activity, mainly due to the presence of *p*-HPEA and verbascoside, which, from previous studies conducted on cooked food products, pork sausages (Balzan et al., 2017) and shrimp (Miraglia et al., 2020), would appear to be able to resist grilling and exert their antioxidant action.

4.3.3 Mutagenicity and genotoxicity of COPs and HCAs

There is also little information in the literature regarding the mutagenicity and genotoxicity of COPs and HACs that are formed in grilled meat products.

The genotoxicity of different meat extracts either not containing added phenols (C) or containing olive phenols (L1-87.5 mg phenols/kg of meat) and sampled after increasing time of storages (0, 6 and 9 days) were assessed on freshly isolated human PBMCs after exposure in complete RPMI medium at 37 °C for 30 min. The results reported in **Table 4.2** show that the solvent (DMSO) did not induce DNA damage on the PBMC while an evident effect was observed by the meat extracts not

containing the phenols (C). The damage was about twice as high as that of DMSO and was not dependent on the storage time. Inclusion of the phenols in the meat during cooking significantly reduced the DNA damage and this effect was more evident at the lower phenol dose (L1) and after 6 days of storage (**Table 4.2**). On the other hand, the different meat extracts did not induce a significant increment of revertants compared to the control (**Table 4.2**). Although the inclusion of phenols slightly reduced the revertants number this effect was not statistically significant.

Table 4.2. Number of revertants per plate induced by preincubation on *S. typhimurium* TA98 strain with different meat extracts (C, L1) and after 0, 6 and 9 days of storage (Ames Test) and genotoxic effect on freshly isolated human PBMCs induced by different meat extracts (C, L1) after 0, 6 and 9 days of storage. The DNA damage is expressed as the % of DNA in the tail (Comet Assay). C, Control (minced beef meat + maltodextrins + starter cultures); L1, minced beef meat + starter cultures + 87.5 mg phenols/kg of meat.

		Storage Time (days)						SEM	*P*		
	Samples	0		6		9			Form	St	Form*St
Comet Assay	DMSO	2.8	C	2.5	B	2.4	B	0.52	**	*NS*	*NS*
	C	5.8	A	5.7	A	6.1	A				
	L1	4.1	a,B	1.9	b,B	3.0	ab,B				
Ames Test	Spontaneous revertants	12.2	*NS*	12.2	*NS*	12.2	*NS*	1.32	*NS*	*NS*	*NS*
	C	20.5	*NS*	21.0	*NS*	13.5	*NS*				
	L1	13.5	*NS*	15.5	*NS*	11.5	*NS*				

Results as reported as means±s.d of 3independent replicates. a-b indicate significant differences (T-student test; $p \leq 0.05$) within the same sample during the shelf-life. A-C indicate significant differences (T-student test; $p \leq 0.05$) among different formulations. **$p < 0.01$. Form, formulation; *NS*, non-significant; St, storage.

It is therefore very important to monitor the progress and formation of the COPs, as these have been shown to exert several *in vitro* and *in vivo* biochemical activities of both physiologic and pathologic relevance (Zmysłowski & Szterk, 2019). Compared to cholesterol, the presence of a further oxygen group makes these compounds more polar and more easily diffusible through cell membranes (Zmysłowski & Szterk, 2017). Therefore, they are much more reactive than cholesterol, in fact they seem to be able to promote and support cytotoxicity, mutagenicity, carcinogenicity, atherogenicity, inflammation, fibrosis, and programmed cell death in several cells and tissues (Lordan et al., 2009; Vejux et al., 2009). COPs, but more generally oxysterols, are involved in some physiological processes, they exert important functions like regulators of the expression of genes involved in lipid and sterol biosynthesis, mediators of reverse cholesterol transport and substrates for the formation of bile acids. However, the oxysterols are potentially involved in the onset and progression of major chronic diseases which inflammation, but also oxidative damage and to a certain extent cell death, are hallmarks and primary mechanisms of progression (Guardiola et al., 1996; Guardiola et al., 2002; Sottero et al., 2009; Biasi et al., 2013a). Therefore, it is now suggested that oxysterols might contribute to the development of numerous other degenerative diseases such

as multiple sclerosis (Diestel et al., 2003; Leoni et al., 2005), age-related macular degeneration and bones disease like osteoporosis (Liu et al., 2005; Malvitte et al., 2006). *In vitro* and *in vivo* studies have also demonstrated an association between different types of oxysterols and the development and progression of cancer of the colon, lung, breast, and bile ducts (Jusakul et al., 2011). Precisely because of these characteristics it is necessary to find strategies to limit the formation of COPs. Biasi et al. (2013b) have tested the phenolic compounds present in Sardinian wine extracts. The results obtained have shown how these compounds are capable to protect against the production of inflammatory cytokines induced by oxysterols in CaCo-2 human enterocyte-like cells.

Regarding HCAs very little is known about their genotoxicity on freshly isolated normal PMBC. The studies of Baumgartner et al. (2012) and Kurzawa-Zegota et al. (2012) have investigated the genotoxic activity of PhIP and IQ on human cryopreserved lymphocytes but the only data available on the genotoxic effect of HCAs on PBMC are those of Fuccelli et al. (2018). Their result proved that all HCAs tested (PhIP, IQ, MeIQx and DiMeIQx) caused a DNA damage in PBMC which was increased by a metabolic activation. In the case of MeIQx and DiMeIQx the genotoxicity was also enhanced by DNA repair inhibitors AraC and HU. They also demonstrated how, in the presence of phenolic compounds deriving from extra virgin olive oil or extracts from olive leaves, the genotoxic action of some HCAs, in particular PhIP, is prevented by very low concentrations of phenolic extracts.

Shaughnessy et al. (2011), instead, analyzed the genotoxicity and mutagenicity of extracts obtained from meat cooked at two different temperatures, low 100°C and high 250°C. The extract from beef cooked at low temperature was not mutagenic and had non-detectable levels of HCAs. On the contrary, the extract from beef cooked at high temperature had high levels of HCAs, with PhIP as a majority compound, and the extract was mutagenic, exhibiting higher mutagenic potency in strain YG1024 compared to strain TA98.

4.3.4 Principal component analysis (PCA)

To better understand which parameters were the most relevant for assessing the effects of phenolic enrichment and storage on the hamburgers, all data were subjected to PCA (**Figure 4.1**).

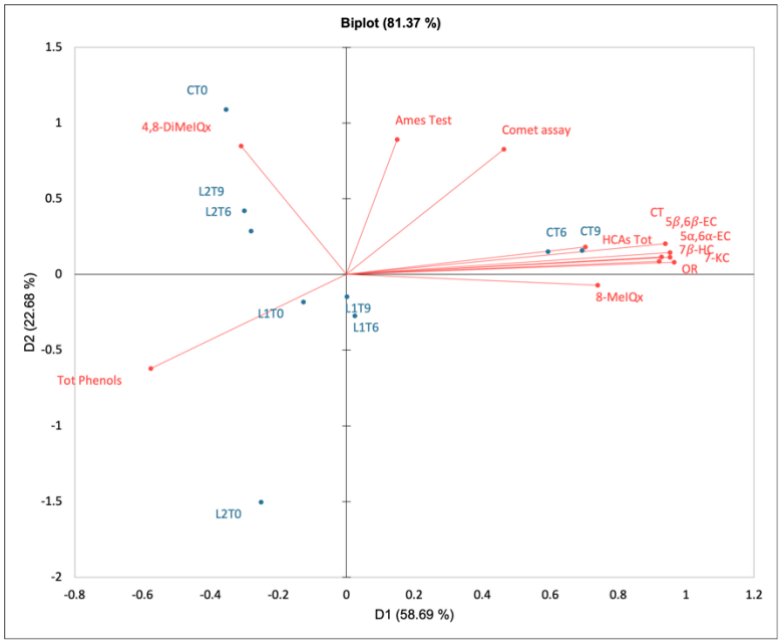

Figure 4.1. Biplot of all parameters. 4,8-DiMeIQx, 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoxaline; 5α,6α-EC, 5α,6α-Epoxy Cholesterol; 5β,6β-EC, 5β,6β-Epoxy Cholesterol; 7β-HC, 7β-Hydroxy Cholesterol; 7-KC, 7-Keto Cholesterol; 8-MeIQx, 2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline; CT, Cholestane Triol; HCAs Tot, Heterocyclic Aromatic Amines; OR, Oxidation Ratio.

The first two components explained 81.37% of the total variance (58.69% for PC1 and 22.68% for PC2). All COPs, OR, HCAs Tot, mutagenicity (Comet assay) and genotoxicity (Ames test) parameters are in the opposite quadrant (2) with respect to total phenols (quadrant 1). Control T6 and T9 samples are more correlated to all COPs, OR and HCAs while L1 and L2 T0 samples are in the same quadrant of Tot Phenols. Regarding mutagenicity and genotoxicity parameters they are located in the same quadrant of CT6 and CT9 samples, but they are not closely related

This distribution therefore explains how the phenolic extract can limit the formation of COPs, but in any case, the quantity of COPs and HCAs formed during grilling at 200 °C did not show a mutagenic and cytotoxic effect.

4.4 CONCLUSIONS

This study demonstrated the efficacy of a microencapsulated formulation of a phenolic extract from OMWW to limit the formation of COPs in grilled beef hamburgers, which had been previously subjected to cold storage for 9 days. The added phenolic compounds underwent a progressive decrease during the shelf-life period, due to both conservation and grilling to which the samples were subjected, but more than 20% and 40% of the added phenols were still retained (for L1 and L2, respectively) after 9 days of storage. Both PE concentrations (87.5 and 175 mg of phenols/kg meat) proved to effectively reduce cholesterol oxides formation, during the burgers' shelf-life. As regards HCAs, following the cooking of the hamburgers in the tested conditions, 200 °C 4 min per

side, these were found in very low values, <0.5 ng/g of meat, both in sample C and in samples L1 and L2, therefore it was not possible to evaluate the effect of PE on this parameter.

Regarding genotoxicity an evident effect was observed by the meat extracts not containing the phenols (C). The damage was about twice as high as that of DMSO and was not dependent on the storage time. Inclusion of the phenols in the meat during cooking significantly reduced the DNA damage and this effect was more evident at the lower phenol dose (L1). For the mutagenicity, the different meat extracts did not induce a significant increment of revertants compared to the control. Although the inclusion of phenols slightly reduced the revertants number this effect was not statistically significant.

In conclusion, this study confirms that PE obtained from the purification of OMWW could be a valid preservative of natural origin to be added to meat products to improve their characteristics from an oxidative point of view and limit the formation of products harmful to health, such as COPs and HCAs.

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Chapter 5. Improved oxidative stability of cooked ham formulated with a reduction of nitrites and enriched with phenol extracts from olive vegetation water.

ABSTRACT

This study aims at evaluating the effect of a phenol-rich extract (PE) obtained from the concentration and purification of olive mill wastewaters on the stability and sensory quality of cooked ham, sliced, packed under modified atmosphere and stored under alternating exposure to fluorescent light at 4±2°C for 30 days. Cooked ham was sampled at different times (0, 15 and 30 days). Storage time caused a progressive decrease of phenols reaching a total loss, considering T30 vs T0, of about 45%. During the shelf-life study, sample S1 (PE 200 mg/kg and NO_2 150 mg/kg) showed the best oxidative stability, with TBARs value closer to 1.0 mg MDA/kg of meat (reference value for rancidity development in cooked pork meat), while the control sample had significantly higher TBARs values (< 3.80 mg MDA/kg of meat). S2 (PE 200 mg/kg and NO_2 35 mg/kg) and S3 (PE 200 mg/kg without NO_2) displayed a similar oxidative trend, with TBARs values below 1.45 mg MDA/kg of meat; therefore, it is possible to hypothesize that the antioxidant activity in both S2 and S3 was mainly due to phenols, while nitrites in S1 were more involved in the development of color and in the microbial stabilization of the product. Regarding COPs and OR%, no significative differences were found between PE and control cooked ham samples.

Keywords: cooked ham; phenolic extract; olive vegetation water; olive by-product; clean label; lipid oxidation; cholesterol oxidation

5.1. INTRODUCTION

The main feature of most of the Italian food products lies in the ancient cultural traditions that led over time to produce a wide variety of processed foods, with local and regional origin. Many of these foods are, in fact, important elements of identification of local realities. This is the case of the Italian meat products which constitute a cultural and commercial wealth to be protected and improved especially when, as in recent years, together with market expansion there is a trend towards a levelling out of the organoleptic and nutritional quality of food products. Ham is a typical meat product belonging to the Italian food tradition having a long heritage. The manufacturing process can be slightly varied according to the tradition of each production area. Cooked ham is prepared from deboned pork legs flavored with a special mixture of aromas (salt, pepper, juniper and laurel) and then steam-cooked at about 70 °C, (addition of nitrites permitted). Ham is part of the Italian

consumption pattern; few data are available on the qualitative aspects of this important production. Safety and quality of meat products during storage depends on microbiological, biochemical and chemical processes. The oxidation of lipids, proteins and pigments are among the most important factors in the development of quality defects. To ensure this, nitrites are added in the formulation. The presence of these preservatives' agents, however, is in the middle of heated debates, both by the scientific community and by consumers. This belief was heightened by the fact that, in 2015, the International Agency for Research on Cancer (IARC) has classified processed meat added with nitrate/nitrite salts as carcinogenic to humans (Group 1), as they can give rise to a series of compounds (N-nitrous compounds (NOCs), nitrosamines and oxidation products), during cooking, subsequent ingestion and digestion that seem to contribute to the onset of colorectal cancer (Bouvard et al., 2015). Independently from the technological advantages, a reduction in the use of nitrates and nitrites has become a matter of primary importance for both industries and consumers. However, their complete or partial replacement with a single natural compound is a challenge, due to their multifunctional characteristics. One alternative approach to the use of chemical additives could be the use of natural antioxidants. Free radical or active oxygen scavenging capacity has been detected in several phenolic compounds. Virgin olive oil (VOO) is a basic component of the Mediterranean diet with a well-established role in contributing to human health, mainly attributed to the antioxidant actions of hydrophilic phenols class (Taticchi et al., 2019). However, it has been shown that almost all the phenols contained in the olive are not found in the oil, but in a by-product that is generated during its production, the olive mill wastewater (OMWV). OMWV is characterized by a high content of sugars, tannins, pectins and phenolic substances and mineral salts (Carrara et al., 2021). This composition makes OMWW an exploitable source of hydrophilic phenols (mainly secoiridoids and its derivates, which are found exclusively in the *Oleaceae* family) which showed a high antioxidant, antimicrobial and anti-inflammatory activity (Servili et al., 2014; Veneziani et al., 2017; Caporaso et al., 2018). Thus, phenols can be recovered from OMWW by using suitable membrane technology (Servili et al., 2011a), and used in meat industries for innovative meat product formulation, to try to reduce/replace nitrites salts in cooked meat products (Galanakis, 2018). Therefore, considering the need of partial/total replacement of nitrites in cooked meat products in a formulation perspective of clean label food products, this study aimed at evaluating the effect of the addition of OMWW extracts rich in phenols on the oxidative stability of cooked ham.

5.2. MATERIAL AND METHODS

5.2.1 OMWW phenol extract

A crude phenolic extract (PE) was obtained from fresh OMWWs of olives harvested in Umbria (Central Italy) from trees of Moraiolo cultivar, by a 3-step membrane filtration of fresh OMWW as previously reported (Ianni et al., 2021). The PE was added with maltodextrin (1: 1, d.w.), as a support, and then spray-dried to get a powder formulation of the PE.

5.2.2. Preparation of phenol-enriched cooked ham

Cooked ham was prepared used fresh pork leg. The injected brine was designed with the aim to have in the product the following common ingredients: sodium chloride (1.5%), glucose (0.2%), ascorbic acid (0.02%), flavours (0.15%), + phenols addition:

Control (maltodextrin (Glucidex 19, France) 0.39% and NO_2 150 mg/kg),
S1 (PE 200 mg/kg and NO_2 150 mg/kg),
S2 (PE 200 mg/kg and NO_2 35 mg/kg),
S3 (PE 200 mg/kg without NO_2).
All these ingredients were mixed into the brine solution.
The meat was softened manually before being syringed (Syringer Günther Pökelprofi PP3) with the brine. Each leg was placed inside a bag and vacuum-packed to avoid losses and pollution during churning; this phase lasted a total of 70 min., alternating 5 min. of motion with 5 min. of rest.
Once the churning was completed, the packaged thighs were left to rest in the cold room for one night, then deprived of the bag they were placed inside the pressure molds and sent to be cooked after weighing.
Hams were cooked in a steam oven (ChefTopTM Unox, Cadoneghe, Italy) with humidity equal to 100% by gradually varying the temperature as per the table below. The timing varied according to the core temperature of the product, as follows:

Oven temperature	°C to the core
40°C	20°C
55°C	40°C
73°C	69°C

At the end of cooking, the legs were cooled in a blast chiller (Attila GN I/I Tecnodom, Vigodarzere, Italy), until they reached 4 °C inside the product. The thighs were then kept refrigerated for one day at 4°C and the post-cooking weight was then determined.

Finally, the cooked thighs were sliced and placed in trays for packaging (70 g) in a protective atmosphere (Alipak 120 - 80% N_2 and 20% CO_2 - ORVED VGP packaging machine). The trays were then placed inside a display counter refrigerated at 4 °C (Carel Industries Spa, Brugine, Padua, Italy) with lighting from 9 am to 20 pm and subsequent darkness (Osram Natura De Luxe L36W/76-1, Munich, Germany).

The sampling was performed at time 0, which corresponds to the production date, and after 15 and 30 days.

5.2.3 Phenols analysis

In the PE. Fifty mg of spray-dried PE were solubilized in 10 mL of a methanol:water mixture (80:20, v/v), filtered with a 0.2 µm polyvinylidene fluoride (PVDF) syringe filter (Agilent Captiva, Agilent Technologies, Santa Clara, CA, USA) and injected into a high-performance liquid chromatograph coupled to a diode array detector (HPLC-DAD Agilent Technologies system Mod. 1100). The HPLC equipment and analytical conditions were those described by Selvaggini et al. (2014). Each measurement was done in duplicate.

In the cooked ham samples. Five grams of cooked ham were mixed with 100 mL of methanol:water (80:20, v/v) containing 20 mg/L of butylated hydroxytoluene (BHT) + 0.2% trichloroacetic acid 1 M. The operations of homogenization, recovery, concentration until a final volume of 40 mL of extract and purification by solid-phase extraction (SPE) from 10 mL of this aqueous extract were carried out as previously described Miraglia et al. (2020). The purified extract was then subjected to HPLC-DAD analysis using the same equipment and conditions of the PE analysis (Selvaggini et al., 2014). Each measurement was done in duplicate.

5.2.4 Chemical analysis

5.2.4.1 Lipid extraction

Lipids were extracted according to Boselli et al. (2001). The extraction was performed on 10 g of cooked ham, which were added with 5α-cholestane (internal standard for the quantification of main lipid classes) (Sigma Chemical, St. Louis, USA). The fat content was determined gravimetrically and expressed as percentage. Three independent replicates were run per sample.

5.2.4.2 Determination of main lipid classes

The qualitative-quantitative profile of the main lipid classes (free fatty acids, FFA; monoacylglycerols, MAG; free sterols, STE; diacylglycerols, DAG; esterified sterols, E-STE; triacylglycerols, TAG) was determined by gas chromatography-flame ionization detection (GC-FID), as reported by Gallina Toschi et al. (2014) and Luise et al. (2018). An aliquot of 20 mg of the lipid extract dissolved in 1 mL of *n*-hexane, was used for this analysis. The internal standard method, with the response factor of each main lipid class (estimated with commercial standards), was used to determine the amount of each lipid class (expressed as g/100 g of lipids). Three independent replicates were run per sample.

5.2.4.3 Determination of total FA

The composition of total fatty acids was determined on 20 mg of lipid extract by GC-FID (Cardenia et al., 2015), after previous methylation and transmethylation. FAME quantification was performed according to the internal standard method (using tridecanoic acid methyl ester) and expressed as a proportion of the identified total FAME (g/100 g). Three independent replicates were run per sample.

Based on the total FA composition, the atherogenic index (AI) and thrombogenic index (TI) were also determined (Ulbricht & Southgate, 1991).

5.2.4.4 Determination of thiobarbituric acid reactive substances (TBARs)

Secondary lipid oxidation was assessed as TBARs on cooked ham (Tarladgis et al., 1960). Two g of each sample were used for this spectrophotometric determination and the absorbance was measured at 530 nm. A 1,1,3,3-tetramethoxypropane standard calibration curve was used for the quantification of TBARs (concentration range of 0.045–0.113 μg/mL; y=0.0077x+0.0072, r^2=0.9998) and the values were expressed as mg MDA/kg meat. Three independent replicates were made per sample.

5.2.4.5 Determination of cholesterol and oxysterols (COPs)

Cholesterol and COPs were extracted by cold saponification of 200 mg of lipid extract, followed by purification with aminopropyl SPE (Cardenia et al., 2015). Silylated cholesterol and COPs were analyzed by Fast GC/MS (Cardenia et al., 2012), using betulinol (Sigma Chemical, St. Louis, USA) and 19-hydroxycholesterol (Steraloids, Newport, Rhode Island, USA) as internal standards, respectively. Mass spectra were acquired in full scan mode (total ion current, TIC), while they were integrated

with single ion monitoring (SIM) mode using the characteristic ions with a high abundance (Cardenia et al., 2012); quantification was carried out by means of calibration curves built for each compound. Cholesterol and total COPs were expressed as mg/kg of meat. Three independent replicates were run per sample. The rate of total cholesterol oxidation (%OR) was also estimated as reported by Cardenia et al. (2015).

5.2.5 Physical analysis

5.2.5.1 Image analysis

The visual analyzer VA400 IRIS (Alpha MOS, France) was applied for visual assessment (color) and to track changes in color over time of cooked ham samples, as described by Barbieri et al, 2016. This imaging system is equipped with a high-resolution CCD (charge-coupled device) camera (resolution 2592x1944p) combined with Alphasoft software for system monitoring, data acquisition and multivariate statistics processing. Each measurement (picture) was done in triplicate.

5.2.6 Sensory analysis

5.2.6.1 Descriptive analysis

The sensory profile of all samples (C, S1, S2, S3) at the 3 different storage times (T0, T1 and T2) was evaluated by a panel of nine fully trained judges of both genders, aged between 20 and 65 years. For the sensory characterization a conventional profiling method was applied (Meilgaard et al., 2007). The sensory attributes, their definition, the profile sheet, the sample preparation and the tests conditions, were the same as described by Barbieri et al., 2016 with some adaptations. In fact, considering that this study aimed to discriminate between different formulations (C and treated samples) and to evaluate changes during storage (T0, T1 and T2), the presence of anomalies (olfactory, gustatory and visual) was also evaluated. Moreover, among appearance descriptors, only pink intensity was taken into consideration. Results were expressed as mean of three replicates.

5.2.6.2 Discriminant test

The triangle test was applied to identify sensory differences between the control sample (C) and the treated samples (S1, S2 and S3) just produced (T0) and to investigating the effect of different storage times, i.e. 15 (T1) and 30 (T2) days. The test was conducted in three different days involving 44 (first day), 40 (second day) and 29 untrained judges, aged between 20 and 65 years and according to the

procedures described by ISO 4120:2007 and the conditions applied by Barbieri et al., 2021. In each session, 6 triad of samples were evaluated.

5.2.7 Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, France) was used to elaborate chemical and sensory data.

The chemical data are reported as mean values of independent replicates of each analytical determination. First, normal distribution of data was tested (p<0.05) with Shapiro-Wilk method. Chemical data were analyzed using two-way analysis of variance (ANOVA), including formulation (Form), storage time (St) and their interaction (Form*St;) as factor. Tukey's honest significance test was performed at a 95% confidence level (p≤0.05), to separate means of statistically different parameters. A principal component analysis (PCA), with a Varimax rotation, was also carried out.

5.3. RESULTS AND DISCUSSION

5.3.1 Evolution of phenolic compounds

The PE used for the preparation of the cooked ham had a total phenol content of 25.7 mg/g of dried product, of which 61.5% was 3,4-DHPEA-EDA (oleacein), 20.6% 3,4-DHPEA (hydroxytyrosol), 13.2% verbascoside and 4.7% *p*-HPEA (tyrosol). As shown in **Table 5.1**, part of the added phenols was lost during storage. In terms of total phenols, there was a loss of 32% and 52% of phenolic compounds in S1 samples after 15 and 30 days of storage, respectively, whereas a major loss was detected in S2 (47 and 66%, respectively) and S3 (28 and 58%) samples, respectively. In particular, the highest variation was observed for 3,4-DHPEA-EDA, which completely disappears in S1 S2 and S3 samples after 15 and 30 days of storage. It can be assumed that, to a certain extent, 3,4-DHPEA-EDA was subjected to hydrolysis, which generates 3,4-DHPEA, as has been found in fermented functional milk (Servili et al., 2011b). As reported by Obied et al. (2008), the degradation mechanism of this oleuropein derivative includes enzymatic and non-enzymatic oxidation and hydrolysis. The increase of 3,4-DHPEA, in presence of 3,4-DHPEA-EDA, has already been observed in different food matrices and at different temperatures (Servili et al., 2011b; Esposto et al. 2015; Taticchi et al., 2017; Balzan et al., 2017), the phenols evolution in S1, S2 and S3 cooked ham strengthens the hypothesis of its hydrolytic origin from the degradation of oleuropein derivatives during storage (Brenes et al., 2001).

Table 5.1. Evolution of phenolic compounds (mg/kg of cooked ham) of cooked ham samples after 0, 15 and 30 days of storage. C, Control (meat + maltodextrine + 150 mg of nitrites /kg of meat); S1, meat + 150 mg of nitrites /kg of meat + 200 mg phenols/kg of meat; S2, meat + 35 mg of nitrites /kg of meat + 200 mg phenols/kg of meat, S3, meat + 200 mg phenols/kg of meat.

		Storage Time (days)						SEM	*P*		
	Samples	0		15		30			Form	St	Form*St
3,4-DHPEA	C	-		-		-		5.90	***	***	***
	S1	63.52	a,B	60.12	b,B	38.15	c,A				
	S2	80.62	a,A	48.40	b,C	24.86	c,C				
	S3	69.09	a,B	66.83	a,A	31.19	b,B				
p-HPEA	C	-		-		-		0.64	***	*	*
	S1	6.15		6.20	C	6.14	B				
	S2	6.59	b	7.29	a,B	6.34	b,B				
	S3	6.90	c	8.35	a,A	8.00	b,A				
VB	C	-		-		-		2.39	***	***	***
	S1	38.01	a,A	30.11	b,A	23.06	c,A				
	S2	31.37	a,B	18.07	b,B	15.96	c,B				
	S3	38.41	a,A	32.91	b,A	23.66	c,A				
3,4-DHPEA-EDA	C	-		-		-		2.87	***	***	***
	S1	33.83	a,A	*n.d.*	b	*n.d.*	b				
	S2	21.56	a,B	*n.d.*	b	*n.d.*	b				
	S3	36.47	a,A	*n.d.*	b	*n.d.*	b				
Total phenols	C	-		-		-		11.17	***	***	***
	S1	141.51	a,B	96.42	b,B	67.35	c,A				
	S2	140.13	a,B	73.76	b,C	47.16	c,B				
	S3	150.86	a,A	108.08	b,A	62.84	c,A				

Results as reported as means and standard error of the mean (SEM) of 2 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) within the same sample during the shelf-life. A-B indicate significant differences (Tukey's test; $p \leq 0.05$) among treatments, * $p<0.5$, *** $p<0.001$. 3,4-DHPEA, hydroxytyrosol; Form, formulation; *p*-HPEA, tyrosol; St, storage; VB, verbascoside; 3,4-DHPEA-EDA, oleacein; *nd*: not determined

Nevertheless, the oxidative degradation of these two phenols has been appointed as the main cause of their decrease (Obied et al., 2008; Di Maio et al., 2011). For 3,4-DHPEA, therefore, two contemporary phenomena would be at the basis of its particular evolution over time: the first is the hydrolysis of 3,4-DHPEA-EDA after which this phenolic alcohol is released in free form, while the second one is the oxidative degradation which leads to its decrement. In the case of samples S1, S2 and S3, in the early stages of storage, 3,4-DHPEA is limitedly involved in oxidation reactions and, therefore, the resultant balance between the decrease on the one hand and the increase on the other, is an increase of the concentration of 3,4-DHPEA. In the more advanced phases, the oxidative degradation prevails in S1, S2 and S3 cooked ham samples, due to the decrease in the concentration of the other more reactive phenols (such as 3,4-DHPEA-EDA). During storage, the concentration of *p*-HPEA and verbascoside did not significantly vary, as already found in other shelf-life studies for cooked meat products (Balzan et al., 2017).

5.3.2 Lipid content and main lipid classes

As reported in **Table 5.2**, the lipid content of cooked ham ranged from 7.44 to 14.54%. Formulation, storage and its interaction, significantly affect the lipid content of the examinate samples. These results were in agree with those reported by Carnovale & Marletta (2013) and Lucarini et al. (2013) for the lipid content of cooked ham. The great variability found between the data could be due to the fact that different types of cooked ham derive from thighs of different animals, therefore with a different deposition of fat.

Table 5.2. Lipid content (%) and main lipid classes profile (% of total lipids) of cooked ham samples after 0, 15 and 30 days of storage. C, Control (meat + maltodextrine + 150 mg of nitrites /kg of meat); S1, meat + 150 mg of nitrites /kg of meat + 200 mg phenols/kg of meat; S2, meat + 35 mg of nitrites /kg of meat + 200 mg phenols/kg of meat, S3, meat + 200 mg phenols/kg of meat.

		Storage Time (days)						SEM	*P*		
	Samples	0		15		30			Form	St	Form*St
Lipid content	C	8.87	b,B	13.52	a,A	13.45	a,AB	0.52	***	***	***
	S1	7.44	b,C	7.47	b,C	9.08	a,C				
	S2	12.80	a,A	10.71	b,B	10.10	b,B				
	S3	8.01	c,B	12.83	b,AB	14.54	a,A				
FFA	C	1.08	ab,A	0.88	b,C	1.57	a,B	0.11	***	***	***
	S1	0.82	b,C	1.15	a,B	1.10	a,C				
	S2	0.96	c,B	1.90	b,A	2.47	a,A				
	S3	0.90	a,B	0.83	b,C	0.87	b,D				
STE	C	1.12	a,A	0.50	b,C	1.19	a,AB	0.07	***	***	***
	S1	0.89	b,C	0.86	b,B	1.04	a,B				
	S2	0.61	b,B	1.03	ab,A	1.42	a,A				
	S3	0.95	a,B	0.50	b,C	0.57	b,C				
DAG	C	5.15	a,A	4.68	b,B	5.12	a,A	0.15	***	***	***
	S1	5.32	ab,A	5.50	a,A	5.46	a,A				
	S2	4.93	b,B	4.90	b,B	5.51	a,A				
	S3	4.92	a,B	4.24	ab,B	4.56	ab,B				
TAG	C	92.82	b,B	93.71	a,A	92.40	b,B	0.29	***	***	***
	S1	93.02	a,B	92.47	b,B	91.84	c,C				
	S2	93.45	a,A	92.72	b,B	91.40	c,C				
	S3	93.46	ab,A	93.95	ab,A	94.00	ab,A				

Results as reported as means and standard error of the mean (SEM) of 3 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) within the same sample during the shelf-life. A-D indicate significant differences (Tukey's test; $p \leq 0.05$) among treatments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. DAG, diacylglycerols; FFA, free fatty acids; Form, formulation; STE, sterols; St, storage; TAG, triacylglycerols.

Regarding main lipid classes (**Table 5.2**), the most abundant class was TAG, followed by DAG, FFA and STE. Formulation, storage and its interaction, significantly affect all lipid classes content of the examinate samples, but without displaying a clear trend.

These differences could be partly due to the formulation of cooked ham as they were prepared with pork thighs from different animals. Leg grooming, which is done manually by an operator before adding ingredients and cooking, may also affect these parameters, depending on the amount of cover fat that is removed.

5.3.3 Total fatty acid profile

Regarding total FA composition (**Table 5.3**), the most represented FA class was monounsaturated fatty acids (MUFA, 82-87%), followed by saturated fatty acids (SFA, 13-16%) and polyunsaturated fatty acids (PUFA, 12-16%). This FA profile agrees with that reported by Garbowska et al. (2015), for cooked ham. Formulation, storage and its interaction, significantly affect the MUFA of the examinate samples. For SFA only storage significantly affect this parameter, while PUFA content is statistically influenced by formulation and the interaction between formulation and storage time, but without displaying a clear trend.

Regarding FA classes ratios (**Tables 5.3**), the PUFA *n*-6/PUFA *n*-3 ratio varied between 4.55 and 7.52, these values agree with those found by Parrini et al (2020) for cooked pork meat products. The PUFA *n*-6/PUFA *n*-3 ratio is a suitable index to compare the nutritional value of food. According to Simopoulos (2006), a low PUFA *n*-6/PUFA *n*-3 ratio (< 4) is desirable for a healthy human diet. Formulation, storage and its interaction significantly impacted this ratio. The trend of this ratio is strongly influenced by the lower content of PUFA *n*-6detected in the S2 and S3 samples, formulated with 35 and 0 mg/kg of NO_2 + 200 mg/kg of PE, respectively. The lower content of PUFA *n*-6 could be due to an oxidation of these which, being fatty acids with many unsaturations, are more susceptible to oxidation. In samples C and S1, on the other hand, NO_2 (150 mg/kg) was able to preserve the PUFA *n*-6 from oxidation more efficiently than PE

The PUFA/SFA ratio is also used for evaluating the nutritional quality of foods lipids and it has been suggested by nutritional guidelines that it should be around 0.4 (Delgado-Pando et al., 2010). In our case, this ratio ranged between 0.70 and 1.31.

The results of the present study agree with the PUFA/SFA ratio reported by Garbowska et al. (2015), for cooked ham. This ratio reflected the behavior of the SFA class, as it was also significantly influenced by storage time and showed a not significant interaction among the factors tested.

The UFA/SFA ratio is useful for observing the oxidative stability of fatty acids in food, as it decreases when UFA oxidize. The UFA/SFA ratio ranged from 6.17 to 8.98. Only storage time influenced this ratio. However, the values obtained in this study could indicate that this high degree of oxidation could be due to the presence of micro holes in the package that contained sliced cooked ham.

Table 5.3. Fatty acid classes (% of total fatty acids), their ratios, atherogenic index (AI) and thrombogenic index (TI) of cooked ham samples after 0, 15 and 30 days of storage. C, Control (meat + maltodextrine + 150 mg of nitrites /kg of meat); S1, meat + 150 mg of nitrites /kg of meat + 200 mg phenols/kg of meat; S2, meat + 35 mg of nitrites /kg of meat + 200 mg phenols/kg of meat, S3, meat + 200 mg phenols/kg of meat.

		Storage Time (days)						SEM	*P*		
	Samples	0		15		30			Form	St	Form*St
SFA	C	14.83	b,B	15.91	a,A	13.23	b,A	0.67	*NS*	***	*NS*
	S1	13.63	b,C	15.00	a,A	12.51	b,B				
	S2	14.63	a,B	11.37	b,C	13.58	b,A				
	S3	15.62	a,A	14.42	b,B	13.77	b,A				
MUFA	C	83.96	B	86.85	a,A	85.75	ab,A	0.38	***	*	***
	S1	82.96	ab,B	83.90	a,B	81.76	b,B				
	S2	86.93	a,A	84.81	b,B	83.69	b,AB				
	S3	86.90	aA	86.01	b,A	85.96	b,A				
PUFA	C	14.26	a,B	11.17	c,D	12.25	b,D	0.38	***	*NS*	**
	S1	15.00	b,A	14.06	b,A	16.37	a,A				
	S2	11.10	b,C	13.16	a,B	14.13	a,B				
	S3	11.30	b,C	12.24	a,C	11.82	b,C				
n-3	C	2.25	a,AB	1.44	b,B	1.41	b,B	0.08	*	**	**
	S1	2.41	a,A	2.22	b,A	2.03	b,A				
	S2	1.64	b,C	2.01	a,AB	2.05	a,A				
	S3	2.05	a,B	1.65	b,B	1.58	b,B				
n-6	C	12.01	a,A	9.73	c,B	10.84	b,C	0.33	***	**	**
	S1	12.59	b,A	11.84	c,A	14.34	a,A				
	S2	9.46	c,B	11.15	b,A	12.08	a,B				
	S3	9.26	b,B	10.60	a,AB	10.25	ab,C				
n-6/*n*-3	C	5.35	c,B	6.76	b,A	7.72	a,A	0.18	*	***	**
	S1	5.23	b,B	5.38	b,B	7.06	a,A				
	S2	5.77	A	5.55	B	5.89	C				
	S3	4.55	C	6.45	a,A	6.51	a,B				
PUFA/SFA	C	0.96	a,B	0.70	b,C	0.93	a,C	0.10	*NS*	**	*NS*
	S1	1.10	b,A	0.95	c,B	1.31	a,A				
	S2	0.76	c,C	1.21	a,A	1.04	b,B				
	S3	0.72	b,C	0.85	a,B	0.86	a,C				
UFA/SFA	C	6.70	b,B	6.17	b,B	7.41	a,AB	0.61	*NS*	**	*NS*
	S1	7.19	b,A	6.55	c,B	7.85	a,A				
	S2	6.70	c,B	8.98	a,A	7.21	b,B				
	S3	6.29	b,B	6.82	b,B	7.11	a,B				
AI	C	0.06		0.07		0.08		0.00	*NS*	*NS*	*NS*
	S1	0.08		0.07		0.07					
	S2	0.07		0.07		0.08					
	S3	0.07		0.06		0.08					
TI	C	0.00		0.00		0.00			*NS*	*NS*	*NS*
	S1	0.00		0.00		0.00					
	S2	0.00		0.00		0.00					
	S3	0.00		0.00		0.00					

Results as reported as means and standard error of the mean (SEM) of 3 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) within the same sample during the shelf-life. A-D indicate significant differences (Tukey's test; $p \leq 0.05$) among treatments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. AI, Atherogenic Index; Form, formulation; MUFA, monounsaturated fatty acids; *NS*, non-significant; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids, St, storage; TI, thrombogenic index; UFA, unsaturated fatty acids.

Based on FA composition, AI and TI were also calculated, which are useful indices for understanding the role of FA composition on both atherogenic and thrombogenic risks. The indices ranged from

0.06 to 0.08 for AI, while is equal to 0.00 for TI. None of the factors here tested (product formulation and storage time) significantly influenced these ratios.

Regarding single FA, the most abundant FA was oleic acid (C18:1 n-9, 67.16-75.58%), followed by stearic acid (C18:0, 5.22-13.91%) and linoleic acid (C18:2, 8.88-13.74%). These values agree with those reported by reported by Garbowska et al. (2015), for cooked ham. No significant differences were found in single FA between control and PE-enriched samples during shelf-life. Slightly differences could be partly due to the variability of the matrix.

5.3.4 Lipid oxidation

Lipid oxidation products were monitored by TBARs and COPs (**Table 5.4**). TBARs ranged from 0.64 to 3.63 mg MDA/kg of meat. Product formulation and storage time significantly influenced this oxidative parameter, and the two factors interaction was significant as well. During the shelf-life study, sample S1 showed the best oxidative stability, with TBARs values closer to 1.0 mg MDA/kg of meat, reference value for rancidity development in cooked pork meat (Gray & Pearson, 1987), while the control sample had significantly higher TBARs values (< 3.80 mg MDA/kg of meat). S2 and S3 displayed a similar oxidative trend, with TBARs values below 1.45 mg MDA/kg of meat; therefore, it is possible to hypothesize that the antioxidant activity in both S2 and S3 was mainly due to phenols, while nitrites in S1 were more involved in the development of color and in the microbial stabilization of the product. The results obtained therefore demonstrate how the PE in synergy with nitrites can limit oxidation, as confirmed by the studies carried out by Balzan et al. (2017), which found TBARs values lower than 1 mg MDA/kg of meat in cooked pork-based sausages formulated with a PE deriving from OMWW.

Cholesterol is an important constituent of cell membranes and, as FA, it is also susceptible to oxidation. The total content of cholesterol (**Table 5.4**) ranged from 1163.0 to 1872.6 mg/kg of meat. These data agree with those reported by Baggio & Bragagnolo (2006) for pork based cooked meat products. Cholesterol content wasn't significantly affected by formulation and storage time.

Table 5.4. TBARs (mg MDA/kg of meat), total cholesterol (mg/kg of meat), COPs (mg/kg of meat) and OR (%) of cooked ham samples after 0, 15 and 30 days of storage. C, Control (meat + maltodextrine + 150 mg of nitrites /kg of meat); S1, meat + 150 mg of nitrites /kg of meat + 200 mg phenols/kg of meat; S2, meat + 35 mg of nitrites /kg of meat + 200 mg phenols/kg of meat, S3, meat + 200 mg phenols/kg of meat.

		Storage Time (days)						SEM	*P*		
	Samples	0		15		30			Form	St	Form*St
TBARs	C	1.19	c,A	2.04	b,A	3.83	a,A	0.17	***	***	***
	S1	1.17	a,A	1.00	b,B	1.02	b,C				
	S2	0.98	b,B	1.20	b,B	1.45	a,B				
	S3	0.64	c,C	1.01	b,B	1.28	a,B				
Cholesterol	C	1244.40	a,BC	1190.40	b,D	1272.50	a,D	57.63	*NS*	*NS*	*NS*
	S1	1163.00	b,C	1531.07	b,B	1872.60	a,A				
	S2	1746.82	a,A	1682.34	b,A	1608.03	b,B				
	S3	1381.31	a,B	1228.60	b,C	1355.80	a,C				
7α-HC	C	0.43	a,C	0.47	a,A	0.07	b,B	0.07	*NS*	**	**
	S1	0.67	a,B	0.05	b,C	0.06	b,B				
	S2	0.95	a,A	0.15	b,B	0.04	b,B				
	S3	0.33	b,D	0.14	b,B	0.77	a,A				
7β -HC	C	0.78	a,B	0.78	a,A	0.17	b,C	0.11	***	***	***
	S1	0.75	b,B	0.49	b,B	2.05	a,A				
	S2	0.73	a,B	0.24	b,B	0.12	b,C				
	S3	0.83	b,A	0.54	b,B	1.63	a,B				
5β,6β-EC	C	0.23	b,B	0.99	a,A	0.46	b,B	0.10	**	***	***
	S1	0.26	b,B	0.40	b,C	1.53	a,A				
	S2	0.56	a,A	0.13	b,D	0.22	b,C				
	S3	0.23	b,B	0.57	b,B	1.35	a,A				
5α,6α-EC	C	0.08	b	0.45	a,A	0.29	b,B	0.04	*	***	*
	S1	0.05	b	0.16	b,B	0.49	a,A				
	S2	0.06	b	0.05	b,C	0.11	a,C				
	S3	0.04	b	0.17	b,B	0.43	a,A				
CT	C	0.13	b,B	0.20	a,A	0.08	b,B	0.03	*	*	*
	S1	0.12	a,B	0.05	b,B	0.12	a,A				
	S2	0.54	a,A	0.07	b,B	0.07	b,B				
	S3	0.11	b,B	0.09	b,B	0.18	a,A				
7-KC	C	0.72	b,A	0.89	b,A	1.30	a,C	0.11	***	***	***
	S1	0.62	b,B	0.34	b,C	2.01	a,A				
	S2	0.62	b,B	0.24	b,C	1.17	a,D				
	S3	0.52	b,C	0.48	b,B	1.62	a,B				
Total COPs	C	2.36	b,B	3.78	a,A	2.37	b,C	0.34	***	***	***
	S1	2.46	b,B	1.50	b,C	6.27	a,A				
	S2	3.46	a,A	0.88	b,D	1.73	b,D				
	S3	2.04	b,C	2.01	b,B	5.99	a,B				
OR	C	0.19	b,A	0.32	a,A	0.19	b,B	0.02	***	***	***
	S1	0.21	b,A	0.10	b,B	0.34	a,A				
	S2	0.20	a,A	0.05	b,C	0.11	b,C				
	S3	0.15	b,B	0.16	b,B	0.44	a,A				

Results as reported as means and standard error of the mean (SEM) of 2 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) within the same sample during the shelf-life. A-D indicate significant differences (Tukey's test; $p \leq 0.05$) among treatments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 5α,6α-EC, 5α,6α-Epoxy Cholesterol; 5β,6β-EC, 5β,6β-Epoxy Cholesterol; 7α-HC, 7α-Hydroxy Cholesterol; 7β-HC, 7β-Hydroxy Cholesterol; 7-KC, 7-Keto Cholesterol; CT, Cholestane Triol; Form, formulation; *NS*, non-significant; OR, Oxidation Ratio; St, storage; TBARs, thiobarbituric arid reactive substances.

Like TBARs, product formulation, storage time and its interaction significantly influenced total COPs content. Total COPs ranged from 0.88 to 6.27 mg/kg meat.

Regarding single COPs profile and amount detected in the present study is similar to the one reported by Balzan et al. (2017) for raw and cooked pork-based sausages. In general, the most abundant COPs were 7-KC >7β-HC> 7α-HC, which demonstrates that COPs in position 7 are formed more than epoxides and triol (Ferioli et al., 2010), because they follow a monomolecular formation mechanism (Cardenia et al., 2013). The formation of 5α,6 -EC and 5β,6β-EC epoxides follow a bimolecular formation mechanism between a cholesterol molecule and a hydroperoxyl radical (Cardenia et al., 2013), confirming the low quantities of epoxides (α-EC 0.08-0.50 mg/kg and β-EC 0.13-1 mg/kg) found in the matrix examined. As regards the triol it was also found in low quantities (< 0.54 mg/kg of meat); this compound tends to form from epoxides, in the presence of H_2O and in an acidic environment by breaking the epoxy ring (Cardenia et al., 2013). In sample C it is possible to note a decrease in COPs at point T30; this could be justified by a shift in equilibrium towards a lower rate of formation of oxysterols, compared to their rate of evolution/reaction with other molecules present in the matrix (e.g., proteins) (Rodriguez-Estrada et al., 2014). Samples S1 and S3 shows the typical oxidation trend, with a slight inflection in the central point of the sampling, and a subsequent slight increase in the final point, while always maintaining very low values. Considering these results, it seems that the phenolic extract does not have a significant effect on the oxidation of cholesterol in this type of product, as sample C shows a lower COPs level than the samples formulated with the extract. This trend could be due to the fact that the extract, to limit cholesterol oxidation, should be able to penetrate inside the cell membrane, a difficult action in the case of cooked ham as this product is made from whole muscle fractions. Syringing and churning in the conditions tested do not appear to be sufficient for this to happen.

The cholesterol oxidation ratio (OR) ranged from 0.05 % to 0.34% (Table 4). Similarly to total COPs, product formulation, storage time and their interactions significantly influenced this ratio. % OR were particularly low in all cooked ham samples (<0.5%). The great variability observed could be attributed to the fat content of each pork leg used to produce the hams subject of the experimentation. Since these matrices are of animal origin, it is often difficult to standardize the finished product, since various factors, including the feeding, age and slaughter weight of the pig, can influence the yields and consequently also the analytical parameters. Furthermore, it is important to emphasize that the samples come from cuts of different thighs with different fat / muscle mass ratios, which were not further trimmed before grinding to obtain the mass sample. Indeed, cholesterol is mainly localized in the cell membranes of the muscular part of the thigh (Garbowska et al., 2015).

5.3.5 PCA of chemical data

To better understand which parameters were the most relevant for assessing the effects of phenolic enrichment and storage on cooked ham, the chemical composition and phenolic composition data were subjected to principal component analysis (PCA) for steam cooked and grilled würstels (**Figure 5.1**). The first two components explained 63.02% of the total variance (34.11% for PC1 and 28.92% for PC2).

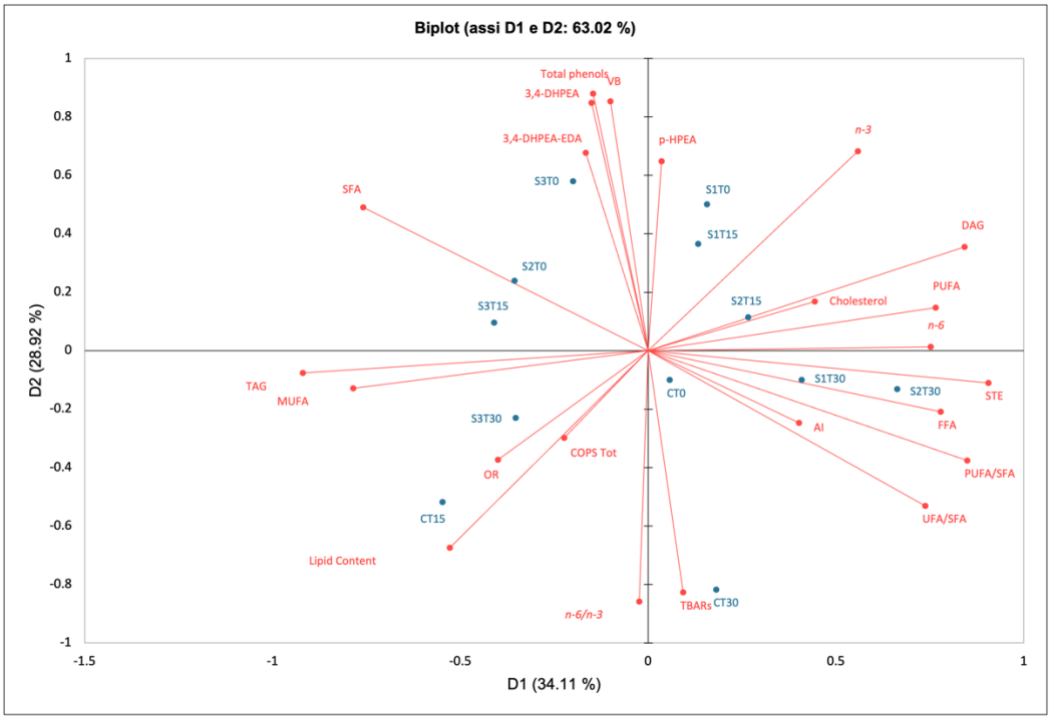

Figure 5.1. Biplot of cooked ham. 3,4-DHPEA, Hydroxytyrosol; 3,4-DHPEA-EDA, decarboxymethyl oleuropein aglycone; AI, Atherogenic Index; COPs, cholesterol oxidation products; DAG, diacylglycerols; E-STE, esterified sterols; FFA, free fatty acids; MAG, monoacylglycerols; MUFA, monounsaturated fatty acids; *p*-HPEA, tyrosol; OR, cholesterol oxidation ratio; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; STE, sterols; TAG, triacylglycerols; TBARs, thiobarbituric acid reactive substances; UFA, unsaturated fatty acids; VB, verbascoside.

COPs and OR are more related to S3T30 and CT15 samples, while TBARs is more related to CT0 and CT30 samples. Total and single phenols are in the same quadrant (1 and 2) of PE T0 and T15 samples.

This conformation is probably due to the great variability of the samples being tested. In fact, cooked hams were obtained from thighs from different animals. It is therefore very difficult to standardize the final product.

5.4 Physical analysis

5.4.1 Image analysis

In this study, the electronic eye was applied to discriminate between different formulations of cooked ham samples (C, S1, S2, S3) stored at 3 times (0, 15, 30 days) by acquisition and subsequent processing of sample' images (Alphasoft software, version 14.0).

In PCA model of instrumental (electronic eye) data, the two most informative dimensions F1 and F2, accounted for 50.43 and 19.96 of data variability, respectively (**Figure 5.2**).

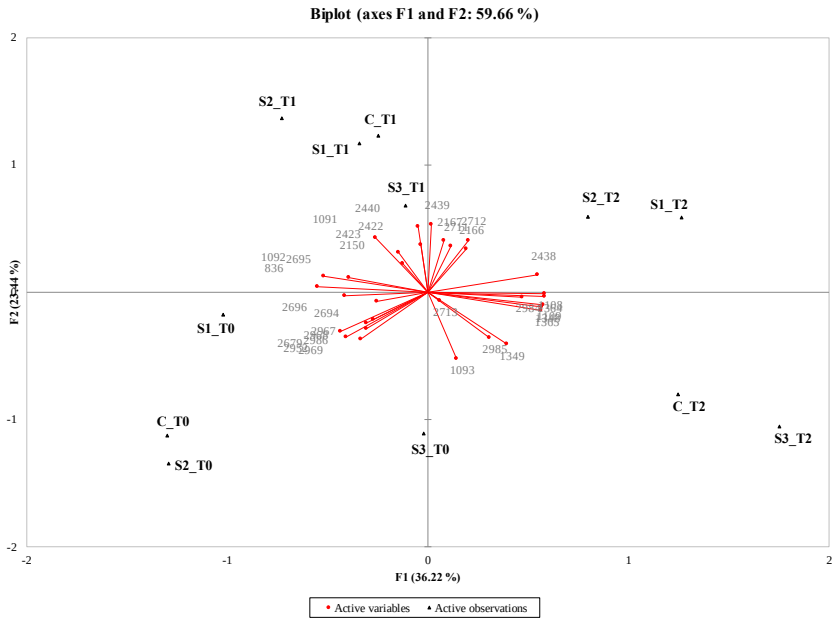

Figure 5.2. Representation of the cases and variables obtained from the principal component analysis (PCA) related to the results of the image analysis (electronic eye) for the 4 samples under examination (control sample, C; formulation with 200 mg/kg of phenols and 150 mg/kg of nitrite, S1; formulation with 200 mg/kg of phenols and 35 mg/Kg of nitrite, S2; formulation with 200 mg/kg of phenols and nitrite-free) evaluated at all the storage times (T0, T1 and T2).

In accordance with sensory results, the samples characterized by the highest intensities of pink (CT0, S1T0, S2T0, S2T1) were placed together between the second and the third quadrant; in the same position, but more shifted towards the positive values of the F1, there were CT1 and S1T1 with intermediate pink intensities. On the other hand, samples positioned between the first and fourth quadrant, are those that showed anomalous colors (CT2, S1T2, S2T2, S3T2) linked to the oxidative process, even if, in some cases, with high intensity of pink (S2T2). The position of samples S3T0 and S3T1, far from the others, confirms its anomalous appearance also detected by the trained panel, that is a color with low intensity of pink, pale and tending to gray.

These results were in agreement with several studies in the literature describing the application of instrumental methods to evaluate the appearance of cooked ham samples and showing a strong correlation between instrumental and sensory data (Valous et al., 2009; Iqbal et al., 2010; Barbieri et al., 2016). In general, it is observed that the surface color value of the control sample gradually

decreases during the storage time. On the contrary, the phenol-enriched samples with nitrite (S1, S2), revealed considerable stability of color up to 15 days of storage. After this time, all samples revealed a qualitative decay with the formation of visual anomalies (discoloration and iridescent, dark, yellowish, greyish areas formation). Similar trends were also reported by other authors (Hawashin et al., 2016; Barbieri et al., 2021) who investigated the effect of the incorporation of olive oil by-products on quality of meat products (beef patties and beef hamburgers, respectively) during cold storage, confirming their effectiveness in stabilize the sensory quality and prolong the shelf-life.

5.5 Sensory analysis

5.5.1 Descriptive analysis

The sensory evaluation of the cooked ham samples was carried out by applying the QDA® method. The list of adopted descriptors included: i) 1 relative to appearance: color intensity (intensity of pink); ii) 3 perceived by orthonasal and retronasal routes: overall aroma (intensity of total aroma of the product), spices and other flavours (intensity of spices and other flavours); smoky (aroma associated with smoked notes in meat products); iii) 2 gustatory: sweet (basic taste), salty (basic taste); iv) 2 relative to the texture: cohesiveness (resistance to the product separation, to be assessed during the first 3-4 bites), juiciness (amount of juice released from the product during mastication).

Table 5.5 provides an overview of the mean values for sensory attributes evaluated by the trained panel and showed significance for 6 out of 8 descriptors.

Concerning the overall aroma, it is possible to notice a general reduction in the intensity of the attribute already after 15 days (T1); this trend was more evident for the control sample (C) than for phenol-enriched and with nitrite ones (S1, S2). S3 (phenol-enriched, nitrite-free) was characterized by the lowest intensity of overall aroma just produced (T0) and also during storage (T1, T2).

Smoky notes perceived by the tasters significantly decrease passing from T0 to T2 for samples C and S3 while S1 and S2 keep them up to T2. Considering all samples just produced (T0), the salty taste was significantly higher for the control sample, however, over time, no changes occurred.

Table 5.5. Mean values (three replicates) of the intensity of the attributes evaluated by QDA® relative to all samples (control sample, C; formulation with 200 mg/kg of phenols and 150 mg/Kg of nitrite, S1; formulation with 200 mg/kg of phenols and 35 mg/kg of nitrite, S2; formulation with 200 mg/kg of phenols and nitrite-free) evaluated at all the storage times (T0, T1 and T2). Values were expressed on a scale from 0 to 100 (0 indicates the absence of perception of the attribute, 100 the maximum perception of the attribute). Different letters ($^{a-f}$) indicate significantly different values from each other (multiple comparison test, Fisher LDS with p <0.05).

Sample	Overall aroma	Spices and flavours	Smoky	Olfactory anomalies	Sweet	Salty	Taste anomalies	Cohesiveness	Jiuiciness	Pink intensity	Visual anomalies
CT0	5.9^{ab}	3.1^{a}	2.3^{ab}	/	4.3^{ab}	4.3^{a}	/	4.9^{bc}	3.9^{a}	5.2^{a}	/
CT1	4.7^{de}	2.2^{abc}	1.0^{c}	Fermented/lactic	4.3^{ab}	3.4^{ab}	Sourish/Rancid	3.6^{d}	3.0^{b}	3.3^{b}	Abnormal coloring (iridescent and/or dark areas)
CT2	5.0^{bcde}	1.7^{bc}	0.9^{c}	Fermented/lactic	4.3^{ab}	3.3^{abc}	Sourish/Rancid	3.7^{d}	2.8^{bc}	3.0^{b}	Abnormal coloring (greyish areas)
S1T0	5.7^{abc}	2.7^{abc}	2.3^{ab}	/	4.5^{ab}	2.6^{bc}	/	6.1^{a}	1.5^{ef}	6.0^{a}	Abnormal coloring (yellowish areas)
S1T1	6.0^{a}	2.5^{abc}	2.4^{ab}	Fermented/lactic	4.0^{ab}	2.8^{bc}	/	5.2^{ab}	1.8^{def}	3.6^{b}	Abnormal coloring (greyish areas)
S1T2	4.9^{cde}	2.7^{ab}	1.7^{abc}	Fermented/lactic	4.1^{b}	2.7^{bc}	Sourich/Rancid	5.3^{ab}	1.8^{def}	3.6^{b}	Abnormal coloring (greyish areas)
S2T0	5.6^{abcd}	2.6^{abc}	2.5^{a}	/	4.5^{ab}	2.5^{bc}	/	4.9^{abc}	1.7^{def}	5.5^{a}	/
S2T1	4.8^{cde}	2.4^{abc}	1.4^{abc}	/	4.4^{ab}	2.7^{bc}	/	4.6^{bcd}	1.8^{def}	6.0^{a}	/
S2T2	4.9^{cde}	2.0^{abc}	1.4^{bc}	Fermented/lactic	4.1^{ab}	2.7^{bc}	Sourish/Rancid	4.2^{bcd}	2.5^{bcd}	6.0^{a}	Abnormal coloring (greyish areas)
S3T0	4.0^{e}	2.0^{abc}	2.0^{abc}	Cooked meat (roast)	4.6^{a}	2.3^{c}	Cooked meat (roast)	5.2^{ab}	1.2^{f}	0.3^{c}	Abnormal coloring (grey)
S3T1	4.0^{e}	1.5^{c}	1.0^{c}	Cooked meat (roast); Fermented/lactic	3.7^{b}	2.8^{bc}	Cooked meat (roast)	4.2^{bcd}	2.2^{cde}	0.6^{c}	Abnormal coloring (grey)
S3T2	4.7^{de}	2.0^{abc}	1.0^{c}	Cooked meat (roast); Fermented/lactic	3.6^{ab}	3.4^{ab}	Cooked meat (roast); Sourish/Rancid	3.9^{cd}	3.0^{bc}	0.6^{c}	Abnormal coloring (grey)
Pr > F(Model)	< 0.0001	0.294	0.007		0.494	0.009		0.0003	< 0.0001	< 0.0001	
Significant	yes	no	yes		no	yes		yes	yes	yes	

Observing the values relating to the texture attributes, the enrichment with phenols did not modify the cohesiveness but allowed it to remain unchanged over time. On the contrary, in the control sample (C), a decrease from T0 to T1 and T2, was detected. Juiciness showed a reduction over time for the C sample but not for S1 and S2 in which the values are not significantly different over the time. An increase in juiciness occurred for S3, probably linked to the decay of the product without nitrites. Regarding the intensity of pink, the panel found a reduction in values for samples C and S1 passing from T0 to T1 and T2. The samples S2 and S3, on the other hand, showed no changes over time of this visual attribute; in the first case, thanks to the presence of phenolic extract and nitrite while in the second, there were no variations over time but its values were the lowest due to the absence of nitrites in its formulation which give the typical pink color of the product (Toldrà et al., 2010).

In addition to the evaluation of the descriptors by linear scale, assessors were asked to indicate the presence of off-flavors and negative sensations. Elaborating the comments provided by assessors, the presence of olfactory anomalies (fermented/lactic) emerged for the C, S1 and S3 at T1 and T2, while for S2, these anomalies appeared only at T2. Our results were in agreement with other studies in the literature, in which the evolution of sensory characteristics of sliced cooked ham packaged in modified atmosphere has been studied; authors indicated discoloration and off-flavours (sourness and rotten as olfactory perceptions) formation among the main defects caused by microbial spoilage that can lead to deterioration of products (Raimondi et al., 2019; Spampinato et al., 2022).

Gustatory anomalies (sourish/rancid) appeared at T1 and T2 for C and only at T2 for the phenol-enriched samples (S1, S2 and S3). Regarding S3, tasters perceived olfactory-gustatory anomalies resembling cooked meat already at time 0 and, over time (T1 and T2). Moreover, an anomalous coloring with the presence of dark, yellowish or gray areas was found on all the samples except for CT0, S2T0 and S2T1.

The presence of PE even if at the lower concentration allowed to S2 sample to be the most effective in delaying the microbiological deterioration and therefore the sensory defects formation (e.g rancid) confirming results from other studies in which phenol-rich extracts from olive mill wastewaters were evaluated on meat products (Nieto et al., 2017; Barbieri et al., 2021).

Samples and sensory attributes (most significant according to ANOVA) evaluated by the panel, were projected into a two-dimensional plane composed of four quadrants to highlight possible

correlations by PCA (**Figure 5.3**). The first two components explained 84.73% of the total variance (49.68% for PC1 and 35.06% for PC2).

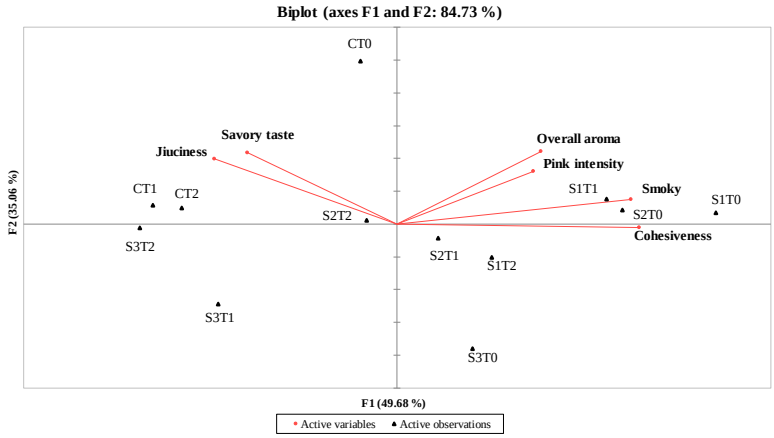

Figure 5.3 Representation of the cases and variables obtained from the principal component analysis (PCA) related to the results of the QDA® for the 4 samples under examination (control sample, C; formulation with 200 mg/kg of phenols and 150 mg/Kg of nitrite, S1; formulation with 200 mg/kg of phenols and 35 mg/kg of nitrite, S2; formulation with 200 mg/kg of phenols and nitrite-free) evaluated at all the storage times (T0, T1 and T2).

The phenol-enriched and with nitrite samples S1T0, S1T1 and S2T0 were located in the first quadrant and characterized by a strong overall aroma, smoky and high pink intensity. The control samples (CT0, CT1 and CT2) and S2T2 were placed in the second quadrant, whose position was influenced by the high intensity of juiciness (CT1, CT2), pink intensity (CT0, S2T2), overall aroma (CT0) and cohesiveness (S2T2). The sample phenol-enriched but without nitrites at 15 and 30 days of storage (S3T1, S3T2) were in the third quadrant being characterized by a low intensity of pink and of attributes perceived by orthonasal and retronasal routes (overall aroma, smoky). Finally, in the fourth quadrant are located: S1T2 that, compared to S1T1, showed a reduction in overall aroma; S2T1 which was very similar S2T2 but less juicy and S3T0, characterized by a higher intensity of smoky and lower of juicy than S3T1 and S3T2.

5.5.2 Discriminant test

The triangle test was carried out to verify the existence of significant differences between the treated sample (S1, S2 and S3) and the control sample (C) at different storage times (0, 15, 30 days), to provide useful information to evaluate the shelf-life of the new formulated products. The test was organized in several sessions in which all possible combinations of the samples to be compared were presented to the involved subjects (**Table 5.6**).

Table 5.6 Sessions, samples, number of subjects involved, number of correct given answers and significance level of the triangle test conducted on cooked ham samples (control sample, C; formulation with 200 mg/kg of phenols and 150 mg/kg of nitrite, S1; formulation with 200 mg/kg of phenols and 35 mg/kg of nitrite, S2; formulation with 200 mg/kg of phenols and nitrite-free) evaluated at all the storage times (T0, T1 and T2). The significance is expressed in terms of α-risk level. *ns* indicates no significant perceptible difference between samples was found.

Session number	Compared samples	Judges number	Corrected answers	Significance
1	CT0 *vs* S1TO	44	28	0.001
2	CT0 *vs* S2T0	44	29	0.001
3	CT0 *vs* S3T0	44	44	0.001
4	S1T0 *vs* S2T0	44	14	*ns*
5	S1T0 *vs* S3T0	44	39	0.001
6	S2T0 *vs* S3T0	44	43	0.001
7	CT1 *vs* S1T1	40	28	0.001
8	CT1 *vs* S2T1	40	17	0.1
9	CT1 *vs* S3T1	40	36	0.001
10	S1T1 *vs* S2T1	40	19	0.05
11	S1T1 *vs* S3T1	40	26	0.001
12	S2T1 *vs* S3T1	40	32	0.001
13	CT2 *vs* S1T2	29	17	0.01
14	CT2 *vs* S2T2	29	23	0.001
15	CT2 *vs* S3T2	29	27	0.001
16	S1T2 *vs* S2T2	29	20	0.001
17	S1T2 *vs* S3T2	29	24	0.001
18	S2T2 *vs* S3T2	29	20	0.001

The sessions 1-6 aimed to compare all samples (C, S1, S2, S3) at time 0 and the results, obtained from the 44 subjects, allowed to detect significant differences between all samples except in the comparison between samples S1T0 and S2T0 (session 4) which were not perceived as different by the interviewed subjects. In particular, S1T0 was differentiated from CT0 by a greater intensity of pink and by texture: it was more consistent and less juicy (session 1), while the CT0 sample resulted different from S2T0 for the less intense overall aroma, pink intensity but more tenderness and juiciness (session 2). Sample CT0 was also discriminated from sample S3 thanks to greater intensity of color intensity, overall aroma and juiciness (session 3). S3 sample was clearly distinguishable from the others; it was nitrite-free and therefore characterized by a lower intensity of overall aroma, juiciness and a paler color, tending to gray (sessions 5, 6).

In sessions 7-12 all samples were compared after 15 days of storage at 4º C (T1) and were significantly different even if in some cases with lower significance values (CT1 vs S2T1; S1T1 vs S2T1). S1T1 and S2T1 were perceived as darker and drier than the CT1 sample (sessions 7, 8); S3T1 was characterized by a completely different color (gray) and lower overall aroma compared to sample C (session 9) and, compared to the other nitrite and phenol-enriched samples, it was also juicier (sessions 11 and 12).

The last sessions (13-18) were carried out to monitor the samples after 30 days of storage at 4º C and to evaluate any differences between them. Also in this case, the interviewees (n = 29) correctly identified the different sample even if, in one case, the significance is lower (CT2 vs S2T2). Sample C was different from S1 and S2 as it was characterized by a less intense pink color, a greater consistency and juiciness but also by the presence of olfactory-gustatory anomalies attributable to rancid and / or fermented (sessions 13, 14). Even after 30 days of storage (T2) the S3 sample was clearly distinguishable from the others (C, S1, S2) due to its anomalous color (gray) and cooked meat aroma (session 15); it was also perceived as more acidulous, juicy and fermented (sessions 17, 18).

Concerning the comparison between S1 and S2 at T2, significant differences were found and attributable to the greater intensity of pink and juiciness of the S2 sample (session 16).

The subjects interviewed were able to discriminate samples under study, confirming what has already been found by the trained judges and commented above: the samples with nitrite and enriched with phenolic extract have a stabilizing effect on the color, reducing the loss of pink intensity over time. This result was particularly evident for S2 (phenols-enriched and with the lowest concentration of nitrites) and confirmed what was reported in the literature by other authors who discussed the use of phenols obtained from different by-products of olives processing as natural additives in meat products (Munekaka et al., 2020; Barbieri et al., 2021).

Moreover, the presence of olfactory and/or gustatory notes attributable to the enrichment with phenols, was not perceived. This aspect is very important since it can impact consumers' acceptability; in fact, meat products enriched with phenols from olive mill wastewaters may have sensory characteristics resembling the source of recovery (bitter and pungent) as a function the extracts concentration (Galanakis, 2018).

5.6. CONCLUSIONS

This study demonstrated the efficacy of a powder formulation of a phenolic extract from olive vegetation water at improving the overall oxidative stability of cooked ham, which has been previously subjected to cold storage for 30 days. Storage time caused a progressive decrease of

phenols reaching a total loss, considering T30 vs T0, of about 45%. Lipid oxidation was assessed by the determination of TBARs and COPs.

During the shelf-life study, sample S1 showed the best oxidative stability, with TBARs value closer to 1.0 mg MDA/kg of meat (reference value for rancidity development in cooked pork meat), while the control sample had significantly higher TBARs values (< 3.80 mg MDA/kg of meat). S2 and S3 displayed a similar oxidative trend, with TBARs values below 1.45 mg MDA/kg of meat; therefore, it is possible to hypothesize that the antioxidant activity in both S2 and S3 was mainly due to phenols, while nitrites in S1 were more involved in the development of color and in the microbial stabilization of the product. It was also reported that nitrites to exert antioxidant activity blocking heme prooxidant activity. Regarding COPs and COR%, no significative differences were found between PE and control cooked ham samples.

In conclusion, this study confirms that OMWW extracts rich in phenols could be an alternative for the reduction of synthetic additives in cooked meat preparations, which would promote the formulation of healthier clean label products and improve the sustainability of the olive oil industry with a circular economy approach, by further valorizing this olive by-product.

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Chapter 6. Improved oxidative stability and sensory characteristics of würstel formulated with a reduction of nitrites and enriched with phenol extracts from olive vegetation water.

ABSTRACT

This study aims at evaluating the effect of a phenol-rich extract (PE) obtained from the concentration and purification of olive mill wastewaters on the stability and sensory quality of beef and pork würstels vacuum packed and stored under alternating exposure to fluorescent light at 4±2°C for 30 days. Würstels were sampled at different times (0, 15 and 30 days) and grilled at 200°C. Manufacturing operations and grilling caused a significant loss on the total phenols (around - 60%); the storage time caused a progressive decrease of phenols reaching a total loss, considering samples after 30 days of chilled storage vs samples immediately after they are produced (T0), of about 30%. In general, the steam-cooked control sample showed an increase in both dienes and trienes during storage, while these parameters remained almost constant in ungrilled PE samples. After grilling, all samples exhibited the classical bell-shape behavior of primary oxidation products. Regarding secondary lipid oxidation, grilled samples had TBARs values that were about 2 times higher than those of steam-cooked würstels, confirming the pro-oxidant effect of grilling. However, surprisingly, TBARs in both steam-cooked and grilled control würstels were lower than those of the phenol-enriched samples during the shelf-life. Regarding COPs and cholesterol oxidation ratio (COR%), no significative differences were found between PE and control steam-cooked würstel samples. Discriminant sensory analysis confirmed the excellent stability of the products along the shelf-life study and no taste anomalies were detected for any product. The key attribute for distinguishing among samples was color, even though a certain difficulty was found for differentiating between samples C (dough + nitrites (150 mg/kg) and maltodextrin) and W2 (dough + PE equivalent to 200 mg/kg of phenols and 150 mg/kg of nitrites).

KEYWORDS: Beef and pork würstels; phenolic extract; olive vegetation water; olive by-product; clean label; lipid oxidation; cholesterol oxidation; sensory analysis.

6.1. INTRODUCTION

Frankfurters are meat-based products, today very widespread all over the world, which were originally born within the gastronomic traditions of Northern Europe, finding their maximum expression in German and Austrian cuisine with multiple variations and traditional recipes (Delgado-Pando et al., 2010). This does not mean that there are illustrious examples of this food also in the

Italian culinary tradition such as, for example, the "Meraner" variety typical of Trentino-Alto Adige, which owes its name to the city of Merano. Since its origins, the frankfurter was born as a functional food for the harsh winter thanks to its high caloric intake, can contain up to 30% fat, with an industry average of about 20%, and salt concentrations of around 2% and higher (Tobin et al., 2012), but this meat preparation is today much discussed. This is mainly due to the belief that these products are formulated using low quality raw materials and are rich in additives and preservatives (i.e., nitrite salt). This belief was heightened by the fact that, in 2015, the International Agency for Research on Cancer (IARC) has classified processed meat added with nitrate/nitrite salts as carcinogenic to humans (Group 1), as they can give rise to a series of compounds (N-nitrous compounds (NOCs), nitrosamines and oxidation products), during cooking, subsequent ingestion and digestion that seem to contribute to the onset of colorectal cancer (Bouvard et al., 2015). Independently from the technological advantages, a reduction in the use of nitrates and nitrites has become a matter of primary importance for both industries and consumers. However, their complete or partial replacement with a single natural compound is a challenge, due to their multifunctional characteristics. One alternative approach to the use of chemical additives could be the use of natural antioxidants. Free radical or active oxygen scavenging capacity has been detected in several phenolic compounds. Virgin olive oil (VOO) is a basic component of the Mediterranean diet with a well-established role in contributing to human health, mainly attributed to the antioxidant actions of hydrophilic phenols class (Taticchi et al., 2019). However, it has been shown that almost all the phenols contained in the olive are not found in the oil, but in a by-product that is generated during its production, the olive mill wastewater (OMWV). OMWV is characterized by a high content of sugars, tannins, pectins and phenolic substances and mineral salts (Carrara et al., 2021). This composition makes OMWW an exploitable source of hydrophilic phenols (mainly secoiridoids and its derivates, which are found exclusively in the *Oleaceae* family) which showed a high antioxidant, antimicrobial and anti-inflammatory activity (Servili et al., 2014; Veneziani et al., 2017; Caporaso et al., 2018). Thus, phenols can be recovered from OMWW by using suitable membrane technology (Servili et al., 2011a), and used in meat industries for innovative meat product formulation, to try to reduce/replace nitrate salts in cooked meat products (Galanakis, 2018).

Therefore, considering the need of partial/total replacement of nitrites in emulsified meat products in a formulation perspective of clean label food products, this study aimed at evaluating the effect of the addition of OMWW extracts rich in phenols on the stability and sensory characteristics of steam cooked and grilled würstels. Specifically, the main objectives were to verify the effectiveness

of a powder formulation of the phenolic extract as preservative agent for the extension of the shelf-life and the oxidative stability on the würstels and to monitor the presence of unacceptable sensory attributes and/or off-flavors during storage.

6.2. MATERIAL AND METHODS

6.2.1 OMWW phenol extract

A crude phenolic extract (PE) was obtained from fresh OMWWs of olives harvested in Umbria (Central Italy) from trees of Moraiolo cultivar, by a 3-step membrane filtration of fresh OMWW as previously reported (Ianni et al., 2021). The PE was added with maltodextrin (1: 1, d.w.), as a support, and then spray-dried to get a powder formulation of the PE.

6.2.2 Preparation of phenol-enriched steam cooked würstels samples

The steam cooked würstels samples were prepared with a mixture of beef (50%), lean pork (20%) and lard (30%) The meat was trimmed with a professional meat mincer (TCW32, Cavalli Meat Processing Machinery, Felino, Italy). The minced meat was then transferred into a C15B cutter (La Felsinea, Padova, Italy) added with ice (20%), salt (1.25%), ascorbic acid (0.02%), aromas (VAN HEES® Wiener Glut GAF) (0.33%), liquid smoke (FP 2110 (E433)) (0.05%) and homogenized until complete emulsion. After, the dough was divided into four batches:

1) Control, dough + nitrites (150 mg/kg) and maltodextrin (0.35 g/100 g, Maltodextrin Glucidex 19, France);

2) W1, dough + PE equivalent to 200 mg/kg of phenols and nitrites (35 mg/kg);

3) W2, dough + PE equivalent to 200 mg/kg of phenols and 150 mg/kg of nitrites;

4) W3, dough + PE equivalent to 200 mg/kg of phenols.

The emulsion was bagged into a collagen bowel using a TreSpade manual bagging machine. After, were cooked in a steam oven according to the following cooking cycle:

1) 45 °C x 30min, Relative Humidity (R.H.) 40%, ventilation 6

2) 60 °C x 60 min , R.H. 50%, ventilation 6

3) 75 °C until reaching the core temperature of 72 °C with R.H. 90% ventilation 6.

After cooling in the blast chiller, würstels were peeled, vacuum-packed and pasteurized at 75 °C for 20 min.

The packs were stored in a Carel display counter fridge (Carel Industries, Spa, Brugine, Padua) at 4 ± 1°C, under alternating exposure to fluorescent light (12 h light/12 h darkness) to simulate retail storage conditions. Steam cooked würstels were sampled at fixed time periods (just after produced,

T0; 15 days of storage, T15; 30 days of storage, T30) and frozen at -80 °C. At the same sampling times, the same number of steam cooked würstels from each batch were grilled in an electrical grilling plate (Fimar FRY1L230M, Rimini, Italy) at 200 °C for 3.30 min per side until the core temperature reached 70 °C.

6.2.3 Phenols analysis

In the PE. Fifty mg of spray-dried PE were solubilized in 10 mL of a methanol:water mixture (80:20, v/v), filtered with a 0.2 µm polyvinylidene fluoride (PVDF) syringe filter (Agilent Captiva, Agilent Technologies, Santa Clara, CA, USA) and injected into a high-performance liquid chromatograph coupled to a diode array detector (HPLC-DAD Agilent Technologies system Mod. 1100). The HPLC equipment and analytical conditions were those described by Selvaggini et al. (2014). Each measurement was done in duplicate.

In the steam cooked würstels. Five grams of steam cooked würstels were mixed with 100 mL of methanol:water (80:20, v/v) containing 20 mg/L of butylated hydroxytoluene (BHT) + 0.2% trichloroacetic acid 1 M. The operations of homogenization, recovery, concentration until a final volume of 40 mL of extract and purification by solid-phase extraction (SPE) from 10 mL of this aqueous extract were carried out as previously described Miraglia et al. (2020). The purified extract was then subjected to HPLC-DAD analysis using the same equipment and conditions of the PE analysis (Selvaggini et al., 2014). Each measurement was done in duplicate.

6.2.4 Chemical analysis

6.2.4.1 Lipid extraction

Lipids were extracted according to Boselli et al. (2001). The extraction was performed on 25 g of steam cooked würstels, which were added with 5α-cholestane (internal standard for the quantification of main lipid classes) (Sigma Chemical, St. Louis, USA). The fat content was determined gravimetrically and expressed as percentage. Three independent replicates were run per sample.

6.2.4.2 Determination of main lipid classes

The qualitative-quantitative profile of the main lipid classes (free fatty acids, FFA; monoacylglycerols, MAG; free sterols, STE; diacylglycerols, DAG; esterified sterols, E-STE; triacylglycerols, TAG) was determined by gas chromatography-flame ionization detection (GC-FID),

as reported by Gallina Toschi et al. (2014) and Luise et al. (2018). An aliquot of 20 mg of the lipid extract dissolved in 1 mL of *n*-hexane, was used for this analysis. The internal standard method, with the response factor of each main lipid class (estimated with commercial standards), was used to determine the amount of each lipid class (expressed as g/100 g of lipids). Three independent replicates were run per sample.

6.2.4.3 Determination of total FA

The composition of total fatty acids was determined on 20 mg of lipid extract by GC-FID (Cardenia et al., 2015), after previous methylation and transmethylation. FAME quantification was performed according to the internal standard method (using tridecanoic acid methyl ester) and expressed as a proportion of the identified total FAME (g/100 g). Three independent replicates were run per sample.

Based on the total FA composition, the atherogenic index (AI) and thrombogenic index (TI) were also determined (Ulbricht & Southgate, 1991).

6.2.4.4 Determination of conjugates dienes and trienes

The analysis of dienoic and trienoic conjugated fatty acid derivates was performed by following the method described in the ISO 3656:2011. The spectrophotometric analysis was performed using a Jasco dual beam spectrophotometer model V-550 UV–vis (Jasco, Tokyo, Japan). A quartz cuvette with an optical path of 10 mm was used. The determination is based on spectrophotometric analysis of 0.1 g of lipid extract diluted in 10 mL of iso-octane. The spectrophotometric investigations were performed at 232 nm for the determination of diene conjugated systems and at 268 nm for the triene conjugated systems

6.2.4.5 Determination of thiobarbituric acid reactive substances (TBARs)

Secondary lipid oxidation was assessed as TBARs on steam cooked and grilled würstels samples (Tarladgis et al., 1960). Four g of each sample were used for this spectrophotometric determination and the absorbance was measured at 530 nm. A 1,1,3,3-tetramethoxypropane standard calibration curve was used for the quantification of TBARs (concentration range of 0.045–0.113 µg/mL; $y=0.0077x+0.0072$, $r^2=0.9998$) and the values were expressed as mg MDA/kg meat. Three independent replicates were made per sample.

6.2.4.6 Determination of cholesterol and oxysterols (COPs)

Cholesterol and COPs were extracted by cold saponification of 200 mg of lipid extract, followed by purification with aminopropyl SPE (Cardenia et al., 2015). Silylated cholesterol and COPs were analyzed by Fast GC/MS (Cardenia et al., 2012), using betulinol (Sigma Chemical, St. Louis, USA) and 19-hydroxycholesterol (Steraloids, Newport, Rhode Island, USA) as internal standards, respectively. Mass spectra were acquired in full scan mode (total ion current, TIC), while they were integrated with single ion monitoring (SIM) mode using the characteristic ions with a high abundance (Cardenia et al., 2012); quantification was carried out by means of calibration curves built for each compound. Cholesterol and total COPs were expressed as mg/kg of meat. Three independent replicates were run per sample. The rate of total cholesterol oxidation (%OR) was also estimated as reported by Cardenia et al. (2015).

6.2.5 Sensory analysis

6.2.5.1 Discriminant test

In this study, the triangle test was applied to identify sensory differences between the control sample (C) and the treated samples (W1, W2 and W3) right after being produced (T0) and to investigate the effect of different storage times, i.e. 0, 15 and 30 days (CT0-CT15-CT30; W1T0-W1T15-W1T30; W2T0-W2T15-W2T30; W3T0-W3T15-W3T30).

Having four samples at three different storage times to be compared, the test was conducted in several successive sessions (two different days) to perform all possible combinations. The sessions were held in the tasting room of the Department of Agricultural and Food Sciences, *Alma Mater Studiorum*-University of Bologna, involving 67 untrained judges, aged between 20 and 67 years; the test were carried out according to the procedures described by ISO 4120:2007.

In this test were evaluated only grilled product. In each session, 6 triad of samples were evaluated. Data processing was performed by comparing the number of correct answers with values reported in a double entry probability table, indicating the minimum number of correct answers corresponding to the number of judges involved in the test or the number of judgments (number of judges for the number of replicas) for the different levels of significance (ISO 4120:2007).

6.2.6 Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, France) was used to elaborate chemical and sensory data. The chemical data are reported as mean values of independent replicates of each analytical

determination. First, normal distribution of data was tested (p<0.05) with Shapiro-Wilk method. Chemical data were analyzed using two-way or three-way analysis of variance (ANOVA), including formulation (Form), storage time (St), grilling (Gr) and their interaction (Form*St; Form*Gr; St*Gr; Form*St*Gr) as factors. Tukey's honest significance test was performed at a 95% confidence level (p≤0.05), to separate means of statistically different parameters. A principal component analysis (PCA), with a Varimax rotation, was also carried out.

6.3. RESULTS AND DISCUSSION

6.3.1 Chemical analysis

6.3.1.1 Evolution of phenolic compounds

The PE used for the preparation of the würstels had a total phenol content of 25.7 mg/g of dried product, of which 61.5% was 3,4-DHPEA-EDA (oleacein), 20.6% 3,4-DHPEA (hydroxytyrosol), 13.2% verbascoside and 4.7% *p*-HPEA (tyrosol).

As shown in **Table 6.1**, part of the added phenols was lost during storage and grilling. In terms of total phenols, there was a loss of 22% and 29% of phenolic compounds in W1 samples after 15 and 30 days of storage, respectively, whereas a major loss was detected in W2 (28 and 34%, respectively) and W3 (23 and 36%) steam cooked samples, respectively. In particular, the highest variation was observed for 3,4-DHPEA-EDA, which completely disappears in W1 W2 and W3 steam cooked samples after 15 and 30 days of storage. It can be assumed that, to a certain extent, 3,4-DHPEA-EDA was subjected to hydrolysis, which generates 3,4-DHPEA, as has been found in fermented functional milk (Servili et al., 2011b). As reported by Obied et al. (2008), the degradation mechanism of this oleuropein derivative includes enzymatic and non-enzymatic oxidation and hydrolysis. The increase of 3,4-DHPEA, in presence of 3,4-DHPEA-EDA, has already been observed in different food matrices and at different temperatures (Servili et al., 2011b; Esposto et al. 2015; Taticchi et al., 2017; Balzan et al., 2017), the phenols evolution in W1, W2 and W3 würstels strengthens the hypothesis of its hydrolytic origin from the degradation of oleuropein derivatives during storage (Brenes et al., 2001). Nevertheless, the oxidative degradation of these two phenols has been appointed as the main cause of their decrease (Obied et al., 2008; Di Maio et al., 2011). For hydroxytyrosol, therefore, two contemporary phenomena would be at the basis of its particular evolution over time: the first is the hydrolysis of 3,4-DHPEA-EDA after which this phenolic alcohol is released in free form, while the second one is the oxidative degradation which leads to its decrement. In the case of samples W1, W2 and W3, in the early stages of storage, hydroxytyrosol is limitedly involved in oxidation reactions

and, therefore, the resultant balance between the decrease on the one hand and the increase on the other, is an increase of the concentration of hydroxytyrosol.

Table 6.1. Evolution of phenolic compounds of steam cooked and grilled würstel samples after 0, 15 and 30 days of storage. C, dough + nitrites (150 mg/kg) and maltodextrin (0.35 g/100 g); W1, dough + PE equivalent to 200 mg kg of phenols and nitrites (35 mg/kg); W2, dough + PE equivalent to 200 mg/kg of phenols and 150 mg/kg of nitrites; W3, dough + PE equivalent to 200 mg kg of phenols.

		3,4-DHPEA	*p*-HPEA	VB	3,4-DHPEA-EDA	Total phenols
		(mg/kg)				
Steam cooked samples		0 days				
	C	-	-	-	-	-
	W1	$77.29\pm0.44^{b,B,X}$	$8.68\pm0.00^{B,X}$	$18.32\pm0.04^{a,B,X}$	$35.27\pm1.91^{a,AB}$	$139.56\pm2.39^{a,B,X}$
	W2	$79.18\pm0.47^{a,B,X}$	$6.63\pm0.00^{a,C,X}$	$20.47\pm0.43^{a,A,X}$	$25.34\pm0.89^{a,B}$	$131.62\pm1.79^{a,B,X}$
	W3	$82.67\pm0.15^{a,A,X}$	$10.21\pm0.00^{a,A,X}$	$21.59\pm0.10^{a,A,X}$	$36.90\pm2.27^{a,A}$	$151.38\pm2.52^{a,A,X}$
		15 days				
	C	-	-	-	-	-
	W1	$82.42\pm1.78^{a,A,X}$	$8.40\pm0.00^{B,X}$	$17.77\pm0.22^{b,C,X}$	nd^{b}	$108.60\pm2.01^{b,B,X}$
	W2	$67.64\pm0.29^{b,B,X}$	$6.91\pm0.00^{a,C,X}$	$19.89\pm0.30^{b,B,X}$	nd^{b}	$94.44\pm0.59^{b,B,X}$
	W3	$85.79\pm1.38^{a,A,X}$	$9.93\pm0.00^{b,A,X}$	$20.89\pm0.16^{b,A,X}$	nd^{b}	$116.61\pm1.54^{b,A,X}$
		30 days				
	C	-	-	-	-	-
	W1	$73.62\pm1.12^{b,A,X}$	$8.09\pm0.00^{B,X}$	$17.42\pm0.31^{b,B,X}$	nd^{b}	$99.12\pm1.43^{c,A,X}$
	W2	$60.35\pm0.15^{c,C,X}$	$5.72\pm0.00^{b,C,X}$	$20.82\pm0.75^{a,A,X}$	nd^{b}	$86.88\pm0.90^{c,B,X}$
	W3	$66.96\pm0.55^{b,B,X}$	$9.62\pm0.00^{b,A,X}$	$20.39\pm0.23^{b,A,X}$	nd^{b}	$96.97\pm0.78^{c,A,X}$
Grilled samples		0 days				
	C	-	-	-	-	-
	W1	$57.72\pm0.48^{a,B,Y}$	$7.59\pm0.00^{a,A,Y}$	16.17 ± 0.13^{Y}	nd^{Y}	$81.48\pm0.62^{a,Y}$
	W2	$61.69\pm0.27^{a,A,Y}$	$6.62\pm0.00^{a,B,Y}$	16.84 ± 0.53^{Y}	nd^{Y}	$85.16\pm0.80^{a,Y}$
	W3	$61.69\pm0.27^{a,A,Y}$	$6.62\pm0.00^{B,Y}$	16.84 ± 0.53^{Y}	nd^{Y}	$85.16\pm0.80^{a,Y}$
		15 days				
	C	-	-	-	-	-
	W1	$47.05\pm0.86^{b,Y}$	$6.57\pm0.00^{b,A,Y}$	$15.86\pm0.51^{B,Y}$	nd^{Y}	$69.48\pm1.37^{b,Y}$
	W2	$44.38\pm1.21^{b,Y}$	$4.66\pm0.00^{b,A,Y}$	$17.05\pm0.24^{A,Y}$	nd^{Y}	$66.09\pm1.45^{b,Y}$
	W3	$40.91\pm0.81^{b,Y}$	$6.86\pm0.00^{A,Y}$	$15.97\pm0.26^{B,Y}$	nd^{Y}	$63.74\pm1.06^{b,Y}$
		30 days				
	C	-	-	-	-	-
	W1	$28.74\pm0.04^{c,C,Y}$	$5.96\pm0.00^{c,A,Y}$	$16.63\pm0.09^{B,Y}$	nd^{Y}	$51.33\pm0.13^{c,B,Y}$
	W2	$38.34\pm0.31^{c,B,Y}$	$4.19\pm0.00^{c,B,Y}$	$18.19\pm1.20^{A,Y}$	nd^{Y}	$60.72\pm1.51^{b,A,Y}$
	W3	$41.72\pm0.00^{b,A,Y}$	$6.03\pm0.00^{A,Y}$	$16.21\pm1.33^{B,Y}$	nd^{Y}	$63.97\pm1.52^{b,A,Y}$
	Factor	F value				
	Form	***	***	***	***	***
	St	***	***	*NS*	***	***
	Gr	***	***	***	***	***
	Form*St	***	***	*	***	***
	Form*Gr	***	***	***	***	***
	St*Gr	***	***	*NS*	***	***
	Form*St*Gr	***	***	*NS*	***	***

Results as reported as means±s.d of 2 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) within the same sample during the shelf-life. A-C indicate significant differences (Tukey's test; $p \leq 0.05$) among treatments, X-Y indicate significant differences (Tukey's test; $p \leq 0.05$) between raw and grilled samples. *$p < 0.05$, *** $p < 0.001$. 3,4-DHPEA, hydroxytyrosol; Form, formulation; Gr, grilling; *NS*, not significantly; *p*-HPEA, tyrosol; St, storage; VB, verbascoside; 3,4-DHPEA-EDA, oleacein; *nd*: not determined

In the more advanced phases, the oxidative degradation prevails in W1, W2 and W3 würstels, due to the decrease in the concentration of the other more reactive phenols (such as 3,4-DHPEA-EDA).

During storage, the concentration of *p*-HPEA and verbascoside did not significantly vary, as already found in other shelf-life studies for cooked meat products (Balzan et al., 2017).
Regarding the behavior of phenolic compounds during cooking, the loss was greater than those found during storage. In all grilled samples, 3,4-DHPEA-EDA completely disappeared, confirming its high susceptibility to high cooking temperatures (Lonzano-Castellon et al., 2020), while a severe loss of hydroxytyrosol was observed as well.

6.3.1.2 Lipid content and main lipid classes

As reported in **Table 6.2**, the lipid content ranged from 14.25 to 19.62% and from 21.25 to 23.88% in steam cooked and grilled würstels, respectively. Formulation and storage did not significantly affect the lipid content of both steam cooked and grilled samples. However, it is possible to note a significant increase in the lipid amount of the grilled samples, due to the loss of water during the heat treatment, consequently increasing the concentration of these components (Baggio & Bragagnolo, 2006).

Regarding main lipid classes (Table 2), the most abundant class was TAG, followed by DAG, FFA, E-STE, STE and MAG in both raw and grilled würstels. While shelf-life did not significantly affect the percentage distribution of the single lipid classes, formulation apparently had an effect on FFA, but without displaying a clear trend.
These differences could be partly due to the formulation of würstels as they were prepared with beef and pork meat and lard from different cuts and animals. After cooking, some significant differences were detected in the total lipid profile (in particular MAG), which may have been influenced by a combined effect of lipolysis and a partial/selective loss of the lipid components during grilling, due to their different melting points.

Table 6.2. Lipid content and main lipid classes profile of steam cooked and grilled würstel samples after 0, 15 and 30 days of storage. C, dough + nitrites (150 mg/kg) and maltodextrin (0.35 g/100 g); W1, dough + PE equivalent to 200 mg/kg of phenols and nitrites (35 mg/kg); W2, dough + PE equivalent to 200 mg/kg of phenols and 150 mg/kg of nitrites; W3, dough + PE equivalent to 200 mg/kg of phenols.

		Lipid content	FFA	MAG	STE	DAG	E-STE	TAG
		(%)	(% of total lipids)					
Steam cooked samples				0 days				
	C	19.62±0.70^{A,Y}	0.88±0.03	0.05±0.00	0.25±0.00	1.26±0.24	0.66±0.02	96.90±0.29
	W1	17.93±0.47^{C,Y}	1.02±0.04	0.06±0.00	0.26±0.00	1.46±0.04	0.68±0.01	96.52±0.19
	W2	17.48±0.47^{C,Y}	0.87±0.04	0.05±0.00	0.26±0.01	1.43±0.01	0.71±0.01	96.67±0.06
	W3	18.14±0.34^{b,B,Y}	0.90±0.01	0.05±0.00	0.26±0.00	1.48±0.02	0.69±0.01	96.62±0.04
				15 days				
	C	19.08±1.86^{A,Y}	1.00±0.03	0.06±0.00	0.24±0.02	1.51±0.02	0.51±0.09	94.67±0.11
	W1	17.56±0.47^{B,Y}	0.94±0.01	0.05±0.00	0.25±0.00	1.54±0.03	0.69±0.00	96.53±0.03
	W2	17.98±0.60^{B,Y}	0.98±0.08	0.06±0.00	0.26±0.00	1.53±0.05	0.69±0.02	96.48±0.12
	W3	14.25±0.64^{c,C,Y}	1.00±0.01	0.06±0.00	0.26±0.00	1.55±0.02	0.69±0.02	96.44±0.04
				30 days				
	C	19.18±0.25^{A,Y}	0.97±0.01	0.05±0.00	0.27±0.00	1.43±0.02	0.60±0.08	94.67±0.11
	W1	17.46±0.17^{A,Y}	1.02±0.04	0.06±0.00	0.30±0.00	1.56±0.04	0.70±0.01	96.37±0.03
	W2	19.60±0.07^{A,Y}	0.84±0.06	0.05±0.00	0.23±0.02	1.46±0.03	0.70±0.01	96.72±0.12
	W3	19.47±0.20^{a,A,Y}	0.97±0.02	0.05±0.00	0.25±0.00	1.52±0.06	0.69±0.01	96.51±0.04
Grilled samples				0 days				
	C	22.81±2.29^{X}	0.91±0.06	0.04±0.00	0.27±0.05	1.03±0.03	0.70±0.09	97.04±0.20
	W1	21.53±0.18^{X}	0.80±0.04	0.03±0.00	0.27±0.00	1.08±0.06	0.66±0.01	97.14±0.13
	W2	21.25±0.45^{X}	0.77±0.05	0.07±0.00	0.27±0.00	1.08±0.04	0.69±0.01	97.12±0.01
	W3	22.72±0.34^{X}	0.80±0.02	0.04±0.00	0.27±0.00	1.29±0.03	0.68±0.02	96.92±0.03
				15 days				
	C	23.44±0.56^{X}	0.77±0.03	0.03±0.00	0.25±0.00	1.31±0.00	0.62±0.01	97.02±0.55
	W1	23.71±1.19^{X}	0.77±0.04	0.03±0.00	0.27±0.00	1.31±0.01	0.70±0.01	96.92±0.05
	W2	22.52±0.59^{X}	0.74±0.00	0.03±0.00	0.27±0.00	1.31±0.00	0.69±0.00	96.97±0.05
	W3	24.02±0.88^{X}	0.83±0.01	0.04±0.00	0.27±0.00	1.48±0.06	0.68±0.01	96.71±0.12
				30 days				
	C	23.88±0.19^{X}	0.77±0.02	0.04±0.00	0.25±0.00	1.42±0.03	0.71±0.01	96.81±0.24
	W1	23.35±0.84^{X}	0.85±0.03	0.04±0.00	0.26±0.00	1.42±0.05	0.61±0.02	96.81±0.10
	W2	21.55±2.09^{X}	0.82±0.01	0.04±0.00	0.26±0.00	1.36±0.02	0.70±0.00	96.82±0.03
	W3	23.32±0.58^{X}	0.89±0.03	0.05±0.00	0.27±0.06	1.51±0.00	0.57±0.02	96.71±0.14
	Factor				F value			
	Form	*NS*	*	*NS*	*NS*	*NS*	*NS*	*NS*
	St	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
	Gr	***	*NS*	***	*NS*	*NS*	*NS*	*NS*
	Form*St	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
	Form*Gr	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
	St*Gr	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
	Form*St*Gr	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

Results as reported as means±s.d of 3 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) within the same sample during the shelf-life. A-C indicate significant differences (Tukey's test; $p \leq 0.05$) among treatments. X-Y indicate significant differences (Tukey's test; $p \leq 0.05$) between steam cooked and grilled samples. *$p < 0.05$, ***$p < 0.001$. DAG, diacylglycerols; E-STE, esterified sterols; FFA, free fatty acids; Form, formulation; Gr, grilling; MAG, monoacylglycerols; STE, sterols; St, storage; TAG, triacylglycerols.

6.3.1.3 Total fatty acid profile

Regarding total FA composition (**Table 6.3**), the most represented FA class was monounsaturated fatty acids (MUFA, 55-62%), followed by saturated fatty acids (SFA, 27-33%) and polyunsaturated fatty acids (PUFA, 9-10%). This FA profile agrees with that reported by Baggio & Bragagnolo (2006) and by Parrini et al. (2020) for steam cooked and grilled frankfurter type würstels.

Table 6.3. Fatty acid classes (expressed as % of total fatty acids) of steam cooked and grilled würstel samples after 0, 15 and 30 days of storage. C, dough + nitrites (150 mg/kg) and maltodextrin (0.35 g/100 g); W1, dough + PE equivalent to 200 mg/kg of phenols and nitrites (35 mg/kg); W2, dough + PE equivalent to 200 mg/kg of phenols and 150 mg/kg of nitrites; W3, dough + PE equivalent to 200 mg/kg of phenols.

		SFA	MUFA	PUFA	*n*-3	*n*-6
		(% total fatty acids)				
Steam cooked samples		0 days				
	C	33.83±1.32^{a,X}	55.97±1.07^{b,Y}	10.21±0.25^{X}	0.88±0.04^{a,X}	9.33±0.22^{X}
	W1	33.61±1.63^{a,X}	56.20±1.25^{b,Y}	10.18±0.38^{a,X}	0.91±0.07^{a,X}	9.28±0.30
	W2	33.82±1.05^{a,X}	56.10±0.96^{b,Y}	10.08±0.10^{X}	0.84±0.08^{a,X}	9.23±0.03^{X}
	W3	32.95±0.99^{a,X}	56.65±0.83^{c,Y}	10.41±0.16^{X}	0.90±0.05^{a,X}	9.51±0.11
		15 days				
	C	27.88±0.49^{b,A}	61.99±0.53^{a,AB}	10.13±0.04	0.60±0.02^{b,C,X}	9.53±0.03^{X}
	W1	26.94±0.44^{c,B,Y}	62.80±0.73^{a,A}	10.26±0.29^{ab}	0.64±0.02^{b,BC,X}	9.62±0.27^{X}
	W2	28.51±0.23^{c,A,Y}	61.26±0.36^{a,A}	10.23±0.18	0.68±0.00^{ab,B,X}	9.56±0.18^{X}
	W3	27.98±0.27^{c,A}	61.71±0.22^{a,A}	10.31±0.05	0.66±0.02^{b,AB,X}	9.65±0.06
		30 days				
	C	28.14±0.49^{b,Y}	61.79±0.21^{a,A}	10.08±0.52	0.59±0.06^{b,X}	9.48±0.46^{X}
	W1	28.57±0.74^{b,Y}	61.59±0.69^{a,A}	9.84±0.08^{b}	0.63±0.03^{b,X}	9.21±0.09^{X}
	W2	29.12±0.18^{b,X}	61.03±0.24^{a,AB}	9.85±0.15	0.55±0.12^{b,X}	9.31±0.03
	W3	29.67±0.29^{b,X}	60.38±0.17^{b,B}	9.95±0.22	0.66±0.04^{b,X}	9.29±0.24
Grilled samples		0 days				
	C	29.57±0.41^{a,Y}	61.35±0.41^{ab,AB,X}	9.08±0.00^{b,C,Y}	0.55±0.02^{b,AB,Y}	8.53±0.02^{b,B,Y}
	W1	29.84±0.51^{Y}	60.44±0.35^{B,X}	9.72±0.19^{AB,Y}	0.59±0.04^{AB,Y}	9.13±0.15^{A}
	W2	29.25±0.51^{Y}	61.48±0.76^{A,X}	9.28±0.25^{b,AB,Y}	0.53±0.08^{B,Y}	8.74±0.17^{b,B,Y}
	W3	29.08±0.45^{a,Y}	60.91±0.34^{AB,X}	10.01±0.11^{b,A,Y}	0.69±0.02^{A,Y}	9.33±0.12^{b,A}
		15 days				
	C	27.64±0.15^{b,B}	61.89±0.05^{a,A}	9.48±0.18^{a,C}	0.56±0.04^{b,B,Y}	8.91±0.17^{ab,B,Y}
	W1	29.25±0.06^{A,X}	61.16±0.21^{B}	9.59±0.18^{BC}	0.63±0.00^{AB,Y}	8.95±0.18^{B,Y}
	W2	28.78±0.30^{B,X}	61.50±0.31^{AB}	9.72±0.02^{ab,B}	0.65±0.02^{A,Y}	9.07±0.01^{b,B,Y}
	W3	28.00±0.09^{b,C}	61.57±0.11^{AB}	10.43±0.06^{a,A}	0.66±0.02^{A,Y}	9.76±0.06^{a,A}
		30 days				
	C	29.32±0.14^{ab,A,X}	60.93±0.03^{b}	9.75±0.11^{a,B}	0.64±0.01^{a,B,Y}	9.11±0.10^{a,B,Y}
	W1	29.23±0.39^{A,X}	61.05±0.48	9.72±0.12^{B}	0.65±0.01^{B,Y}	9.07±0.12^{B,Y}
	W2	28.83±0.38^{AB,Y}	61.18±0.37	9.98±0.09^{a,B}	0.64±0.02^{B,Y}	9.35±0.09^{a,B}
	W3	28.22±0.19^{ab,B,Y}	61.37±0.17	10.41±0.10^{a,A}	0.69±0.01^{A,Y}	9.72±0.10^{a,A}
	Factor			**F value**		
	Form	*NS*	*NS*	*NS*	*NS*	*NS*
	St	*NS*	*NS*	*NS*	*NS*	*NS*
	Gr	*NS*	*NS*	*NS*	*NS*	*NS*
	Form*St	*NS*	*NS*	*NS*	*NS*	*NS*
	Form*Gr	*NS*	*NS*	*NS*	*NS*	*NS*
	St*Gr	*NS*	*NS*	*NS*	*NS*	*NS*
	Form*St*Gr	*NS*	*NS*	*NS*	*NS*	*NS*

Results as reported as means±s.d. of 3 independent replicates. a–c indicate significant differences (Tukey's test; $p \leq 0.05$) between the same sample during the shelf-life, A-C indicate significant differences (Tukey's test; $p \leq 0.05$) between treatments, X-Y indicate significant differences (Tukey's test; $p \leq 0.05$) between steam cooked and grilled samples. ***$p < 0.001$. Form, formulation; Gr, grilling; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; St, storage.

No significant effect of formulation, storage time, grilling and its interactions was observed on FA profile of both steam cooked and grilled würstels (Table 3). Grilling may have induced modifications in the FA composition of würstels by oxidation of unsaturated FA and/or selective melting of lipids (Baggio & Bragagnolo, 2006), these two effects add up to the selective degradation of phenols in the grilled PE-enriched products (**Table 6.1**), as oleacein completely disappeared during shelf-life of steam cooked samples and after grilling.

Table 6.4. Ratios of fatty acid classes, atherogenic index (AI) and thrombogenic index (TI) of steam cooked and grilled würstel samples after 0, 15 and 30 days of storage. C, dough + nitrites (150 mg/kg) and maltodextrin (0.35 g/100 g); W1, dough + PE equivalent to 200 mg/kg of phenols and nitrites (35 mg/kg); W2, dough + PE equivalent to 200 mg/kg of phenols and 150 mg/kg of nitrites; W3, dough + PE equivalent to 200 mg/kg of phenols.

		***n*-6/*n*-3**	**PUFA/SFA**	**UFA/SFA**	**AI**	**TI**
Steam cooked samples			**0 days**			
	C	14.62±0.23^{b}	0.30±0.09	1.96±0.32	0.43±0.01	0.80±0.06
	W1	14.24±0.49^{b}	0.30±0.00	1.98±0.63	0.43±0.01	0.99±0.04
	W2	14.02±1.04	0.30±0.08	1.96±0.06	0.43±0.03	0.93±0.07
	W3	14.64±0.47^{b}	0.32±0.07	2.04±0.99	0.32±0.00	0.76±0.07
			15 days			
	C	15.79±0.48^{a,A}	0.36±0.07	2.59±0.53	0.31±0.08	0.76±0.18
	W1	14.96±0.24^{a,AB}	0.38±0.06	2.71±0.73	0.31±0.04	0.73±0.05
	W2	14.08±0.35^{B}	0.36±0.00	2.51±0.38	0.33±0.06	0.78±0.15
	W3	14.66±0.44^{a,AB}	0.37±0.06	2.57±0.27	0.34±0.03	0.81±0.05
			30 days			
	C	16.00±0.96^{a}	0.36±0.01	2.55±0.61	0.32±0.02	0.77±0.07
	W1	14.68±0.69^{a}	0.34±0.01	2.50±0.75	0.34±0.01	0.79±0.06
	W2	17.63±4.37	0.34±0.06	2.43±0.28	0.41±0.02	0.96±0.04
	W3	14.14±1.09^{a}	0.34±0.06	2.37±0.34	0.35±0.00	0.83±0.00
Grilled samples			**0 days**			
	C	15.54±0.60	0.31±0.01	2.38±0.41	0.34±0.01	0.83±0.06
	W1	15.62±0.82^{a}	0.33±0.05	2.35±0.52	0.36±0.01	0.89±0.04
	W2	16.59±2.14	0.32±0.08	2.42±0.76	0.43±0.03	0.83±0.07
	W3	13.60±0.56	0.34±0.08	2.44±0.45	0.32±0.00	0.86±0.07
			15 days			
	C	15.82±1.03	0.33±0.06	2.49±0.19	0.35±0.08	0.76±0.18
	W1	14.11±0.29^{b}	0.33±0.02	2.42±0.23	0.35±0.04	0.73±0.05
	W2	13.86±0.45	0.34±0.06	2.47±0.31	0.35±0.06	0.78±0.15
	W3	14.69±0.34	0.37±0.07	2.57±0.13	0.34±0.03	0.81±0.05
			30 days			
	C	14.21±0.18	0.33±0.02	2.41±0.14	0.32±0.02	0.77±0.07
	W1	14.04±0.34 ^{b}	0.33±0.06	2.42±0.50	0.34±0.01	0.79±0.06
	W2	14.64±0.52	0.35±0.03	2.47±0.42	0.31±0.02	0.76±0.04
	W3	14.11±0.22	0.37±0.05	2.54±0.23	0.35±0.00	0.83±0.00
	Factor			**F value**		
	Form	*NS*	*NS*	*NS*	*NS*	*NS*
	St	*NS*	*NS*	*NS*	*NS*	*NS*
	Gr	*NS*	*NS*	*NS*	*NS*	*NS*
	Form*St	*NS*	*NS*	*NS*	*NS*	*NS*
	Form*Gr	*NS*	*NS*	*NS*	*NS*	*NS*
	St*Gr	*NS*	*NS*	*NS*	*NS*	*NS*
	Form*St*Gr	*NS*	*NS*	*NS*	*NS*	*NS*

Results as reported as means± s.d of 3 independent replicates. a–c indicate significant differences (Tukey's test; $p \leq 0.05$) between the same sample during the shelf-life, A-C indicate significant differences (Tukey's test; $p \leq 0.05$) between treatments, X-Y indicate significant differences (Tukey's test; $p \leq 0.05$) between steam cooked and grilled samples. ***$p < 0.001$. AI, Atherogenic Index; Form, formulation; Gr, grilling PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids, St, storage; TI, thrombogenic index; UFA, unsaturated fatty acids.

Regarding FA classes ratios (**Table 6.4**), the PUFA *n*-6/PUFA *n*-3 ratio varied between 14.02 and 17.63 in steam coked würstels, while it ranged from 13.60 to 16.59 in grilled ones; these values agree with those found by Baggio & Bragagnolo (2006) and Parrini et al (2020) for steam cooked and grilled frankfurter type würstels. The PUFA *n*-6/PUFA *n*-3 ratio is a suitable index to compare the nutritional value of food. According to Simopoulos (2006), a low PUFA *n*-6/PUFA *n*-3 ratio (< 4) is desirable for a healthy human diet. Formulation, grilling, storage and its interactions not significantly impacted this ratio.

The PUFA/SFA ratio is also used for evaluating the nutritional quality of foods lipids and it has been suggested by nutritional guidelines that it should be around 0.4 (Delgado-Pando et al., 2010). In our case, this ratio ranged between 0.30 and 0.38 in both steam cooked and grilled würstels samples.

The results of the present study agree with the PUFA/SFA ratio reported Baggio & Bragagnolo (2006) for steam cooked and grilled frankfurter type würstels. This ratio reflected the behavior of the PUFA class, as it was also significantly influenced by formulation, storage time, grilling and showed a not significant interaction among all the factors tested.

The UFA/SFA ratio is useful for observing the oxidative stability of fatty acids in food, as it decreases when UFA oxidize. The UFA/SFA ratio ranged from 1.96 to 2.71 and from 2.35 to 2.54 in steam coked and grilled würstels samples, respectively. These values agree with those reported by Baggio & Bragagnolo (2006) for steam cooked and grilled frankfurter type würstels. All the factors here tested (product formulation, storage time and grilling) and their interactions not significantly influenced this ratio. However, the values obtained in this study indicates that the oxidative stability of this product is largely due to the emulsion that distinguishes this type of product, in fact, no statistically significant differences were found between C and PE samples.

Based on FA composition, AI and TI were also calculated, which are useful indices for understanding the role of FA composition on both atherogenic and thrombogenic risks. The indices ranged from 0.31 to 0.43 and from 0.73 to 0.99 for AI and TI, respectively. None of the factors here tested (product formulation, storage time and grilling) significantly influenced these ratios.

Regarding single FA, the most abundant FA in both steam-cooked and grilled samples was oleic acid (C18:1 n-9, 51.65-59.5 %), followed by palmitic acid (C16:0, 19.03-24.36 %), linoleic acid (C18:2, 8.20-9.53 %) and stearic acid (C18:0, 6.48-9.28 %). These values agree with those reported by Baggio & Bragagnolo (2006) for steam cooked and grilled frankfurter type würstels and with those reported by Delgado-Pando et al. (2010) for frankfurter and pork fat. No significant differences were found in single FA between control and PE-enriched samples during shelf-life nor after grilling. Slightly

differences could be partly due to the variability of the matrix, which depends both on the type of feeding of the animals from which the fat and lean components used in the formulation of the samples derive, but also on the trimming of these components. For the formulation of the würstels analyzed in this study both beef and pork meet were used, therefore, some of the fatty acids identified in the samples have the trans isomer; these, in fact, are natural components of animal fats. They are formed by biohydrogenation, by the action of the enzymes of polygastric animals' rumen microbiota, and by the action of enzymes, such as isomerase and hydrogenase. In the meat of non-ruminant animals, in fact, the quantity of trans fatty acids is generally low and depends on their presence in feed (Baggio & Bragagnolo, 2006).

6.3.1.4 Lipid oxidation

Primary lipid oxidation products were monitored by conjugates dienes (K_{232}) and trienes (K_{270}) while TBARs, and COPs were determined as secondary oxidation products (**Table 6.5**).

As suggested by Dominguez et al. (2019), for an evaluation of dienes and trienes in meat products, the evaluation of the trends at the wavelengths of 232 nm (dienes) and 268 nm (triene) was carried out.

K_{232} ranged from 3.63-6.04 and 3.27-6.66 in steam cooked and grilled samples, respectively. Product formulation and grilling significantly influenced this oxidative parameter and the two- three factors interaction was significant as well. K_{268} ranged from 0.19-0.50 and 0.34-1.21 in steam cooked and grilled samples, respectively. Product formulation, storage time and grilling significantly influenced this oxidative parameter; and the two- three factors interaction was significant as well.

Table 6.5. K_{232} (conjugates dienes), K_{268} (conjugates trienes), TBARs, cholesterol, COPs and COR of steam cooked and grilled würstel samples after 0, 15 and 30 days of storage. C, dough + nitrites (150 mg/kg) and maltodextrin (0.35 g/100 g); W1, dough + PE equivalent to 200 mg/kg of phenols and nitrites (35 mg/kg); W2, dough + PE equivalent to 200 mg/kg of phenols and 150 mg/kg of nitrites; W3, dough + PE equivalent to 200 mg/kg of phenols.

		K_{232}	K_{268}	TBARs (mg MDA/kg meat)	Cholesterol	7α-HC	7β-HC	5β,6β-EC	5α,6α-EC	7-KC	COPsTot	COR (%)
						(mg/kg meat)						
Steam cooked samples						**0 days**						
	C	4.92±0.44^{a,Y}	0.50±0.02^{c,A,Y}	0.23±0.02^{B,Y}	770.40±49.90^{c,C,Y}	0.13±0.00^{b,Y}	0.25±0.00^{Y}	0.16±0.00	0.10±0.00	0.38±0.00^{Y}	1.05±0.03^{B,Y}	0.18±0.05^{a,A,Y}
	W1	4.39±0.21^{a,X}	0.29±0.02^{a,B,Y}	0.20±0.03^{c,B,Y}	829.53±72.70^{b,B,Y}	0.14±0.00^{b,Y}	0.26±0.00^{Y}	0.16±0.00	0.10±0.00	0.31±0.00^{Y}	1.08±0.02^{a,B,Y}	0.16±0.00^{a,A,X}
	W2	4.70±0.31^{a,Y}	0.41±0.01^{a,A,Y}	0.15±0.10^{c,B,Y}	1042.73±113.77^{b,A,Y}	0.13±0.00^{b,Y}	0.25±0.00^{Y}	0.13±0.00	0.06±0.00	0.33±0.00^{Y}	1.01±0.05^{b,C,Y}	0.10±0.04^{b,B,Y}
	W3	4.05±0.33^{Y}	0.19±0.01^{c,C,Y}	0.51±0.11^{a,A,Y}	1011.00±59.26^{a,A,Y}	0.14±0.00^{c,Y}	0.30±0.00^{Y}	0.12±0.00	0.06±0.00	0.38±0.00^{Y}	1.12±0.02^{b,A,Y}	0.11±0.02^{b,B,Y}
						15 days						
	C	4.25±0.42^{c,A,Y}	0.45±0.02^{b,A,Y}	0.21±0.03^{C,Y}	1241.16±18.65^{a,A}	0.27±0.00^{a,A,Y}	0.30±0.00^{Y}	0.12±0.00	0.07±0.00	0.37±0.00^{Y}	1.14±0.07^{A,Y}	0.09±0.00^{b,B,Y}
	W1	4.25±0.32^{a,A,Y}	0.38±0.04^{a,B,Y}	0.37±0.05^{b,B,Y}	957.60±3.57^{a,B,Y}	0.18±0.00^{b,B,Y}	0.24±0.00^{Y}	0.15±0.00	0.06±0.00	0.29±0.00^{Y}	0.91±0.03^{b,C,Y}	0.09±0.00^{b,B,Y}
	W2	3.03±0.21^{b,B,Y}	0.34±0.06^{b,B,Y}	0.41±0.07^{b,B,Y}	1166.19±113.77^{a,A}	0.23±0.00^{a,B,Y}	0.21±0.00^{Y}	0.12±0.00	0.07±0.00	0.30±0.00^{Y}	0.94±0.02^{c,C,Y}	0.08±0.00^{b,B,Y}
	W3	4.97±0.58^{A}	0.38±0.01^{a,B,Y}	0.58±0.09^{a,A,Y}	856.56±89.33^{b,C}	0.23±0.00^{b,B,Y}	0.29±0.00^{Y}	0.12±0.00	0.07±0.00	0.31±0.00^{Y}	1.03±0.05^{c,B,Y}	0.12±0.01^{b,A,Y}
						30 days						
	C	4.69±0.22^{b,B,X}	0.50±0.04^{a,A,X}	0.18±0.04^{B,Y}	1174.73±14.96^{b,A,X}	0.27±0.00^{a,Y}	0.31±0.00^{Y}	0.14±0.00	0.07±0.00	0.38±0.00^{Y}	1.17±0.06^{A,Y}	0.10±0.04^{b,B,Y}
	W1	2.63±0.97^{b,C,Y}	0.22±0.03^{b,C,Y}	0.60±0.12^{a,A,Y}	609.43±20.48^{c,B,Y}	0.27±0.00^{a,Y}	0.29±0.00^{Y}	0.11±0.00	0.06±0.00	0.29±0.00^{Y}	1.02±0.08^{a,B,Y}	0.17±0.00^{a,A,Y}
	W2	3.74±0.45^{b,B,Y}	0.39±0.07^{b,B,Y}	0.55±0.09^{a,A,Y}	583.24±85.99^{c,B,Y}	0.26±0.00^{a,Y}	0.27±0.00^{Y}	0.13±0.00	0.08±0.00	0.31±0.00^{Y}	1.06±0.00^{a,B,Y}	0.18±0.05^{a,A,Y}
	W3	6.04±0.29^{A,Y}	0.38±0.03^{b,B,Y}	0.48±0.11^{b,A,Y}	765.43±25.21^{c,B,Y}	0.27±0.00^{a,Y}	0.37±0.00^{Y}	0.13±0.00	0.08±0.00	0.33±0.00^{Y}	1.19±0.01^{a,A,Y}	0.16±0.00^{a,A}
Grilled samples						**0 days**						
	C	6.66±0.50^{a,A,X}	1.21±0.14^{a,A,X}	0.71±0.22^{a,A,X}	856.90±18.90^{c,C,X}	1.41±0.03^{a,B,X}	1.53±0.00^{a,B,X}	0.19±0.00	0.14±0.00	1.38±0.06^{a,B,X}	4.99±0.00^{a,B,X}	0.56±0.07^{a,A,X}
	W1	3.85±0.35^{b,C,Y}	0.45±0.05^{C,X}	0.56±0.13^{b,B,X}	1449.95±112.31^{a,A,X}	0.36±0.00^{c,D,X}	0.39±0.00^{c,D,X}	0.23±0.00	0.11±0.00	0.51±0.00^{b,D,X}	1.61±0.07^{c,D,X}	0.11±0.00^{b,B,Y}
	W2	5.91±0.02^{a,B,X}	0.66±0.02^{a,B,X}	0.55±0.09^{c,B,X}	1173.80±114.64^{a,B,X}	1.65±0.00^{a,A,X}	1.99±0.00^{a,A,X}	0.26±0.00	0.11±0.00	1.50±0.00^{a,B,X}	6.34±0.03^{a,A,X}	0.56±0.08^{a,A,X}
	W3	4.72±0.78^{b,C,X}	0.58±0.08^{b,B,X}	0.84±0.18^{c,A,X}	1551.22±104.43^{a,A,X}	0.58±0.00^{b,C,X}	0.70±0.00^{b,C,X}	0.27±0.00	0.26±0.00	0.72±0.00^{b,C,X}	2.52±0.01^{b,C,X}	0.16±0.04^{b,B,X}
						15 days						
	C	4.39±0.65^{b,X}	0.53±0.15^{b,B,X}	0.60±0.15^{b,B,X}	1119.90±45.33^{a,A}	0.92±0.01^{b,A,X}	1.05±0.04^{b,A,X}	0.20±0.00	0.10±0.00	1.35±0.05^{a,A,X}	3.62±0.04^{b,A,X}	0.33±0.05^{b,A,X}
	W1	4.86±0.24^{a,X}	0.50±0.03^{B,X}	0.59±0.22^{b,B,X}	992.45±102.13^{b,B}	0.60±0.00^{b,B,X}	0.64±0.00^{b,C,X}	0.17±0.00	0.09±0.00	0.77±0.00^{a,C,X}	2.27±0.04^{b,B,X}	0.25±0.05^{a,B,X}
	W2	4.06±0.42^{b,X}	0.50±0.08^{b,B,X}	0.93±0.11^{b,A,X}	1010.73±68.00^{a,A}	0.57±0.00^{b,B,X}	0.61±0.00^{b,C,X}	0.19±0.00	0.28±0.00	0.71±0.00^{b,C,X}	2.36±0.07^{b,B,X}	0.23±0.06^{b,B,X}
	W3	4.87±0.54^{a}	0.76±0.01^{a,A,X}	1.16±0.17^{b,A,X}	870.44±59.02^{b,B}	0.62±0.00^{a,B,X}	0.74±0.00^{a,B,X}	0.17±0.00	0.09±0.00	0.94±0.00^{a,B,X}	2.57±0.09^{b,B,X}	0.30±0.00^{a,A,X}
						30 days						
	C	3.27±0.38^{c,B,Y}	0.34±0.06^{c,C,Y}	0.42±0.40^{c,C,X}	913.65±119.26^{b,B,Y}	0.42±0.00^{c,B,X}	0.47±0.00^{c,C,X}	0.16±0.00	0.09±0.00	0.50±0.00^{b,C,X}	1.65±0.06^{c,D,X}	0.18±0.00^{c,B,X}
	W1	4.64±0.26^{a,A,X}	0.49±0.04^{B,X}	1.13±0.09^{a,B,X}	967.29±40.05^{b,B,X}	0.56±0.00^{a,A,X}	0.70±0.00^{a,A,X}	0.22±0.00	0.16±0.00	0.76±0.00^{a,A,X}	2.40±0.03^{a,C,X}	0.25±0.04^{a,A,X}
	W2	4.29±0.09^{b,A,X}	0.42±0.02^{b,B,X}	1.54±0.24^{a,A,X}	838.03±15.77^{b,B,X}	0.35±0.00^{c,C,X}	0.43±0.00^{c,C,X}	0.34±0.00	0.24±0.00	0.52±0.00^{c,C,X}	1.88±0.00^{c,B,X}	0.22±0.00^{b,A,X}
	W3	4.32±0.08^{b,A,X}	0.51±0.04^{b,B,X}	1.26±0.16^{a,B,X}	1464.03±147.00^{a,A,X}	0.43±0.00^{c,B,X}	0.63±0.00^{c,B,X}	0.70±0.00	0.23±0.00	0.65±0.00^{c,B,X}	2.64±0.01^{a,A,X}	0.18±0.00^{b,B}
	Factor						**F value**					
	Form	*	***	***	*	*	*NS*	***	*	*	***	**
	St	*NS*	***	***	***	*	*	***	*NS*	**	***	**
	Gr	***	***	***	***	***	***	***	***	***	***	***

Form*St	*	***	***	***	**	*	***	*	*	***	***
Form*Gr	***	***	**	***	*	*NS*	***	*	*NS*	***	***
St*Gr	**	***	**	***	***	**	***	*NS*	*	***	***
Form*St*Gr	***	***	**	**	**	*	***	*	*	***	***

Results as reported as means± s.d of 3 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) between the same sample during the shelf-life, A-D indicate significant differences (Tukey's test; $p \leq 0.05$) between treatments, X-Y indicate significant differences (Tukey's test; $p \leq 0.05$) between steam cooked and grilled samples. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 5α,6α-EC, 5α,6α-Epoxy Cholesterol; 5β,6β-EC, 5β,6β-Epoxy Cholesterol; 7α-HC, 7α-Hydroxy Cholesterol; 7β-HC, 7β-Hydroxy Cholesterol; 7-KC, 7-Keto Cholesterol COR, cholesterol oxidation ratio; COPs, cholesterol oxidation products; Form, formulation; Gr, grilling; St, storage; TBARs, thiobarbituric acid reactive substances.

In general, the steam-cooked control sample showed an increase in both dienes and trienes during storage, while these parameters remained almost constant in steam-cooked PE samples. Compared grilled and steam cooked samples, values of specific extinction are slightly higher in grilled ones. This is probably due to the pro-oxidant action of grilling, which supplies energy to the system favoring degradation phenomenon (Balzan et al., 2017). For steam cooked würstels, C (150 mg/kg of nitrites) and sample W2 (150 mg/kg of nitrites + 200 mg/kg of PE) show a high oxidative stability, explained by the presence of sodium nitrite in their formulation creates a synergistic effect with PE to limit oxidation. Otherwise, W3 sample exhibits high specific extinction values at 232 nm. This condition is explained by the total absence of sodium nitrite, entirely replaced by PE extract (200 mg/kg). Delgado et al. (2016) underline how polyphenols can be a source of α-dicarbonyl compounds capable, during the Strecker degradation (a fundamental phase of the Maillard reaction), to react with amino acids that lead to the formation of aromatic compounds including pyrazines, pyridines and Strecker aldehydes. In fact, the temperatures to which the samples are exposed both during the production process (70 °C at the core) and during grilling (200 °C) can cause a loss of phenols, canceling their antioxidant action.

In case of grilled samples, the specific extinction at the 232 nm tends to decrease over the shelf-life. High temperatures to which these samples were exposed which certainly led to an evolution of the oxidative phenomenon towards more advanced stages. However, it is possible to hypothesize an explanation for the elevated levels recorded by the control sample and by the W2 sample at time 0. In this case, the presence of sodium nitrite arrested the lipid oxidation during the early stages of conservation resulting in a higher concentration of conjugated dienes and trienes rather than other reactive species which would indicate a more advanced oxidative phase. At the same time, the W1 and W3 samples show a slightly different trend. In fact, for these products the specific extinction tends to increase between day 0 and 14, and then decreases slightly until day 28. This condition would find a valid explanation in the role of phenols which, net of their degradation during storage and firing, guarantee good oxidative stability in the medium to long term. The difficult interpretation of these data must, however, be contextualized to a further characteristic instance of these samples, inherent in the abundant use of beef meat in the formulation. In fact, ruminant meat is more pigmented and richer in unsaturated fatty acids (**Table 6.3**) and makes this type of meat more susceptible to oxidation. The concentration of these fatty acids, however, largely depends on the animal's diet (Baggio & Bragagnolo, 2006). However, the level of unsaturated fatty acids such as linoleic acid is always lower than that of vegetable oils and therefore leads to a lower formation of

conjugated dienes. Therefore, considering the emulsion as a complex system in which numerous factors interact and considering the different potential origins of the meats used, it is possible to assume a certain variability between the samples.

The specific extinction values at 268 nm, associated with the presence of conjugated trienes, confirm what has been seen previously, underlining the high oxidative stability of these products in particular C and W2 sample. Otherwise, for samples W1 and W3 there is a slightly different trend with values that tend to increase from day 0 to 15 and then decrease again until day 30. Therefore, it is possible to hypothesize how the phenolic extract can limit lipid oxidation in the first two weeks of storage, stopping it at the initial stages, while as the storage time expands, the trienes tend more to form new reactive species and their concentration to decrease. For grilled samples, C and W2 show higher specific extinction values at time 0, potentially indicating that nitrite is able to limit oxidation in the early stages. However, in both cases, the prolongation of storage determines the evolution of these molecules towards secondary oxidation compounds. On the contrary, the W3 and W1 samples show low specific extinction values at time 0 which tend to increase during the shelf-life up to day 14, and then decrease again up to day 28. This trend would confirm that phenols are able to guarantee a certain stability. This effect would be more pronounced in the W1 sample where these molecules act in synergy with the sodium nitrite present in the formulation.

Regarding secondary lipid oxidation, TBARs ranged from 0.15-0.60 and 0.55-1.54 mg MDA/kg in steam cooked and grilled würstels, respectively. In general, grilled samples had TBARs values that were about 2 times higher than those of steam-cooked würstels. Product formulation, storage time and grilling significantly influenced this oxidative parameter and the two- three factors interaction was significant as well.

Surprisingly, TBARs in both steam-cooked and grilled control samples were lower than those of the other samples during the shelf-life. This behavior might be partly due to the thermolysis and hydrolysis of phenols during the cooking treatments; moreover, during grilling at 220°C, phenols might have been involved in the Strecker reaction, preventing them from exerting their antioxidant role (Delgado et al., 2016). The results obtained therefore demonstrate how the PE, even with a low concentration of nitrites (35 mg kg in sample W1) is able to limit oxidation, as confirmed by the studies carried out by Balzan et al. (2017), which found TBARs values lower than 1 mg MDA/kg of meat in cooked pork-based würstels formulated with a PE deriving from OMWW. Indeed, it has been shown that TBAR values above 1 mg MDA/kg of meat would favor the development of rancidity in cooked pork meat (Gray & Pearson, 1987).

Cholesterol is an important constituent of cell membranes and, as FA, it is also susceptible to oxidation. The total content of cholesterol (**Table 6.5**) ranged from 770.40 to 1241.16 mg/kg in steam cooked würstels and from 838.06 to 1515.22 mg/kg in grilled ones. These data agree with those reported by Baggio & Bragagnolo (2006) for steam cooked and grilled frankfurter type würstels. Cholesterol content was significantly affected by formulation, storage time and grilling. All the two three factors' interactions, in fact, were statistically significant. This trend could be justified by the fact that, for the formulation of these products, pork and bovine meat was used, in addition grilling significantly increased the cholesterol content of the würstels, which could be ascribed to the water loss and consequent dehydration of the product during grilling (Baggio & Bragagnolo, 2006).

Like TBARs, product formulation, storage time and grilling significantly influenced total COPs content and the two- three factors interaction was significant as well, but without displaying a clear trend. Total COPs ranged from 0.91-1.19 mg/kg meat and from 1.61-6.34 mg/kg meat in steam cooked and grilled würstels, respectively. As expected, grilling increased the content of total COPs in all samples. However, no PE concentration-dependent effect was found.

Regarding single COPs profile and amount detected in the present study is similar to the one reported by Balzan et al. (2017) for raw and cooked pork würstels, where oxidation products deriving from monomolecular reaction pathway (i.e. 7-derivatives) were more abundant than those generated by bimolecular ones (i.e. epoxy and triol derivatives); in particular triol, whose formation is known to be favored by water in acid conditions, was not detected. The most abundant COPs was 7-KC, followed by 7β-HC, 7α-HC, 5β,6β-EC and 5α,6α-EC. The cholesterol oxidation ratio (COR) ranged from 0.08 % to 0.18% and from 0.11% to 0.56% in steam cooked and grilled samples (**Table 5**), respectively. Similarly to total COPs, product formulation, storage time, grilling and their interactions significantly influenced this ratio. It must be pointed out that COR% of most grilled samples was 2.2 times higher than their corresponding steam cooked samples

As emerged from the study, the use of phenol extracts from agricultural by-products in synergy with nitrites allows to limit lipid oxidation and the formation of potentially harmful compounds such as cholesterol oxidation products. Successful results were obtained by using a phenolic extract rich in 3,4-DHPEA, *p*-HPEA, verbascoside and 3,4-DHPEA-EDA, in raw and cooked pork würstels (Balzan et al., 2017).

6.3.1.5 PCA of chemical data

To better understand which parameters were the most relevant for assessing the effects of phenolic enrichment, cooking treatment and storage on würstels, the chemical composition and phenolic composition data were subjected to principal component analysis (PCA) for steam cooked and grilled würstels (**Figure 6.1**).

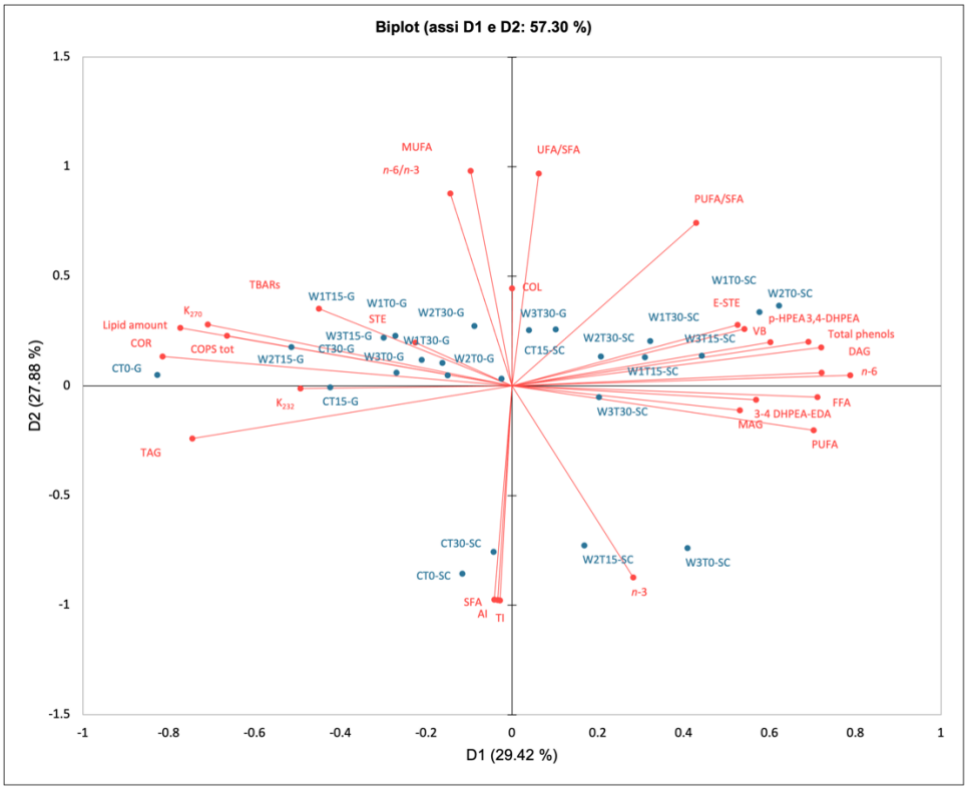

Figure 6.1. Biplot of steam cooked and grilled würstels. 3,4-DHPEA, Hydroxytyrosol; 3,4-DHPEA-EDA, decarboxymethyl oleuropein aglycone; AI, Atherogenic Index; COPs, cholesterol oxidation products; COR, cholesterol oxidation ratio; DAG, diacylglycerols; E-STE, esterified sterols; FFA, free fatty acids; MAG, monoacylglycerols; MUFA, monounsaturated fatty acids; *p*-HPEA, tyrosol; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; STE, sterols; TAG, triacylglycerols; TBARs, thiobarbituric acid reactive substances; TI, thrombogenic index; UFA, unsaturated fatty acids; VB, verbascoside.

The first two components explained 57.30% of the total variance (29.42% for PC1 and 27.88% for PC2). All oxidative parameters (COPs, TBARs, COR, K_{232} and K_{270}) are in the quadrant 1, while total phenols and the single phenolic compounds (3,4-DHPEA, *p*-HPEA and VB), except 3,4-DHPEA-EDA, were in quadrant 2.

Grilled samples are well separated from steam cooked ones and are located in different quadrants. Control T0 grilled samples are more correlated to COPs tot and COR, despite being in the same quadrant as CT0, samples W1, W2 and W3 seem to be more related to the STE variable.

Control steam cooked samples are more correlated with SFA ratio, while W1, W2 and W3 steam cooked samples are more related to total and single phenols except 3,4-DHPEA-EDA. This could be

due to the susceptibility of phenols to high temperatures, which led to a greater loss of phenols in W1, W2 and W3 samples compared to the corresponding steam cooked samples over the shelf-life (**Table 1**). In all grilled and in the T15 and T30 PE steam cooked samples; in fact, 3,4-DHPEA-EDA completely disappeared.

This conformation of the PCA indicates that between the different samples there are no differences, neither at the compositional level, nor as regards the oxidative stability. This is mainly due to the correct execution of the emulsion which allowed to formulate a very stable product. From other side manufacturing operations and grilling caused a significant loss on the total phenols (around -60%); the storage time caused a progressive decrease of phenols reaching a total loss, considering T30 vs T0, of about 30%.

6.3.2 Sensory analysis

6.3.2.1 Discriminant test

The triangle test was carried out to verify the existence of significant differences between the three phenol-enriched steam cooked würstels (W1, W2 and W3) and the control sample (C) and between each type of sample stored at different times (0, 15 and 30 days), to provide useful information to evaluate the shelf-life of the new clean label formulated products. The test was organized in several sessions in which all possible combinations of the samples to be compared were presented to the involved subjects (**Table 6.6**). The sessions 1-18 aimed to compare all samples (C, W1, W2, W3) at time 0, 15 and 30 and the results, obtained from the 34 subjects, showed significant differences except for the CT0-W2T0 comparison. In particular, the C sample was recognized as different from W1 because it was characterized by a more intense cooked beef flavor and different color, pinker than PE steam cooked würstels samples (sessions 1, 7, 13), while the C sample resulted like W2, except for flavor, W2 in fact result more aromatic than C (sessions 2, 8, 14). The W3 sample was discriminated from C for the color, in fact W3 was characterized by grey color, due to the total absence of nitrites in formulation (sessions 3, 9, 15). W1 and W2 samples were different for color, W2 is pinker than W1, due to the major concentration of nitrites in W2 formulation (150 mg/kg vs 35 mg/kg) (sessions 4, 10, 16). Even in the case of the comparison between W1 and W3 samples, the color is the main attribute that allows to discriminate between the two samples, W3, in fact, was characterized by grey color, due to the total absence of nitrites in formulation, and a lover intensity of tenderness and juiciness (sessions 5, 11, 17). Finally, W2 and W3 samples they stand out not only for the color, W3 grayer than W2, but also for the more pleasant taste of W2 and for the less homogeneous consistency of W3 (sessions 6, 12, 18).

Table 6.6. Sessions, samples, number of subjects involved, number of correct given answers and significance level of the triangle test conducted on grilled steam cooked würstels (C, dough + nitrites (150 mg/kg) and maltodextrin (0.35 g/100 g); W1, dough + PE equivalent to 200 mg/kg of phenols and nitrites (35 mg/kg); W2, dough + PE equivalent to 200 mg/kg of phenols and 150 mg/kg of nitrites; W3, dough + PE equivalent to 200 mg/kg of phenols), at all the storage times (T0, T15 and T30).

Session n.	Compared samples	Judges n.	Correct answers	Significance
1	CT0 vs. W1T0	34	21	0.05-0.01
2	CT0 vs. W2T0	34	13	*n.s*
3	CT0 vs. W3T0	34	29	0.05-0.01
4	W1T0 vs. W2T0	34	21	0.05-0.01
5	W1T0 vs. W3T0	34	28	0.05-0.01
6	W2T0 vs. W3T0	34	29	0.05-0.01
7	CT15 vs. W1T15	34	28	0.05-0.01
8	CT15 vs. W2T15	34	11	*n.s*
9	CT15 vs. W3T15	34	27	0.05-0.01
10	W1T15 vs. W2T15	34	21	0.05-0.01
11	W1T15 vs. W3T15	34	24	0.05-0.01
12	W2T15 vs. W3T15	34	29	0.05-0.01
13	CT30 vs. W1T30	34	29	0.05-0.01
14	CT30 vs. W2T30	34	11	*n.s*
15	CT30 vs. W3T30	34	31	0.05-0.01
16	W1T30 vs. W2T30	34	19	0.05-0.01
17	W1T30 vs. W3T30	34	27	0.05-0.01
18	W2T30 vs. W3T30	34	32	0.05-0.01
19	CT0 vs. CT15	33	14	*n.s*
20	CT0 vs. CT30	33	15	*n.s*
21	CT15 vs. CT30	33	12	*n.s*
22	W1T0 vs. W1T15	33	21	0.05-0.01
23	W1T0 vs. W1T30	33	11	*n.s*
24	W1T15 vs. W1T30	33	15	*n.s*
25	W2T0 vs. W2T15	33	13	*n.s*
26	W2T0 vs. W2T30	33	20	0.05-0.01
27	W2T15 vs. W2T30	33	8	*n.s*
28	W3T0 vs. W3T15	33	10	*n.s*
29	W3T0 vs. W3T30	33	11	*n.s*
30	W3T15 vs. W3T30	33	13	*n.s*

The significance is expressed in terms of α-risk level. *n.s.* indicates no significant perceptible difference between samples was found.

In sessions 19-21, a comparison between the control samples at the 3 storage times, was carried out. No significant differences were found between samples during shelf life, this indicates that the tasters were unable to distinguish between the different samples. Further sessions were conducted for the evaluation of the phenol-enriched samples at the 3 storage times. Interviewees (n=33) found statistically significant differences between W1T0 and W1T15 (session 22) due to color and taste, in fact at T15 samples presented a less intense color and more intense taste. No significant differences in W1 at 15 and 30 days of storage, were perceived (sessions 23 and 24).

Concerning W2 sample, significant differences were detected when comparing the sample just produced (T0) with those at 30 days of storage (session 26), while no differences were perceived when comparing W2T0 and W2T15 (session 25) and W2T15 and W2T30 (sessions 26). In the significant session, samples were differentiated by taste more intense at T30. For W3 samples, no significant differences were found between samples during shelf life (sessions 28-30).

6.4. CONCLUSIONS

This study demonstrated the efficacy of a powder formulation of a phenolic extract from olive vegetation water at improving the overall oxidative stability and sensory quality of steam cooked and grilled pork and beef würstels, which had been previously subjected to cold storage for 30 days. Manufacturing operations and grilling caused a significant loss on the total phenols (around - 60%); the storage time caused a progressive decrease of phenols reaching a total loss, considering T30 vs T0, of about 30%. In general, the steam-cooked control sample showed an increase in both dienes and trienes during storage, while these parameters remained almost constant in ungrilled PE samples. After grilling, all samples exhibited the classical bell-shape behavior of primary oxidation products. Regarding secondary lipid oxidation, grilled samples had TBARs values that were about 2 times higher than those of steam-cooked würstels, confirming the pro-oxidant effect of grilling. However, surprisingly, TBARs in both steam-cooked and grilled control würstels were lower than those of the phenol-enriched samples during the shelf-life. This behavior might be partly due to the thermolysis and hydrolysis of phenols during the cooking treatments; moreover, during grilling at 220°C, phenols might have been involved in the Strecker reaction, preventing them from exerting their antioxidant role. Regarding COPs and COR%, no significative differences were found between PE and control steam-cooked würstel samples. Grilled samples showed higher values than steam cooked ones, with 3.5- and 2.27-fold higher values for control and PE samples respectively, confirming the grilling pro-oxidant effect. However, no PE concentration-dependent effect was found. It must be pointed out that COR% of most grilled samples was 2.2 times higher than their corresponding steam cooked samples. Discriminant sensory analysis confirmed the excellent stability of the products along the shelf-life study and no taste anomalies were detected for any product. The key attribute for distinguishing among samples was color, even though a certain difficulty was found for differentiating between samples C and W2.

In conclusion, this study confirms that OMWW extracts rich in phenols could be an alternative for the reduction of nitrites in cooked meat preparations, which would promote the formulation of

healthier clean label products and improve the sustainability of the olive oil industry with a circular economy approach, by further valorizing this olive by-product.

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Chapter 7. Development and characterization of a new functional olive-based spreadable food

ABSTRACT

During the production of olive oil, large quantities of by-products are produced, including pomace, which contains high amounts of bioactive compounds. Considering the environmental impact of the olive oil production chain and the interest in more sustainable production systems, it would be appropriate to promote the enhancement of these by-products in various fields, including food. In this regard, the pomace is being studied for the creation and formulation of new foods. However, it needs to undergo a de-bittering process before it can be used, in order to reduce the strong bitter taste attributable to its high polyphenol content. The aim of this work was to characterize an olive paste obtained from the fermentation of pomace and added with probiotics from an instrumental and sensorial point of view. This innovative product was analysed from the compositional point of view, from the oxidative and colour stability. Finally, to ascertain the palatability of the product, a descriptive sensory test (Flash Profile) was performed with untrained consumers.

KEYWORDS

Olive paste, Olive pomace, by-products of oil processing, probiotics, phenolic compounds, oxidative stability, hydrolysis, shelf-life, flash Profile

7.1. INTRODUCTION

The oil industry is considered a very important sector in the Mediterranean Agro-food chain since olive oil is a fundamental part of the various local diets as it possesses peculiar organoleptic and health characteristics that have caused an increase in consumption and production. Olive oil has excellent nutritional properties, and its consumption is increasing globally, pushing countries such as Argentina, Australia, the United States to emerge as producers by promoting olive cultivation (Maffia et al., 2020). As regards the production of olive oil (OO), according to the latest data released by the International Olive Council (COI) for the 2020/2021 campaign, it is estimated a world production of olive oil of 3.3 million tons (3% more than the last year). The main world leader is Spain (1.6 million tons) followed by Greece (265 thousand tons), Italy (255 thousand tons, 30% less than the previous year's campaign) and Tunisia (120 thousand tons) (ISMEA, 2020). The increasing production can be explained by the success of the Mediterranean diet, which is associated with a lower incidence of atherosclerosis, cancers and cardiovascular and neurodegenerative diseases

(Banias et al., 2017). With the world population increasing, the demand for greater food production is also growing and this constitutes a challenge for agriculture, aiming for greater sustainability of the agri-food supply chains. To be sustainable, the OO supply chain requires a process of continuous improvement, in which the most impacting techniques, those harmful to the environment, are progressively replaced with more sustainable ones (Maffia et al., 2020). The production of olive, however, involves the consumption of large quantities of resources and the generation of emissions into the air that significantly downgrade the natural environment. In addition, OO production also generates various waste (wood, branches, leaves) and by-products (olive pomace, vegetation water, olive stones) in large quantities (Donner & Radić, 2021) that are included in the category of agricultural livestock waste (ALW) (Abbattista et al., 2021). Among the three different extraction processes (traditional hydraulic pressure, two and three phase centrifugation processes) there is a truly remarkable amount of production of by-products: in the traditional process, from a ton of olives used there is a production of 200 kg of OO with a waste of 200-400 kg of pomace and 400-600 kg of vegetation water. In the three-phase production process, on the other hand, for the same quantity of OO produced, 500-600 kg of pomace and 1000-1200 kg of vegetable water are obtained (Donner & Radić, 2021). These by-products, in addition to representing an economic problem for producers, pose serious environmental concerns; their partial reuse, like that of all agronomic production residues, represents one of the objectives to be pursued (Abbattista et al., 2021). The enhancement of the by-products of the olive industry must be a general commitment to the environment but not only, as the reuse of waste constitutes a resource in many fields. In fact, if until now the waste and by-products of olives have been more than anything else converted into products with low added value (bioenergy or fertilizers), there are several initiatives that already successfully market products with high added value (e.g., extracts obtained from waste for their use in the cosmetic field, to produce handicraft products, etc.) (Donner & Radić, 2021). The virgin pomace constitutes the solid by-product of olive processing, formed by the fibrous part of the fruit, from the stone, of approx. 5% of residual oil and a quantity of water that can vary, according to the type of centrifugation used. The pomace, in fact, can appear from humid to semi-solid; when using two-phase centrifugation systems, the water content is between 50% and 70%, while the water content is reduced with three-phase centrifugation systems (35-40%) and with those traditional discontinuous pressure vessels (with 20-25% water) (Abbattista et al., 2021). Pomace represents 35-40% of the total weight of olive processing in the mill and is considered as the main residue of the olive oil extraction production process (Otero et al., 2021). The approximate composition of the

pomace can be summarized as follows: water (60-70%), lignin (13-15%), cellulose and hemicellulose (18-20%), oil retained in the pulp (2.5-3%). While the main organic compounds are sugars (3%), volatile fatty acids (C2-C7) (1%), polyalcohols (0.2%), proteins (1.5%), polyphenols (0.2%), pigments (0.5%), vitamin E (α-tocopherol, β-tocopherol, α-tocotrienol and γ-tocopherol) present however mainly in the form of α-tocopherol (> 2.6 mg/100 g), in the lipid fraction is oleic acid content (approx. 75%), followed by palmitic, linoleic and stearic acids (Abbattista et al., 2021). Finally, the phenolic content of virgin pomace depends on the oil extraction system used; the virgin pomace obtained from three-phase extraction plants have approx. 48% of phenolic compounds, while in the pomace obtained from the two-phase system there is a higher content of phenolic compounds since the fraction of vegetation water is also present. The most present phenolic compounds are hydroxyphenylethanol (or tyrosol, *p*-HPEA), dihydroxyphenylethanol (or hydroxytyrosol, 3,4-DHPEA) the aglyconic forms of oleuropein, demethyloleuropein and ligostroside such as 3,4-DHPEA-EA (or 3,4-DHPEA-elenolic acid mono-aldehyde or oleuropein-aglycone mono-aldehyde), 3,4-DHPEA-EDA (or 3,4-DHPEA-elenolic acid di-aldehyde, or oleuropein-aglycone of -aldehyde), *p*-HPEA-EDA (*p*-HPEA-elenolic acid di-aldehyde or ligstroside-aglycone di-aldehyde), in addition to verbascoside (Proietti et al., 2012). To improve the characteristics of the olive pomace to be treated and added to the product, various chemical and biological methods have been described (Medouni-Haroune et al., 2018). In general pomace, before being made edible must undergo pre-treatments that modify some sensory characteristics and decrease or eliminate the bitterness contained in it. The main responsible for the typical bitter taste of olives is oleuropein which from a chemical point of view is a glycoside formed by an ester of elenolic acid and 3,4-DHPEA; this compound can undergo hydrolysis in alkaline conditions (by chemical means or chemical de-bittering) or by enzymes such as β-glucosidase (biological method) (Lanza & Poiana, 2012). Biological method consists in a fermentation controlled by inoculating selected microorganisms, capable of growing in the presence of high levels of phenolic bioactive compounds (Durante et al., 2019). In general, for olives and by-products, a lactic fermentation is carried out using homo and heterofermentative lactic ferments and/or yeasts. The lactobacilli mainly considered in starter selection studies are *L. plantarum* and *L. pentosus* (Corsetti et al., 2012). During this process, oleuropein is hydrolyzed following the combined action of acidity, derived from lactic fermentation, and the hydrolytic activity of the enzyme β-glucosidase produced especially by bacteria of the *L. plantarum* species (Lanza & Poiana, 2012). This enzyme breaks down oleuropein into glucose and aglycone; the first is used by the bacterium as a source of sugar for its activity, the second however has an inhibiting effect on the

lactic bacteria themselves. The aglycone is then attacked by the esterase enzyme which splits it into elenolic acid and 3,4-DHPEA (Lanza & Poiana, 2012). With this method it is not possible to totally decrease the level of "bitterness" as in the chemical system, consequently that slight bitter taste remains which, in the case of olives, is always appreciated by the consumer (Lanza & Poiana, 2012). The purpose of this work, therefore, was to evaluate the composition and stability of the lipid fraction of an innovative product obtained from the pomace deriving from the processing of olives to produce virgin olive oil. Specifically, it was a green olive pomace paste suitably fermented by two strains of *Lactiplantibacillus* (Lpb.) *pentosus* with a de-bittering function and, subsequently, pasteurized. Furthermore, following the pasteurization, two different formulations of probiotics (*Lpb. plantarum*) of human origin and isolated from fermented foods were added. First, the amount of fat extracted from the olive paste was determined. Subsequently, this innovative product was characterized by the compositional point of view, by the oxidative and color stability. Finally, to ascertain the palatability of the product, a descriptive sensory test, the Flash Profile, was performed with untrained consumers.

7.2. MATERIAL AND METHODS

7.2.1 Preparation of fermented olive paste

7.2.1.1 Bacterial cultures

A mixture consisting of two strains of *Lactiplantibacillus* (*Lpb*.) *pentosus* was used as starter cultures, to start fermentation, with a de-bittering function (Tofalo et al., 2014; patent no. 0001428559). After deamarization, two different formulations of probiotics were added: P1, probiotic strain of human origin *Lpb. plantarum* IMC513 (Synbiotec srl, Camerino, Italy); P2, a mixture of two *Lpb plantarum* strain, isolated from fermented foods. All the strains used belong to the crop collection of the Faculty of Biosciences and Agro-Food Technologies of the University of Teramo.

7.2.1.2 Preparation of the fermentation

The fermentations were carried out in the laboratories of the Faculty of Biosciences and Agro-Food Technologies of the University of Teramo. For the chemical analysis, the fermentation was set up, on a laboratory scale, by aliquoting the frozen olive paste, coming from Peranzana *cultivar* olives, and then left to thaw overnight at room temperature in jars of about 800 g. The jars were added with 1% salt, with glucose (20 g/L), yeast extract (10 g/L), and then inoculated with *Lpb*. *pentosus*, setting up two parallel fermentations. All the inoculations were carried out with the same

concentration equal to 10^7 CFU/mL, incubated at 30°C for 3 days. Once the incubation time of 3 days was reached, the samples were inoculated with the two different probiotic formulations (P1 and P2); for the correctness of the comparison between the different products, a non-inoculated control was provided. All the samples were monitored with chemical-physical (pH, acidity and A_w) and microbiological analyzes at different times (0, 3, 5 and 10 days). The times 5 days and 10 days refer to 5 and 10 days from the inoculation of the probiotics. For the present thesis project, aliquots of the samples fermented at T0, 5 and 10 days were taken and approx. 100 g of green olive paste frozen upon removal, and subsequently stored in the freezer.

For the sensory analysis, fermentation was set up with de-bittering starter microorganisms as described above, but without the subsequent inoculation of the probiotics P1 and P2. After 3 days of incubation, the fermented matrix was suitably aliquoted in two sterile 250 g jars, and subsequently pasteurized at 90°C for 15 min. All the samples were monitored with chemical-physical (pH, acidity and A_w) and microbiological analyzes at two different times (T0, and after 3 days). For the sensory analysis, two different fermentations were carried out (one week apart) with the same conditions. The fresh samples (aliquoted after fermentation) were sent in two different shipments and sensorially evaluated in two separate sessions.

7.2.2 Phenols analysis

Five grams of fermented olive cream were mixed with 100 mL of methanol:water (80:20, v/v) containing 20 mg/L of butylated hydroxytoluene (BHT) + 0.2% trichloroacetic acid 1 M. The operations of homogenization, recovery, concentration until a final volume of 40 mL of extract and purification by solid-phase extraction (SPE) from 10 mL of this aqueous extract were carried out as previously described Miraglia et al. (2020). The purified extract was then subjected to HPLC-DAD analysis using the same equipment and conditions of the reported by Selvaggini et al. (2014). Each measurement was done in duplicate.

7.2.3 Chemical analysis

7.2.3.1 Lipid extraction

Lipids were extracted according to Boselli et al. (2001). The extraction was performed on 25 g of olive cream paste, which were added with 5α-cholestane (internal standard for the quantification

of main lipid classes) (Sigma Chemical, St. Louis, USA). The fat content was determined gravimetrically and expressed as percentage. Three independent replicates were run per sample.

7.2.3.2 Determination of main lipid classes

The qualitative-quantitative profile of the main lipid classes (free fatty acids, FFA; monoacylglycerols, MAG; free sterols, STE; diacylglycerols, DAG; esterified sterols, E-STE; triacylglycerols, TAG) was determined by gas chromatography-flame ionization detection (GC-FID), as reported by Gallina Toschi et al. (2014) and Luise et al. (2018). An aliquot of 20 mg of the lipid extract dissolved in 1 mL of *n*-hexane, was used for this analysis. The internal standard method, with the response factor of each main lipid class (estimated with commercial standards), was used to determine the amount of each lipid class (expressed as g/100 g of lipids). Three independent replicates were run per sample.

7.2.3.3 Determination of total FA

The composition of total fatty acids was determined on 20 mg of lipid extract by GC-FID (Cardenia et al., 2015), after previous methylation and transmethylation. FAME quantification was performed according to the internal standard method (using tridecanoic acid methyl ester) and expressed as a proportion of the identified total FAME (g/100 g). Three independent replicates were run per sample.

Based on the total FA composition, the atherogenic index (AI) and thrombogenic index (TI) were also determined (Ulbricht & Southgate, 1991).

7.2.3.4 Determination of conjugates dienes and trienes

The analysis of dienoic and trienoic conjugated fatty acid derivates was performed by following the method described in the ISO 3656:2011. The spectrophotometric analysis was performed using a Jasco dual beam spectrophotometer model V-550 UV–vis (Jasco, Tokyo, Japan). A quartz cuvette with an optical path of 10 mm was used. The determination is based on spectrophotometric analysis of 0.1 g of lipid extract diluted in 10 mL of iso-octane. The spectrophotometric investigations were performed at 232 nm for the determination of diene conjugated systems and at 268 nm for the triene conjugated systems

7.2.3.5 Determination of sterols

Sterols were extracted by cold saponification of 200 mg of lipid extract, silylated and analyzed by Fast GC/MS (Cardenia et al., 2012), using betulinol (Sigma Chemical, St. Louis, USA) as internal standard. Mass spectra were acquired in full scan mode (total ion current, TIC), while they were integrated with single ion monitoring (SIM) mode using the characteristic ions with a high abundance (Cardenia et al., 2012; Inchingolo et al., 2014); quantification was carried out by means of calibration curves built for each compound. Sterols were expressed as mg/kg of olive cream. Three independent replicates were run per sample.

7.2.4 Colorimetric analysis

The determination of the color was carried out on the samples of olive paste (C, P1, P2) at each sampling time (T0, T5, T10) without pre-treatment. The spectrophotometric analysis was performed using a Jasco dual beam spectrophotometer model V-550 UV–vis (Jasco, Tokyo, Japan). A quartz cuvette with an optical path of 10 mm was used., Numerical parameters L *, a * and b * were obtained after processing the physical information by means of the instrument software. Three independent replicates were run per sample.

7.2.5 Sensory analysis

7.2.5.1 Flash profile

A classical flash profile was performed by a group of ten subjects (7 females; mean age=30 years; 4 subjects were trained for tasting other food products, while six were students at the Department of Agricultural and Food Sciences at *Alma Mater Studiorum*-University of Bologna) according to the method described by Dairou & Sieffermann (2002). Briefly, three sessions in which five samples of olive pastes were presented simultaneously were carried out. During the first session, an explanation of the procedure was given to the assessors; then, the assessors were asked to individually generate the sensory descriptors that best described the differences among the presented samples, avoiding the use of hedonic terms. Subsequently, during the second and the third sessions, assessors were requested to rate and to rank the olive paste samples for each descriptor, by placing the sample code on a linear unstructured scale anchored at the left side (low intensity) and on the right side (high intensity). Results with identical scores were allowed for ranking. Both rating and ranking were performed in duplicate. During each session 20 g of each sample were served into a disposable glass with a disposable teaspoon. Samples were served at room temperature (between 25 and 27°C). Samples were coded using 3-digit random numbers.

Subjects rinsed their mouth with mineral water and a slice of apple between samples. All the sessions were performed in individual sensory booth in the sensory room at the Department of Agricultural and Food Sciences at *Alma Mater Studiorum*-University of Bologna (Viale Fanin 40, Bologna, Italy).

7.2.6 Statistical analysis

The software XLSTAT 7.5.2 version (Addinsoft, France) was used to elaborate chemical and sensory data. The chemical data are reported as mean values of independent replicates of each analytical determination. First, normal distribution of data was tested (p<0.05) with Shapiro-Wilk method. Chemical data were analyzed using two-way or three-way analysis of variance (ANOVA), including formulation (Form), storage time (St) and their interaction (Form*St) as factor. Tukey's honest significance test was performed at a 95% confidence level (p≤0.05), to separate means of statistically different parameters. A principal component analysis (PCA), with a Varimax rotation, was also carried out. A consensus configuration for the flash profile data was provided by Generalized Procrustes Analysis (GPA).

7.3. RESULTS AND DISCUSSION

7.3.1 Chemical analysis

7.3.1.1 Evolution of phenolic compounds

As shown in **Table 7.1**, part of the added phenols was lost during storage. In terms of total phenols, there was a loss of 24% and 47% of phenolic compounds in C samples after 5 and 10 days of storage, respectively, whereas a minor loss was detected in P1 (40 and 36%, respectively) and P2 (32 and 35%) samples, respectively. In particular, the highest variation was observed for 3,4-DHPEA-EDA, which completely disappears in P1 and P2 samples after 15 and 30 days of storage. It can be assumed that, to a certain extent, 3,4-DHPEA-EDA was subjected to hydrolysis, which generates 3,4-DHPEA, as has been found in fermented functional milk (Servili et al., 2011). As reported by Obied et al. (2008), the degradation mechanism of this oleuropein derivative includes enzymatic and non-enzymatic oxidation and hydrolysis.

The increase of 3,4-DHPEA, in presence of 3,4-DHPEA-EDA, has already been observed in different food matrices (Esposto et al. 2015; Taticchi et al., 2017; Balzan et al., 2017), the phenols evolution in P1 and P2 fermented olive paste strengthens the hypothesis of its hydrolytic origin from the degradation of oleuropein derivatives during storage (Brenes et al., 2001). Nevertheless, the

oxidative degradation of these two phenols has been appointed as the main cause of their decrease (Obied et al., 2008; Di Maio et al., 2011).

Table 7.1. Evolution of phenolic compounds of fermented olive paste cream, with (P1 and P2) and without (C) probiotic addition, samples after 0, 5 and 10 days of storage.

Samples	3,4-DHPEA	*p*-HPEA	VB	3,4-DHPEA-EDA	Total phenols
		(g/kg)			
		0 days			
C	0.72±0.02^{b}	1.65±0.07^{a}	2.08±0.13^{a}	1.76±0.19^{a}	6.22±0.40^{a}
P1	0.72±0.02^{c}	1.65±0.07^{a}	2.08±0.13^{a}	1.76±0.19^{a}	6.22±0.40^{a}
P2	0.72±0.02^{c}	1.65±0.07^{a}	2.08±0.13^{a}	1.76±0.19^{a}	6.22±0.40^{a}
		5 days			
C	0.87±0.00^{a,C}	1.62±0.05^{a,A}	1.56±0.05^{b,A}	0.64±0.01^{b,A}	4.68±0.11^{b,A}
P1	1.48±0.01^{b,B}	1.47±0.01^{b,B}	0.78±0.01^{b,B}	*nd*^{b,B}	3.73±0.03^{b,B}
P2	1.90±0.00^{a,A}	1.45±0.01^{b,B}	0.83±0.00^{b,B}	*nd*^{b,B}	4.18±0.01^{b,C}
		10 days			
C	0.20±0.01^{c,B}	1.37±0.01^{b,B}	1.33±0.05^{c,A}	0.36±0.04^{c,A}	3.26±0.12^{c,B}
P1	1.74±0.01^{a,A}	1.49±0.01^{b,A}	0.71±0.03^{b,B}	*nd*^{b,B}	3.94±0.05^{b,A}
P2	1.73±0.00^{b,A}	1.47±0.03^{b,A}	0.80±0.04^{b,B}	*nd*^{b,B}	4.00±0.08^{c,A}
Factor			F value		
Form	***	*NS*	***	***	*NS*
St	***	***	***	***	***
Form*St	***	***	***	*	*

Results as reported as means±s.d of 2 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) within the same sample during the shelf-life. A-C indicate significant differences (Tukey's test; $p \leq 0.05$) among treatments. *$p < 0.05$, *** $p < 0.001$. 3,4-DHPEA, hydroxytyrosol; Form, formulation; *NS*, not significantly; *p*-HPEA, tyrosol; St, storage; VB, verbascoside; 3,4-DHPEA-EDA, oleacein; *nd*: not determined

For hydroxytyrosol, therefore, two contemporary phenomena would be at the basis of its particular evolution over time: the first is the hydrolysis of 3,4-DHPEA-EDA after which this phenolic alcohol is released in free form, while the second one is the oxidative degradation which leads to its decrement. In the case of samples P1 and P2, in the early stages of storage, hydroxytyrosol is limitedly involved in oxidation reactions and, therefore, the resultant balance between the decrease on the one hand and the increase on the other, is an increase of the concentration of hydroxytyrosol. In the more advanced phases, the oxidative degradation prevails in P1 and P2 samples, due to the decrease in the concentration of the other more reactive phenols (such as 3,4-DHPEA-EDA).

During storage, the concentration of *p*-HPEA not significantly vary, as already found in other shelf-life studies for meat products (Balzan et al., 2017).

3.1.2 Lipid content and main lipid classes

As reported in **Table 7.2**, the lipid content of fermented olive paste cream ranged from 2.85 to 3.15%. Only formulation significantly affect the lipid content of the examinate samples. Regarding main lipid classes (**Table 7.2**), the most represented was TAG (92.16-93.58%), followed by DAG (3.48-3.73%), FFA (1.60-2.21%), STE (0.84-0.93%), E-STE (0.23-0.32%) and finally MAG (0.11-0.17%). In the examinate samples, formulation, significantly affect only E-STE content, storage significantly affect FFA, MAG and TAG content, while STE and DAG were not significantly affected nor by formulation and storage, nor by its interaction.

Table 7.2. Lipid content and main lipid classes profile of fermented olive paste cream, with (P1 and P2) and without (C) probiotic addition, samples after 0, 5 and 10 days of storage.

Samples	Lipid content (%)	FFA	MAG	STE	DAG	E-STE	TAG
				(% of total lipids)			
0 days							
C	3.21±0.04	1.60±0.07^{B}	0.17±0.04	0.89±0.02	3.48±0.29	0.28±0.03	93.58±0.33^{A}
P1	3.21±0.04	1.60±0.07^{B}	0.17±0.04	0.89±0.02	3.48±0.29	0.28±0.03	93.58±0.33^{A}
P2	3.21±0.04	1.60±0.07^{B}	0.17±0.04	0.89±0.02	3.48±0.29	0.28±0.03	93.58±0.33^{A}
5 days							
C	2.99±0.04^{ab}	2.07±0.15^{A}	0.13±0.00^{a}	0.92±0.01	3.49±0.05	0.23±0.03^{b}	93.16±0.18^{AB}
P1	3.15±0.02^{a,A}	2.12±0.18^{A}	0.11±0.00^{b,B}	0.85±0.02	3.48±0.07	0.28±0.03^{ab}	93.16±0.26
P2	2.83±0.02^{b,B}	2.21±0.29^{A}	0.12±0.01^{a}	0.89±0.11	3.73±0.35	0.32±0.02^{a,A}	92.74±0.78
10 days							
C	3.00±0.01^{a}	2.19±0.16^{A}	0.16±0.02^{a}	0.93±0.05^{a}	3.71±0.13	0.25±0.03	92.76±0.38^{B}
P1	2.88±0.02^{b,B}	2.11±0.09^{A}	0.11±0.01^{b,B}	0.84±0.02^{b}	3.60±0.06	0.25±0.03	93.08±0.17
P2	2.85±0.01^{b,B}	2.17±0.07^{A}	0.13±0.00^{ab}	0.87±0.01^{ab}	3.66±0.04	0.24±0.01^{B}	92.93±0.11
Factor				**F value**			
Form	*NS*	*NS*	*NS*	*NS*	*NS*	*	*NS*
St	***	***	***	*NS*	*NS*	*NS*	***
Form*St	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

Results as reported as means±s.d of 3 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) within the same sample during the shelf-life. A-C indicate significant differences (Tukey's test; $p \leq 0.05$) among treatments. *$p < 0.05$, ***$p < 0.001$. DAG, diacylglycerols; E-STE, esterified sterols; FFA, free fatty acids; Form, formulation; MAG, monoacylglycerols; NS, not significantly; STE, sterols; St, storage; TAG, triacylglycerols.

Regarding TAG, no statistically significant differences were found for the individual samples at the same time of monitoring. There is only a statistically significant decrease in the C samples along the shelf-life, while, in the P1 and P2 samples there is no significant change, but there is a decreasing trend along the shelf-life. C samples are those that are added only with starter cultures capable of de-bittering the olive paste, acting mainly on oleuropein (OLE), a bitter glycoside, responsible for the bitter taste of olives. In fact, during the de-bittering process, lactic bacteria hydrolyze OLE, thanks to the intervention of β-glucosidase, into glucose and aglycones, which are subsequently degraded by an esterase into the corresponding non-bitter phenols, 3,4-DHPEA and elenolic acid. In literature, there are few information on the effects of this fermentation process on the lipid composition trend in fermented olives, so it can only be hypothesized that the decrease of TAG in C samples is due to hydrolysis of the latter thanks to the presence of lipolytic enzymes released from fruits or from the environmental microbiota (Chabane et al., 2019). Also, in samples P1 and P2 TAGs show a decrease, even if not statistically significant; this decrease could be attributable to the presence of lipolytic enzymes that hydrolyze the TAGs with the consequent increase in DAG and FFA. The lipolytic activity as well as the esterase activity have been confirmed for the strain used for fermentation in samples P1 and P2, or for *Lpb. plantarum* (Esteban-Torres et al., 2015); the presence of this microorganism could help to hydrolyze TAGs by creating an acid environment during fermentation, with the release of DAG and FFA. Furthermore, even the yeasts, present in the formulation of the olive paste, have shown in some studies to have esterase and lipolytic activity during the fermentation of table olives (Botta & Cocolin, 2012). Therefore, they could also contribute to the hydrolytic phenomenon since the lipases produced by these microorganisms vary the characteristics of the fat in the fruit, changing the composition in free fatty acids (Botta & Cocolin, 2012). These aspects have not been investigated in the present thesis but could be investigated in further microbiological studies. Regarding FFA, there are no significant differences between the different samples at the same monitoring time. However, during the shelf-life, a statistically significant increase can be noted both in samples C and in those inoculated with probiotics (P1 and P2). During fermentation, the presence of lactic bacteria such as *Lpb. plantarum*, allow to acidify the product thanks to the production of lactic acid, and produce other compounds such as organic acids, short-chain fatty acids and other nutritional compounds that have a high potential for modulating health (Garcia-Gonzalez et al., 2021). Probably the increase in FFA along the shelf-life is also due to this acidification phenomenon that occurs in the sample, which would favor a chemical lipolysis of the TAGs with the consequent release of FFA. In the case of STE and E-

STE, the data prove to be quite stable both for each sample over the different monitoring times and over the shelf-life. The variations, although present and statistically significant, are minimal. The reactions seem to be in equilibrium. Regarding DAG, no statistically significant differences were found in the samples at the same time of monitoring. Along the shelf-life, on the other hand, a tendency to increase in this class of compounds was found for all 3 types of samples, especially at T10, thus confirming the hydrolytic action against TAG, both starter microorganisms and prebiotics added during formulation. Analyzing the MAG, they show a significant variation at T5 and T10 between the individual samples and, furthermore, an increase relative to the P1 sample over the shelf-life. This trend seems to be due to the hydrolysis phenomena on the TAGs. In the C samples there are no significant variations, but there is an increasing trend during the shelf-life probably due to a hydrolytic phenomenon caused by the TAGs. In agreement with the data of the present study, also Chabane et al. (2019) evaluated parameters such as DAG and TAG for olive samples fermented with natural method, in the absence of chemical agents, and subjected to sampling and monitoring after 60, 120 and 150 days from fermentation. In the experiment by Chabane et al. (2019), the level of DAG tended to increase over monitoring times, while the TAGs to decrease. This phenomenon, together with an increase in free acidity, was due to a hydrolysis of TAGs, with consequent release of DAG and FFA, by lipolytic enzymes present in olives and / or by inoculated microorganisms (Chabane et al., 2019).

7.3.1.3 Total fatty acid profile

Regarding total FA composition (**Table 7.3**), the most represented FA class was monounsaturated fatty acids MUFA (69.79-73.06%), followed by saturated fatty acids SFA (17.32-21.33%) and polyunsaturated fatty acids PUFA (8.87-9.62%). Formulation and storage significantly affect the *n*-6 content of the examinate samples. For SFA, MUFA and PUFA only storage significantly affect this parameter, while *n*-3 content is not statistically influenced by formulation and the interaction between formulation and storage time, but without displaying a clear trend. In literature there are not many data regarding the FA composition of fermented olive paste, therefore the comparisons will be made with olive oil obtained from olives of the same cultivar and pomace oil. The olive oil obtained from Peranzana cultivar olives, the same cultivar from which the olive paste used for the formulation of the samples object analyzed in this work, is characterized by 14-22% of SFA, 67-77% of MUFA (with prevalence of acid oleic 60-70%) and 8-10% of PUFA (Servili et al., 2012). As regards the samples being analyzed, there is a high content of PUFA, like that reported for the olive oil; this

could be due to the action of lactic bacteria which, during fermentation, could favor the increase of polyunsaturated compounds.

Table 7.3. Fatty acid classes (expressed as % of total fatty acids) of fermented olive paste cream, with (P1 and P2) and without (C) probiotic addition, samples after 0, 5 and 10 days of storage.

Samples	SFA	MUFA	PUFA	*n*-3	*n*-6
			(% of total fatty acids)		
			0 days		
C	21.33±0.94	69.79±0.78	8.87±0.17	0.77±0.01	8.11±0.16
P1	21.33±0.94	69.79±0.78	8.87±0.17	0.77±0.01	8.11±0.16
P2	21.33±0.94	69.79±0.78	8.87±0.17	0.77±0.01	8.11±0.16
			5 days		
C	21.02±1.10	70.01±0.94	8.96±0.15	0.77±0.02	8.19±0.14
P1	17.32±3.05	73.06±2.67	9.62±0.39^{A}	0.84±0.10	8.78±0.33^{A}
P2	20.52±0.16	70.00±0.82	9.47±0.71	0.80±0.05	8.67±0.67
			10 days		
C	20.40±0.42	70.26±0.74	9.34±0.39	1.02±0.39	8.32±0.01
P1	19.91±0.99	70.96±0.86	9.13±0.14^{AB}	0.78±0.01	8.35±0.13^{AB}
P2	21.07±0.77	69.92±0.69	9.01±0.09	0.77±0.00	8.24±0.09
Factor			**F value**		
Form	*NS*	*NS*	*NS*	*NS*	*
St	*	*	*	*NS*	*
Form*St	*NS*	*NS*	*NS*	*NS*	*NS*

Results as reported as means±s.d of 3 independent replicates. no significant differences (Tukey's test; $p \leq 0.05$) within the same sample during the shelf-life. no significant differences (Tukey's test; $p \leq 0.05$) among treatments. *$p < 0.05$. Form, formulation; MUFA, monounsaturated fatty acids; NS, not significantly; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; St, storage; UFA, unsaturated fatty acids.

Furthermore, in the samples, a higher MUFA content was detected than that of olive oil, but in accordance with the characteristics of the pomace oil, which has a higher MUFA content, mainly due to the presence of oleic acid which represents 56-85% of the total fatty acids (Mateos et al., 2020). Regarding MUFA and SFA, no significant differences are observed between the samples at the same time of monitoring and even along the shelf-life, while for the PUFA, significant differences are noted only along the shelf-life for the P1 sample. This variation could be partly due to the presence of the inoculated lactic acid bacteria. In this regard, it can be emphasized that lactic bacteria can produce conjugates of linoleic acid, helping to increase polyunsaturated compounds (Park & Kim, 2011). This production was found more precisely by *Lpb. plantarum* on different plant

matrices (Park & Kim, 2011). *Lpb. plantarum* is the probiotic strain used in the samples of the present work. As these samples are rich in MUFA, they are less exposed to oxidative phenomena. In fact, MUFA, acting in synergy with the presence of vitamin E, a powerful fat-soluble antioxidant, protect long-chain fatty acids from oxidative damage (Nunes et al., 2018). In addition, high levels of unsaturated fatty acids (UFA), 78.67-82.68%, mainly due to the abundance of oleic acid (C18: 1) and are responsible for positive effects on the health such as reduction of cardiovascular disease and cancer (Chabane et al., 2019).

Table 7.4. Ratios of fatty acid classes, atherogenic index (AI) and thrombogenic index (TI) of fermented olive paste cream, with (P1 and P2) and without (C) probiotic addition, samples after 0, 5 and 10 days of storage.

Samples	*n*-6/ *n*-3	PUFA/SFA	UFA/SFA	AI	TI
			0 days		
C	10.60±0.13	0.42±0.56	3.69±0.94	0.14±0.00	0.31±0.00
P1	10.60±0.13	0.42±0.56	3.69±0.94	0.14±0.00	0.31±0.00
P2	10.60±0.13	0.42±0.56	3.69±0.94	0.14±0.00	0.31±0.00
			5 days		
C	10.67±0.04	0.43±0.63	3.76±1.10	0.13±0.01	0.28±0.02
P1	10.51±1.01	0.56±1.72	4.77±3.05	0.12±0.01	0.27±0.03
P2	10.77±0.17	0.46±0.44	3.87±0.85	0.12±0.01	0.27±0.03
			10 days		
C	8.87±2.75	0.46±0.41	3.90±0.78	0.13±0.00	0.28±0.01
P1	10.76±0.08	0.46±0.56	4.02±0.99	0.14±0.00	0.31±0.00
P2	10.73±0.09	0.43±0.43	3.75±0.77	0.14±0.00	0.31±0.00
Factor			**F value**		
Form	*NS*	*NS*	*NS*	**	**
St	*NS*	*	*	**	**
Form*St	*NS*	*NS*	*NS*	*NS*	*NS*

Results as reported as means± s.d of 3 independent replicates. no significant differences (Tukey's test; $p \leq 0.05$) between the same sample during the shelf-life, no significant differences (Tukey's test; $p \leq 0.05$) between treatments. *$p < 0.05$., **$p < 0.01$. AI, Atherogenic Index; Form, formulation; NS, not significantly; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids, St, storage; TI, thrombogenic index; UFA, unsaturated fatty acids.

Regarding FA classes ratios (**Tables 7.4**), the PUFA *n*-6/PUFA *n*-3 it should not exceed the 4: 1 ratio, according to the INRAN guidelines (Valencia et al., 2008), to optimize bioavailability, metabolism, and incorporation of fatty acids into membrane phospholipids (Valencia et al., 2008). Excessive

PUFA *n*-6 content, and high *n*-6/*n*-3 ratios can promote different types of pathogenesis, including cardiovascular disease, cancer, autoimmune and inflammatory diseases, while the increase in n-3 PUFA (consequently low ratios of PUFA *n*-6/*n*-3) exerts suppressive effects on these pathologies (Domìnguez et al., 2017).

In the present study for the n-3, n-6 and *n*-6/n-3, no statistically significant differences are found either for the samples at the same time of monitoring, nor along the shelf-life. The parameters are quite stable*: n*-3 has a percentage range between 0.77 and 1.02% and *n*-6 has values between 8.11-8.78%, so the *n*-6/*n*-3 ratio varies between 8.77 and 10.76 and therefore does not fall within the values recommended by INRAN (Valencia et al., 2008). The PUFA/SFA ratio, from a nutritional point of view, is one of the fundamental parameters currently used to evaluate the nutritional quality of the lipid fraction of foods. In this regard, the INRAN guidelines recommend a PUFA/SFA ratio between 0.4 and 1 (Valencia et al., 2008). It has been reported that an increase in the dietary PUFA/SFA ratio can lead to a reduction in plasma total cholesterol. From the data obtained in the present study, a PUFA/SFA ratio of about 1 can be seen, which remains constant in all types of samples at the various monitoring times and throughout the shelf-life; only storage time significantly influenced this ratio. The UFA/SFA ratio is very useful instead for observing the oxidation trend: if the ratio decreases during the shelf-life, it indicates a decrease in the UFA level which could be related to their oxidation. Stable trends in the UFA/SFA ratio (3.69-4.77) are observed in this study, with no significant variation between either individual samples C, P1 and P2 at the same monitoring time (T0, T5 and T10), storage time, therefore, significantly affect this ratio. Some studies also clarify that the *n*-6/*n*-3 ratio may not be totally suitable for evaluating the nutritional value of fat since it implies that all SFA, in the same way, can induce an increase in cholesterol levels, not taking into account instead of the anti-cholesterol effects of MUFA, in particular of C18: 1. Ulbricht & Southgate (1991) introduced two other indices: atherogenic index (AI) and thrombogenic index (TI) to "mathematically quantify" the propensity of fats to cause atherosclerosis and / or the formation of thrombus. These are formulas that assign where possible a risk or benefit factor to each fatty acid or each category of fatty acids, in relation to the different contribution of these in favoring or preventing the onset of morbid events. Basically, more the AI and TI values increase, more the food risk due to the negative characteristics of fat increases. The recommended values for these two indices in the diet should be low and less than 1 (Ulbricht & Southgate, 1991). In the present study, these two parameters remain below 1 (<0.2 for AI and <0.4

for TI) for all samples. Both indices have stable values, significantly influenced by formulation and storage time, but not from their interaction.

The most abundant fatty acid is oleic acid (C18:1, 65.37-68.23%), followed by palmitic acid (C160, 9.80-11.10%), by butyric acid (C4:0, 5.27-8.93%) and linolenic acid (C18:2, 7.64-8.32%). No statistically significant changes in the individual fatty acids between the samples were observed at the different monitoring times (T0, T5 and T10) nor along the shelf-life. Particular attention could be paid to the presence of butyric acid in a fairly evident percentage which remains constant throughout the shelf-life. From a study carried out on the identification of volatile compounds present in the headspace of olive oil, it appears that the winy flavor typical of olive oil defects could be connected, in addition to the presence of high percentages of acetic acid, also due to the presence of butyric acid in considerable quantities (Zhu et al., 2016). In fact, as will be seen in the following paragraphs, one of the attributes found in the sensory analysis for some samples is the acetic/lactic fermented flavor which could be confirmed by the presence of butyric acid in the analyzed samples. The butyric acid found in the olive paste could come from the metabolism of lactic bacteria. Lactic bacteria use glycosides as a carbon source to initiate lactic fermentation and produce lactic acid; if the product is not pasteurized, during storage it may undergo a further fermentation phase with the development of other heterofermentative lactic bacteria which, by metabolizing lactic acid, produce butyric acid (Lanza et al., 2016). The presence of long-chain PUFA *n*-3 is also not characteristic for olive oils; the increase recorded in the samples subject of this experimentation could be due to lactic bacteria which during fermentation are able to promote, thanks to the action of desaturase and elongase enzymes, the formation of long-chain fatty acids through desaturation and lengthening of the hydrocarbon chain (Ogawa et al., 2005). It can therefore be said that the composition of the olive paste analyzed in this thesis has a significant and important percentage of MUFA due to the presence of oleic acid (C18: 1 65.37-68.23%), typical of olives and which remains also in the by-products of oil processing. MUFA, as previously mentioned, contribute to increasing oxidative stability (Nunes et al., 2018). Finally, it is possible to state that, also from previous studies on fermented table olives, the composition of fatty acids and their trend depend more on the ripeness and state of the olive fruit and not on processing (López-López et al., 2009). In the experiment by Nunes et al. (2018) the fatty acid profile of an olive paste was performed. The study showed a very high percentage of MUFA (76.25%), followed by SFA (14.49%) and PUFA (9.26%) in accordance with the results of this study. Furthermore, 75% of oleic acid (C18: 1) was found as the most abundant fatty acid, in accordance with what emerged in the present

experiment. The olive paste analyzed in the study by Nunes et al. (2018) was not fermented, so the presence of butyric acid was not detected.

7.3.1.4 Lipid oxidation, colorimetric analysis and sterol composition

The determination of conjugated dienes and trienes was performed by spectrophotometric examination in the ultraviolet. This method, envisaged at Community level by the REG. (EC) 2472/97, is widely used for the detection of commercial fraud involving vegetable oils, in particular extra virgin olive oil (EVOO). This type of analysis is also useful for monitoring the primary oxidation of lipids: it is based on the reactions that can occur during the formation of hydroperoxides, primary products of lipid oxidation, in which the hydroperoxyl radicals produced in the initiation and propagation phases monomolecular, they then tend to stabilize through a rearrangement of the double bonds, forming conjugated dienes and trienes (Dominguez et al., 2019).

Table 7.5. K_{232} (conjugates dienes), K_{268} (conjugates trienes), ΔK, L*, a*, b* and sterols of fermented olive paste cream, with (P1 and P2) and without (C) probiotic addition, samples after 0, 5 and 10 days of storage.

Samples	K_{232}	K_{270}	Δk	L*	a*	b*	Camp	Stig	β-sito	Δ5-aven	Ste Tot	Camp/stig
									mg/kg of olive cream			
						0 days						
C	0.53±0.04^{a}	0.08±0.01^{c,B}	0.00±0.00	40.22±0.58^{ab}	1.28±0.13^{b}	10.59±0.19^{b}	2.34±0.25	0.57±0.11	95.91±4.70	0.01±0.00	98.83±11.33	4.13±0.18
P1	0.53±0.04^{b}	0.08±0.01^{c,C}	0.00±0.00	40.22±0.58^{b}	1.28±0.13^{b}	10.59±0.19^{c}	2.34±0.25	0.57±0.11	95.91±4.70	0.01±0.00	98.83±11.33	4.13±0.18
P2	0.53±0.04^{b}	0.08±0.01^{c,B}	0.00±0.00	40.22±0.58^{b}	1.28±0.13^{c}	10.59±0.19^{b}	2.34±0.25	0.57±0.11	95.91±4.70	0.01±0.00	98.83±11.33	4.13±0.18
						5 days						
C	0.30±0.03^{c}	0.08±0.04^{b,B}	0.00±0.00	40.79±0.24^{a,B}	2.55±0.01^{a,A}	11.76±0.19^{a,B}	3.44±0.56	0.83±0.07	141.17±5.95	0.01±0.01	145.45±18.43	4.16±0.32
P1	0.26±0.01^{c}	0.23±0.02^{b,B}	0.00±0.00	46.82±0.08^{a,A}	2.15±0.06^{a,B}	16.35±0.15^{a,A}	2.21±0.24	0.79±0.06	125.14±5.18	0.01±0.00	128.14±13.26	2.78±0.15
P2	0.31±0.05^{c}	0.28±0.03^{a,A}	0.00±0.00	46.81±0.16^{a,A}	2.43±0.08^{a,A}	16.69±0.14^{a,A}	2.39±0.35	0.95±0.11	102.05±4.25	0.01±0.00	105.40±9.39	2.51±0.23
						10 days						
C	0.50±0.05^{b,C}	0.18±0.00^{a,B}	0.01±0.00	39.55±0.46^{b,B}	2.57±0.11^{a,A}	10.94±0.34^{b,C}	2.28±0.22	0.49±0.04	110.53±2.86	0.01±0.00	113.31±7.99	4.62±0.13
P1	1.15±0.07^{a,A}	0.29±0.03^{a,A}	0.00±0.00	45.68±0.71^{a,A}	2.09±0.14^{a,B}	15.32±0.37^{b,B}	3.59±0.43	1.60±0.17	117.39±6.65	0.01±0.00	122.58±13.32	2.25±0.30
P2	1.01±0.03^{a,B}	0.25±0.02^{a,A}	0.00±0.00	46.77±0.51^{a,A}	2.14±0.04^{b,B}	16.76±0.44^{a,A}	2.65±0.13	1.01±0.08	83.15±1.91	0.01±0.00	86.81±3.94	2.62±0.11
Factor						**F value**						
Form	***	***	*NS*	***	***	***	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
St	***	***	*NS*	***	***	***	*NS*	*NS*	*	*NS*	*NS*	*NS*
Form*St	***	***	*NS*	***	***	***	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

Results as reported as means± s.d of 3 independent replicates. a–c indicates significant differences (Tukey's test; $p \leq 0.05$) between the same sample during the shelf-life, A-B indicate significant differences (Tukey's test; $p \leq 0.05$) between treatments. *$p < 0.05$, ***$p < 0.001$. β-sito, β- sitosterol; Δ5-aven, Δ5-avenasterol; a*, red index; b* yellow index; Camp, campesterol; Form, formulation; L*, luminosity; NS, not significantly; Stig, stigmasterol; Ste Tot; total sterols; St, storage.

Conjugated dienes and trienes are very unstable molecules that tend to react with other compounds, with the products of lipid oxidation, to form even more complex compounds (e.g., aromatic compounds, dimers and polymers). Therefore, while identifying low levels of dienes and trienes, it is not certain that these are indicative of an absolute absence of oxidative phenomena, given that they may already have become other species of secondary oxidation, both volatile and non-volatile. Similarly, in a completely opposite way, even high levels of these compounds do not necessarily represent a negative result since, to confirm it, the degradation phenomenon should be evaluated to understand, that is, if the oxidation has stopped at that level or has evolved in other directions. In the present study (**Table 7.5**) K_{232} ranged from 0.26 to 1.15, product formulation and storage time significantly influenced this oxidative parameter, and the two factors interaction was significant as well. K_{270} ranged from 0.08-0.29, like conjugate dienes, product formulation and storage time significantly influenced this oxidative parameter, and the two factors interaction was significant as well. It is possible to note for C samples a significant variation in all three monitoring times (T0, T5 and T10). A similar trend can be found for samples P1 and P2, which show a variation in T5 and an increase in dienes conjugated to T10. In addition, a significant increase in T10 is found in the different samples (C, P1 and P2) compared. The increase in the formation of conjugated dienes in the P1 and P2 samples compared to the controls (in the absence of probiotics), is probably due to the oxidation of fatty acids, together with a decrease in the antioxidant activity of the phenolic compounds present in the samples. From the oleuropein, during fermentation, 3,4-DHPEA and *p*-HPEA are obtained, which have been shown to have antioxidant activity (Difonzo et al., 2019, Difonzo et al., 2021) and could slow down the formation of conjugated dienes. Specific extinction values at 270 nm (conjugated trienes) on the fermented olive paste was also measured (**Table 7.5**). As regards the C samples, a stability of the production of conjugated trienes is noted from T0 to T5, to then increase to T10. P1 shows a constant and statistically significant increase from T0 to T10, while P2 shows an increase in the formation of conjugated trienes from T0 to T5, which then tends to stabilize at T10. Furthermore, there is a variation at T5 between the C samples and those inoculated with probiotics (P1 and P2), and at T10 between the C and inoculated samples (P1 and P2). Unsaturated fatty acids can undergo the formation of hydroperoxides with consequent slippage of the double bond and the formation of a conjugated diene system; an even more intense oxidation of these compounds can form conjugated trienes, following the decomposition of a hydroperoxide deriving from linoleic acid or the formation of ketone compounds. The data shows a greater formation of conjugated trienes in the samples with probiotic inoculation compared to the controls,

which show a probable oxidative phenomenon of fatty acids and phenols. In fact, the action and impact of phenolic compounds, such as oleuropein, which releases other antioxidant compounds such as 3,4-DHPEA and *p*-HPEA during fermentation, should also be investigated (Lanza et al., 2016; Difonzo et al., 2019, Difonzo et al., 2021). Finally, ΔK index was calculated, nor product formulation or storage time, nor the two factors interaction significantly influenced this oxidative parameter. The ΔK index can be considered as a summary of the oxidative stability of the samples examined, while not finding bibliographic references for use for olive paste. **Table 7.5** shows the ΔK trend, allowing you to have an overall view of the oxidative stability of the olive paste fermented with the starter cultures (C) and with the addition of the different probiotic formulations (P1 and P2). By analyzing the trend of this parameter there are no statistically significant differences for samples C, P1 and P2 during the same monitoring time (T0, T5 T10) and not even along the shelf-life. This could indicate a certain oxidative stability of the samples even in contact with the different probiotic formulations over the different monitoring times, but these data should be integrated with other secondary oxidation parameters (e.g., volatile compounds) to get a more complete picture of the oxidative trend of the products. However, it is also important to consider the pasteurization process to which all the samples were subjected before being inoculated with the two probiotic formulations, which may have influenced the oxidative trend in some way. In the study by Chabane et al. (2019) oxidative parameters, such as the specific extinction values at 232 nm and 270 nm, were evaluated for samples of fermented olives (natural method, in the absence of chemical agents) and subjected to sampling and monitoring after 60, 120 and 150 days from fermentation. In accordance with the results of the thesis in question, the study also reported an increase in specific extinction at 232 nm (after 60 days from 1.45 to 1.51% with a subsequent decrease) and at 270 nm (after 2 months from 0.10 to 0.12%), thus reflecting a small and not statically significant increase in oxidation in fermented olives during storage.

The color changes that the olives undergo during processing are due to the transformation of their pigments. At the beginning of the ripening stages of the fruit, the color changes from an intense green to a green/yellow; the color of the olives is due to the presence of both chlorophylls a and b and carotenoids. In the processing of Spanish-style table olives (chemical debittering process), the chlorophylls degrade towards the corresponding derivatives free of Mg (mostly pheophytins) with gray-brownish colors and the carotenoids towards the corresponding derivatives with 5,8-furanosic groups in their molecule. Because of all these pigment transformations, Spanish style olives have a characteristic color, much appreciated by consumers. For the natural fermentation on green olives

(organic), however, the transformation of the pigment during the processing of the olives is not known and the final brownish color that results could be related to chlorophylls and transformations of carotenoid pigments but also to oxidation of polyphenolic compounds (Ramìrez et al., 2015). Therefore, for the present experimentation, it was considered appropriate to carry out the color analysis for the samples of fermented olive paste, to evaluate the change in the color of the different samples and therefore observe the possible variations in the pigments and the oxidative state of the phenolic compounds, of which the matrix used for the formulation of the samples was rich. For all 3 tested parameters, L*, a* and b*, product formulation and storage time was statistically significant, and the two factors interaction was significant as well. Regarding L* C samples show stable values during shelf-life, while samples P1 and P2 show an increase in brightness. The olive paste for these samples appears with a lighter brown than the controls which instead turn out to be a darker green. The brightness could be affected by the oxidation of chlorophyll pigments during fermentation and/or by the oxidation of phenolic compounds. For a* was noted an increase in all samples, especially at T5, that remain stable at T10. The red index appears to have a similar trend in all three samples (C, P1 and P2). This behavior could be linked to the fact that at T0 there is only the presence of starters that perform the de-bittering. This trend for all three samples may be due to the oxidative state of the phenolic compounds or to the change that the chlorophyll undergoes during fermentation. Finally for b*, the trend of this index for samples C is stable over shelf-life and is different from the trends of the other two samples P1 and P2. The trends of this index for P1 and P2 in fact show, an increase at T5 to then remain stable at T10. The increase that is noted in samples P1 and P2 could be attributable to the presence of probiotics added to the samples and not to the presence of only the starters (as in samples C) that carry on the fermentation and probably contribute to favoring the oxidation of both the compounds phenolics and pigments, including chlorophyll. These reactions could therefore contribute to the color change of the samples towards brown tones, with the consequent increase in the red index (a*). From a general analysis of color data and from previous studies it can be stated that the color trend in olives or olive paste, as in the case of this experiment, varies according to the type of fermentation process. In this case, as expected, in the presence of biological fermentation, the samples tend to change from a dark green color to brown tones; in the experiment of Ramirez et al. (2015), on the other hand, the olives fermented through alkaline chemical fermentation recorded a color tending to yellow-golden, more appreciated also by consumers. The color tends to vary, albeit slightly in the present study, due to the transformation of the pigments. From the study by Ramirez et al. (2015) it appears that, as

already clarified above, most phenolic compounds are also involved in the color changes of olives. It is well known, in fact, that the enzymatic and chemical oxidation of *o*-diphenolic compounds can form dark colored compounds (Ramìrez et al., 2015). Furthermore, a direct correlation was seen between the activity of polyphenol oxidase (PPO) and the browning rate of a raw homogenate of five varieties of Italian olives (Ramìrez et al., 2015). The main phenolic compound of olives as well as of olive paste is oleuropein. The fundamental step to sweeten the olives involves the elimination of this bitter component to obtain a more palatable product. It has therefore been hypothesized that the mechanism of the browning reaction in olives consists primarily of an enzymatic release of 3,4-DHPEA from the oleuropein by the action of β-glucosidase and esterase enzymes. Subsequently, this *o*-diphenol is oxidized by PPO (polyphenoloxidase), forming brown compounds (Ramìrez et al., 2015). In addition, by evaluating the variation of pigments in naturally fermented green table olives, it appeared that the main transformations were due to the acid pH originating from the fermentation process (Ramìrez et al., 2015). In particular, it appeared that the chlorophyll pigments present in the olives at the end of the process were without Mg. The presence of Mg in the chromophore group of chlorophyll compounds is responsible for the green colors of these pigments; however, when Mg^{2+} is replaced by $2H^{+}$, the green colors turn into tones ranging from gray to brownish/green (Ramìrez et al., 2015). Therefore, all Mg-free chlorophyll derivatives show similar colors even if other structural changes have occurred in the molecule such as the oxidative opening of the isocyclic ring (Ramìrez et al., 2015). As regards the carotenoid fraction, no appreciable differences were found between the samples under study (Ramìrez et al., 2015). In accordance with the data of the present experiment, it can be stated that the color change may be due to these variations on the chlorophyll pigments and on the oxidative state of the phenolic compounds, which were probably also influenced by the HTST pasteurization process (72 °C for 15 sec) to which all the samples were subjected before inoculation of the probiotics. Phytosterols are minor components of vegetable and olive oils and are found in cell membranes. According to the literature, once taken with the diet, they perform a cholesterol-lowering action since they compete with the absorption of cholesterol in the intestine (Ostlund, 2007). To have this cholesterol-lowering effect, according to EFSA, 1.5 to 2.4 g of sterols per day must be taken (EFSA, 2021). Furthermore, the composition of phytosterols in samples such as oil represents a useful marker for detecting any adulterations: the authenticity of a product is important both from the health point of view and from the point of view of commercial value (Azadmard-Damirchi, 2010). In this regard, the minimum value established by the EU regulation for the "extra virgin" olive oil category is 1000 mg/kg (Haddada et

al., 2007). In addition, for the EVOO category, the EU regulation reports the minimum values of the composition of the different phytosterols that must be present in the oil (EEC, 1991): campesterol ≤ 4.0%; stigmasterol <campesterol; β-sitosterol ≥ 93.0%. On the other hand, by analyzing the phytosterol composition of pomace oil, it contains a higher level of phytosterols (1800-3000 mg kg) than the rest of olive oils, with very high β-sitosterol values (> 93%), and in a lower percentage of campesterol and stigmasterol (Mateos et al., 2020). From the literature it emerges that the processing practices applied to olives can affect the composition and content of phytosterols (Ruiz Méndez et al., 2008). In general, fermented olives can contain from 200 to 520 mg/kg of phytosterols. The main phytosterols present in the lipid fraction of (fermented) table olives are β-sitosterol, Δ5-avenasterol, campesterol. More recent epidemiological and experimental studies suggest that dietary phytosterols, β-sitosterol particularly, may offer protection from the most common cancers. **Table 7.5** shows phytosterols profile in the samples of fermented olive paste and their trend along the shelf-life. Product formulation storage time and its interaction not statistically significant affect single phytosterol and total phytosterol, except for β-sitosterol content, that was affected only by storage time. From the data it appears that β-sitosterol is the most present compound (83.15-141.17 mg/kg), followed by campesterol (2.21-3.59 mg/kg), stigmasterol (0.49-1.60 mg/kg) and Δ5-avenasterol (0.01 mg/kg). Based on the sterol content found in the analyzed olive paste (0.84-0.93%), it would be necessary to assimilate approx. 200 g of olive paste per day to have a cholesterol-lowering effect. As can be seen from the table, there are no significant differences in the trend of different phytosterols along the shelf-life. The values of stigmasterol in all tested samples are lower than those of campesterol; this suggests that the olive paste samples (as for the oils) come from healthy fruits according to Haddada et al. (2007). In fact, previous studies (Koutsaftakis et al., 1999) investigate how the trend of phytosterols is influenced by the type of oil extraction during its processing: the stigmasterol content increases when extracted with (traditional) pressing systems. On the other hand, there is a higher ratio of campesterol/stigmasterol in oils extracted with two and three-phase decanter centrifugation. It is interesting to note how the campesterol/stigmasterol ratio reaches a high value in the period of December, which indicates that this is the optimal ripening phase for the olive harvest. It is also important to underline that, in the early stages of olive ripening, the campesterol content is very close to 4.5%, a value higher than the maximum limit set by EC legislation, and then decreases as the ripening continues (Koutsaftakis et al., 1999). The analysis of the olive paste shows a campesterol/stigmasterol ratio of 2.25-4.62. The values of stigmasterol (0.49-1.60 mg/kg) are always lower than the values of campesterol (2.21-3.59

mg/kg); this could be an indicator of the fact that the olives from which the experimented pasta was obtained were processed in optimal conditions. The values of campesterol not exceeding 4.5% also indicate that the olives were not harvested in the early stages of ripening. In addition, the pomace from which the olive paste was obtained comes from processing with more modern systems (two and three-phase method) therefore, in accordance with what was previously reported, the values obtained of the campesterol/stigmasterol ratio are higher than to a processing with traditional systems (Haddada et al., 2007; Koutsaftakis et al., 1999).

7.3.1.5 PCA of chemical and colorimetric data

To better understand which parameters were the most relevant for assessing the effects of formulation and storage on fermented olive paste, chemical composition and phenolic composition data were subjected to principal component analysis (PCA) (**Figure 7.1**).

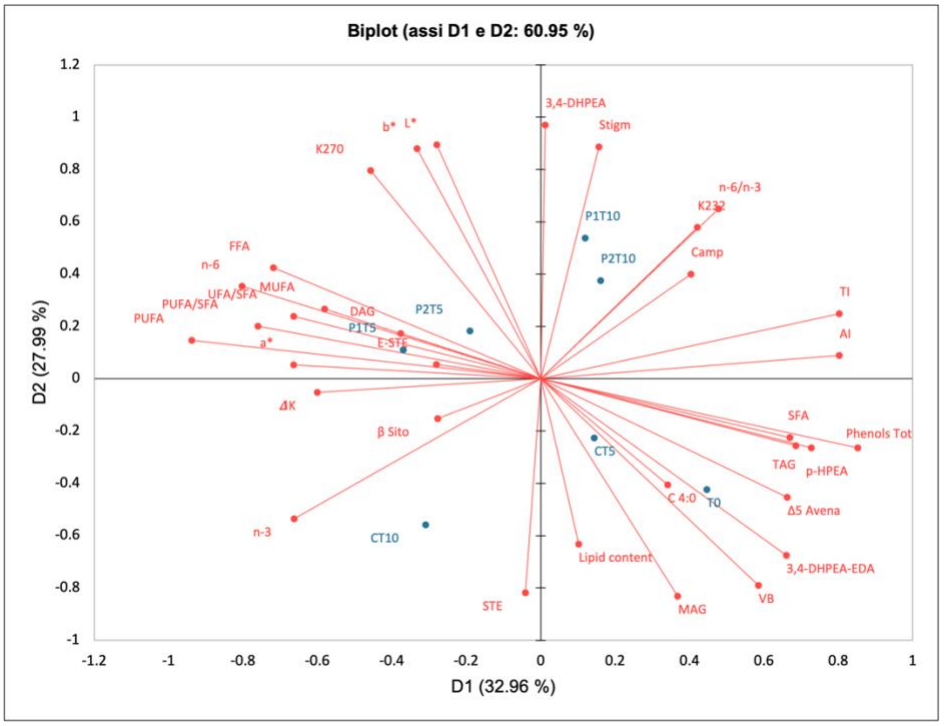

Figure 7.1. Biplot of fermented olive cream. β-sito, β- sitosterol; Δ5-aven, Δ5-avenasterol; 3,4-DHPEA, Hydroxytyrosol; 3,4-DHPEA-EDA, decarboxymethyl oleuropein aglycone; a*, red index; AI, Atherogenic Index; b* yellow index; Camp, campesterol; DAG, diacylglycerols; E-STE, esterified sterols; FFA, free fatty acids; L*, luminosity; MAG, monoacylglycerols; MUFA, monounsaturated fatty acids; *p*-HPEA, tyrosol; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; STE, sterols; Stig, stigmasterol; TAG, triacylglycerols; TI, thrombogenic index; UFA, unsaturated fatty acids; VB, verbascoside.

The first two components explained 60.95% of the total variance (32.96% for PC1 and 27.99% for PC2). The control at T0 is well separated from all the other samples, while the control samples at T5 and T10 are in closer quadrants and are well separated from the rest of the other samples (P1 and

P2). C samples are mainly characterized by the variables TAG, MAG, STE, Δ5-avenasterol, lipid content, C 4: 0 (butyric acid) and single phenols. The latter is also present in this cluster due to the lipolytic activity of the lactic bacteria inoculated as a starter. As regards the samples P1 and P2, the closeness is more evident between P1T5 and P2T5 and between P1T10 and P2T10. P1 and P2 at T5 are characterized by variables concerning the classes of fatty acids and their ratios (such as FFA, MUFA, PUFA, *n*-6, UFA/SFA, PUFA/SFA), while at T10 they are characterized by the variables minority sterols (such as campesterol, stigmasterol), K_{232} which provides information on the formation of conjugated dienes resulting from the oxidation process, and 3,4-DHPEA, that deriving from the oxidation of oleuropein, which disappears completely along the shelf-life of the samples added with probiotics, and are more abundant in this samples. This conformation could demonstrate that the different formulations of the added probiotics do not necessarily imply many differences in the various parameters treated, but it is mostly the storage times that differentiate the different types of samples.

7.3.2 Sensory evaluation

In the first session each subject created between 13 and 27 terms. Then, the subjects selected their definitive list of attributes, containing between 7 and 10 terms. At the end of the session a discussion was made in order to understand the definition of each selected attribute, since the majority of the attribute were identified by all the assessors. In particular, the list of the 16 attributes found by the tasters is reported in the **Table 7.6**.

Table 7.6. List of the 16 descriptors identified by the 10 assessors during the first session of flash profile

Descriptor	Definition	Reference
Green[1]	Intensity of green color	Difonzo et al., 2019
Brown[1]	Intensity of brown color	
Oily phase[1]	Presence/separation of a solid phase (olive paste) and an oily phase (oil)	Cosmai et al, 2017
Rancid[2]	Characteristic flavor common to all fats that have suffered an oxidative process, due to their prolonged contact with air, light and heat. This flavor is unpleasant and irreversible	Monteleone et al., 2012

Lactic fermented[2]	Olfactory sensation reminiscent of the smell of butter or cheese (butyric or lactic acids)	López-López et al., 2018
Acid fermented[2]	Characteristic flavor reminiscent of that of wine or vinegar. It is essentially due to an aerobic fermentation process of the olives or the remains of olive paste in not washed properly, which leads to the formation of acetic acid, ethyl acetate and ethanol	López-López et al., 2018
Fermented[2]	Olfactory sensation (perceived directly or retronasally) that reminiscent of the smell of decaying organic matter (putrid), of butter or cheese (butyric), of wine or vinegar (acetic acid), of the combination of different fatty acids which overall recalls the smell of leather rotten	Monteleone et al., 2012
Olive[2]	Odour/aroma of fresh green or ripe fruit of olive	Monteleone et al., 2012; Cosmai et al., 2018; López-López et al., 2018; Difonzo et al., 2019
Brine[2]	Flavor of olives preserved in brine	
Bitter[3]	Elementary taste characteristic perceived by the goblet-shaped papillae that form the lingual V	Monteleone et al., 2012; López-López et al., 2018
Acid[3]	Taste sensation produced by acidic substances (e.g. acid citric or tartaric acid) perceived during chewing	Monteleone et al., 2012
Salty[3]	Taste sensation produced by saline solutions (e.g. sodium chloride) perceived during chewing	Monteleone et al., 2012; López-López et al., 2018
Granularity[4]	Characteristic that measures the ease of perception of the particles (granules) that form in the sample before reducing it to a bolus. The particles that form the	BS ISO 11036:2020

	compound can be ranked on a scale according to theirs size	
Fibrous[4]	Sensation caused by the geometric structure and linked to the perception of the shape and orientation of the product particles; fibrousness refers to the elongated conformation of the particles, oriented in the same direction	ISO 11036:1994
Adhesiveness[4]	Mechanical attribute related to the work needed that the tongue does to detach a food glued to the palate and teeth	BS ISO 11036:2020
Creaminess[4]	Sensation associated with fat content and can be described as a velvet, smooth and not rough or dry feeling, with a velvety coating on the tongue and palate	Jellema et al., 2005

1 Appearance, 2 Odour, 3 Taste, 4 Texture

Following the discussion, the data analysis was carried out with the sensory attributes that for most of the tasters (at least 80% of them) identified in describing the samples. Among the attributes that made the tasters agree to consent, which can be defined as characterizing, we find green, brown, oily phase, fermented, olive, bitter, sour, salty, granularity, fibrousness, adhesiveness, and creaminess. In fact, from the discussion it emerged that the analyzed samples could be ordered, in terms of intensity, by 12 attributes. In particular, the color (green, brown), certainly linked to the presence of chlorophylls or to the oxidative state of the olive paste; the bitter attribute, associated with the presence of bitter substances, mainly polyphenols, which is assumed to be more intense in samples in which the incomplete de-bittering does not occur chemically but rather microbiologically (Lanza & Poiana, 2012). The sensation of fermented is linked to the term of anomalous fermentation which contains all those olfactory sensations that recall the smell of decomposing organic matter, aged cheese, rotten eggs, sludge, deriving from the establishment of parallel fermentations caused by the development of contaminating microorganisms (Lanza & Poiana, 2012). The creaminess of the sample, on the other hand, appears to be associated with the fat content (Jellema et al., 2005). Data analysis was done with the sensory attributes which are widely used by panelists in describing

samples (at least by the 80% of the assessors), those were green, brown, oily phase, fermented, olive, bitter, acid, salty, granularity, fibrous, adhesiveness and creaminess. The distribution of the five samples is reported in **Figure 7.2**, which shows the location of the five products and the consensus, evaluated by the ten subjects. The five products were distributed in three quadrants. Samples C (biologically debittered sample) and C4 (commercial sample 4) were distant from all the others. In particular, sample C, has opposite characteristics compared to samples C1, C2 and C3 (commercial sample 1,2 and 3) in the first component and is located in a separate cluster in the second main component. C4 is also located in a cluster opposite to C with respect to the first component. This suggests that C1, C2 and C3 have more characteristics in common than C and C4.

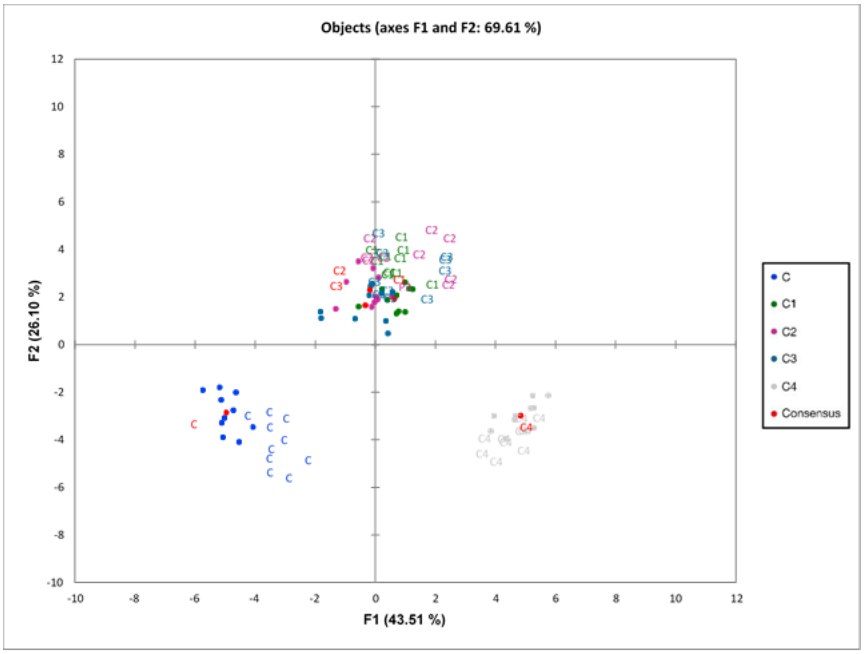

Figure 7.2 Principal component analysis (PCA) of the distribution of all the samples on the Cartesian plane, according to the evaluations expressed by each assessor for each sample. This PCA highlights the positioning of each judge with respect to the consensus (median of the group).

In **Figure 7.3** were showed the consensual configuration of the green olive paste samples after GPA, which explained the 69,61% of the total variability in two dimensions. This represented an interesting result if it is considered that the panel was not trained. Sample C was characterized by *creamy*, *sour*, *fermented* and *bitter* attributes. Sample C1 was strongly characterized by attributes such as the presence of an oily phase, *graininess, fibrous* and *olive* flavor. Samples C2 and C3 were described by the attributes *brown* and *salty*. Finally, sample C4 was characterized by *green* and *adhesiveness*.

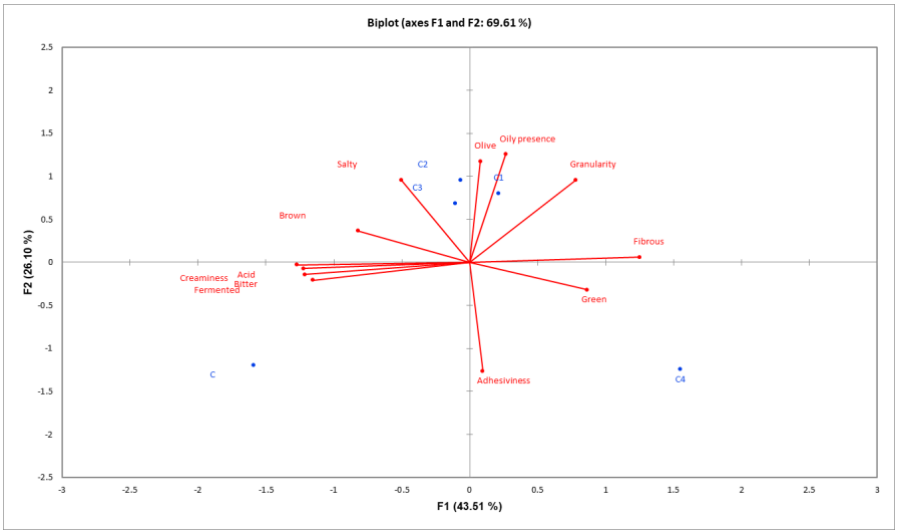

Figure 7.3 Biplot of the six olive pastes and the terms used to describe the samples made by General Procrustes Analysis (GPA) of data from flash profile.

Finally, it was possible to draw a Spider graph for each sample. The main purpose of this graph is to highlight the perceived intensity level of the descriptors of interest of the product studied in order, therefore, to have a direct comparison between the various products, enhancing the predominant characteristics (Randazzo et al., 2014).

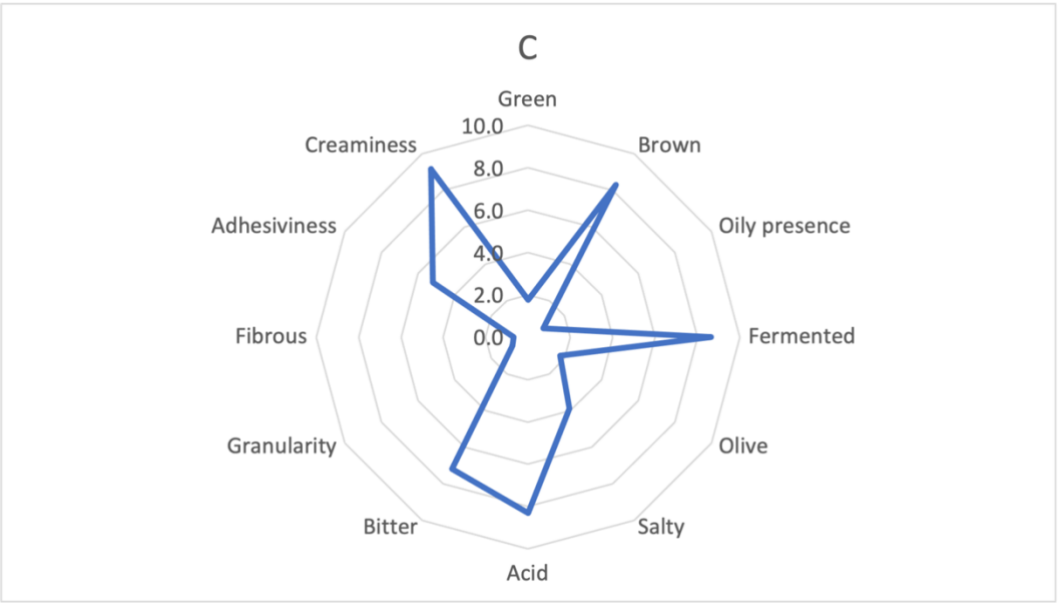

Figure 7.4. Spider graph of C sample

Figure 7.4 shows the spider web plot for sample C. The sample of the PRIN project analyzed, added with the sole presence of starter cultures, results to have a low intensity for the green, fibrous and flavor attributes of olives, while a ' higher intensity for brown, fermented, sour, bitter and creamy attributes. The high intensity of the bitter attribute could be due to the partial de-bittering of the

polyphenols present in the sample. As already mentioned, in fact, organic fermentation is unable to totally decrease the level of "bitterness" as in the chemical system, consequently a slight bitter taste remains which, in the case of table olives, is generally appreciated by the consumer (Lanza & Poiana, 2012). The creaminess is to be attributed to the fat content (Jellema et al., 2005). The fermented attribute could also be confirmed by the presence of butyric acid (Zhu et al., 2016) in the sample analyzed in previous chemical analyzes.

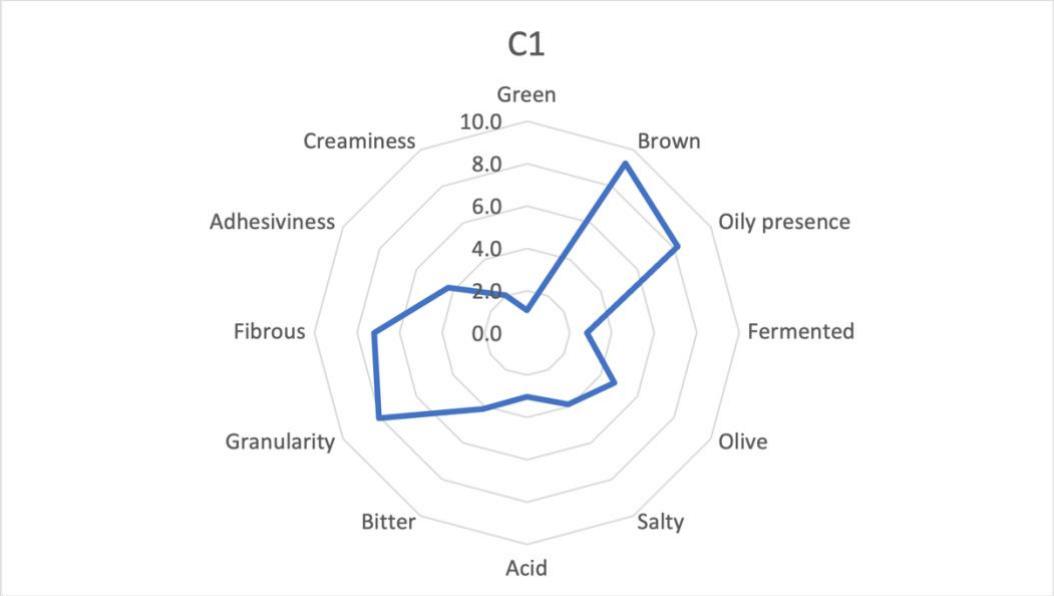

Figure 7.5. Spider graph of C1 sample

Figure 7.5 shows the spider graph for sample C1. It too has a high intensity for the brown attribute. In addition, it is characterized by the presence of an oily phase, fibrousness and graininess. Finally, it has a low intensity for attributes such as green, fermented, acid, bitter and creaminess. We therefore notice some differences compared to the previous sample as creaminess is not one of the characterizing attributes but, on the contrary, the sample appears with a fibrous and grainy appearance.

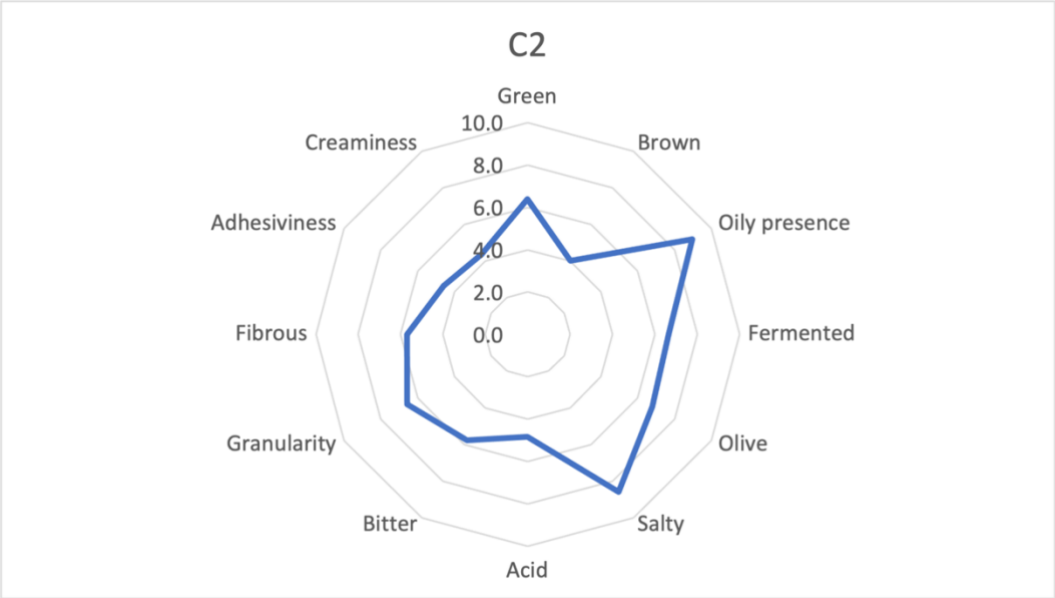

Figure 7.6. Spider graph of C2 sample

Figure 7.6 shows the spider web plot for sample C2. It has a low intensity for attributes such as brown, sour, bitter; a higher intensity for attributes such as oily, salty and green phase. It also differs strongly in its salty notes, rather than in the presence of attributes such as bitter and / or acid.

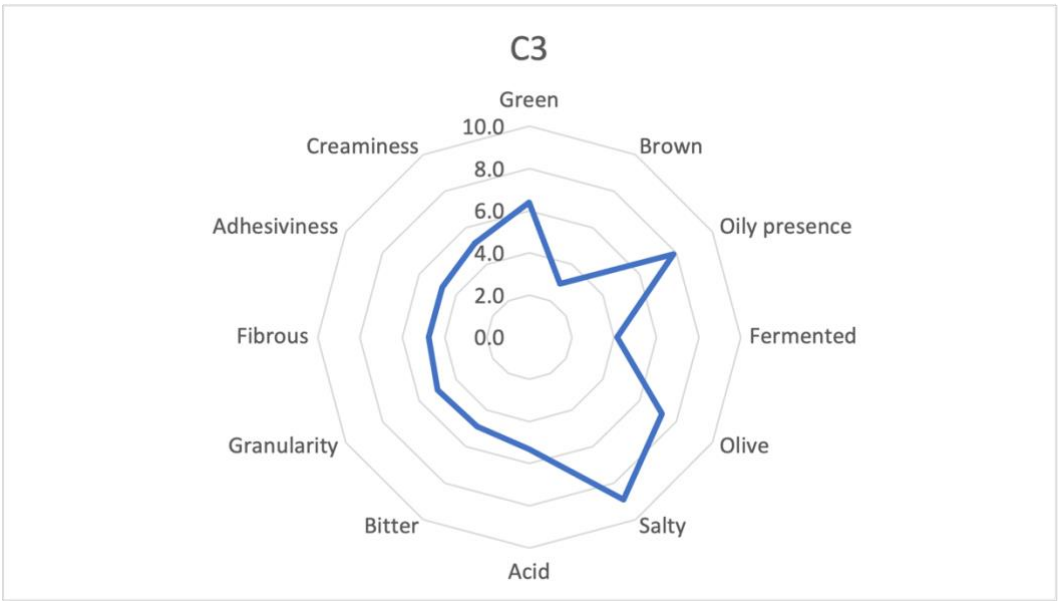

Figure 7.7. Spider graph of C3 sample

Figure 7.7 shows the spider web plot for sample C3. This sample has a high intensity for attributes such as salty and oily phase, while a low intensity was detected for the brown and fermented attributes.

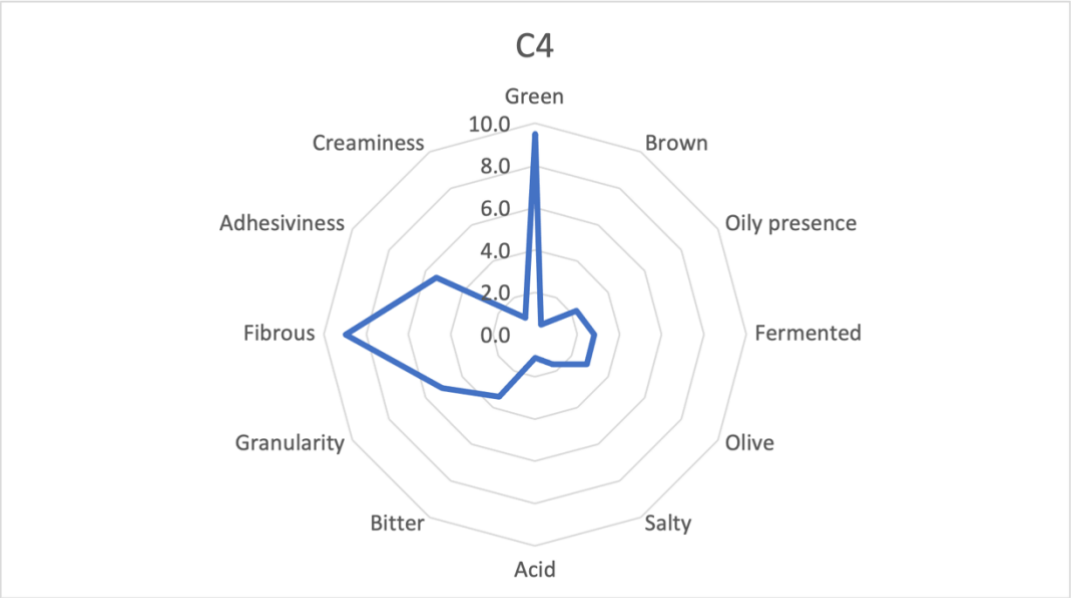

Figure 7.8. Spider graph of C4 sample

Figure 7.8 shows the spider web plot for sample C4. The sample, as can also be seen from the graph shown in **Figure 7.3**, also in this case is very different from the other samples. In fact, it has a low intensity for attributes such as brown, fermented, olive and acid taste, while it denotes a high intensity for attributes such as green and fibrousness.

Figure 7.9 shows the samples tested during the FP among which we highlight the first, C, belonging to the PRIN project and all the others purchased on the market.

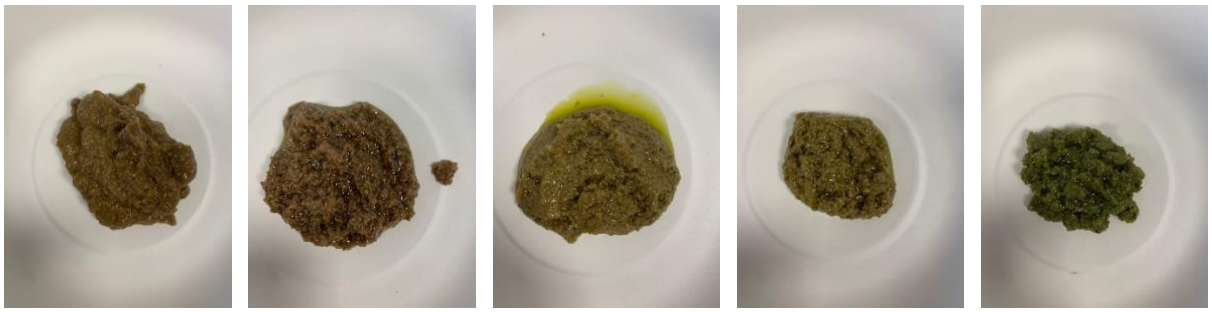

Figure 7.9. Samples tested during the FP among which we highlight the first, C, belonging to the PRIN project and all the others purchased on the market.

In conclusion, the various samples tested show different characteristics. The brown color, found mostly in samples C and C1, could be due to fermentation and the action of polyphenols oxidase enzymes which helps to create brown compounds, as already seen in the color analysis; in fact, the mechanism of the browning reaction in olives consists in an enzymatic release of 3,4-DHPEA from

the OLE by the action of β-glucosidase and esterase enzymes during fermentation (Ramìrez et al., 2015). Furthermore, the bitter attribute, characteristic of the first two samples, does not necessarily denote an incomplete de-bittering of the olive paste but could be due to the presence of some phenolic compounds, such as cresol, at the end of fermentation. This was demonstrated by Randazzo et al. (2014) who analyzed table olives fermented with the Spanish method with and without the addition of probiotics. In the study, all the samples found the presence of cresol, even if the olives with the addition of probiotics were the ones richest in this compound and, in fact, showed a more pronounced intensity of the unpleasant odor and taste descriptors, compared to the controls (only with presence of starters as in the case of sample C).

7.4. CONCLUSIONS

The purpose of this study was to evaluate the composition and oxidative stability of an innovative product obtained from pomace deriving from the processing of olives for the production of virgin olive oil. Specifically, it was a green olive pomace paste fermented using two strains *of Lactiplantibacillus (Lpb.) pentosus* with a de-bittering function and, subsequently, pasteurized. Following the pasteurization, two different formulations of probiotics (*Lpb. plantarum*) of human origin and isolated from fermented foods were added. In terms of total phenols, there was a loss of 24% and 47% of phenolic compounds in C samples after 5 and 10 days of storage, respectively, whereas a minor loss was detected in P1 (40 and 36%, respectively) and P2 (32 and 35%) samples, respectively. In particular, the highest variation was observed for 3,4-DHPEA-EDA, which completely disappears in P1 and P2 samples after 15 and 30 days of storage. From the determination of the total lipid profile, it emerged that the class of compounds most represented is that of TAGs (92.16-93.58%), followed by DAGs (3.48-3.73%), FFAs (1, 60-2.21%), by STE (0.84-0.93%), by E-STE (0.23-0.32%) and finally by MAG (0.11-0.17%). It can be said that in the samples analyzed a decrease in TAGs was found with a consequent increase in DAGs and long shelf-life FFAs, which would be attributable to a phenomenon of hydrolysis by probiotic strains or yeasts that have been shown to have lipolytic activity. As for the composition in total fatty acids, MUFA are the most abundant class (69.79-73.06%), followed by SFA(17.32-21.33 %) and PUFA (8.87-9.62%). The most present fatty acid is oleic acid (65.37-68.23%), followed by palmitic acid (9.80-11.10%), butyric acid (5.27-8.93 %) and linolenic acid (7.64-8.32%). The important MUFA content helps to increase the oxidative stability of the product. The significant presence of butyric acid, not typical for olive oil, could come from the metabolism of heterofermentative lactic bacteria which, by metabolizing lactic acid, produce this compound. All the ratios of fatty acids used as nutritional indices (PUFA/SFA, PUFA *n*-6/*n*-3) are

similar between the various samples and almost stable during the shelf-life; moreover, the atherogenic and thrombogenic indices are very low and fall within the recommended values (<1). Regarding oxidative stability, all products have shown high stability along the shelf-life; the specific extinction values at 232 nm show an increase over the shelf-life for samples inoculated with probiotics. From the color analysis it can be seen how this parameter tends to vary passing to browner tones during storage, which could be attributed to both the transformations of the chlorophylls and the oxidation of the polyphenolic compounds. Furthermore, the pasteurization treatment to which the samples were subjected before being inoculated with probiotics could also have influenced the specific extinction values and the color change over the shelf-life. From the analysis of phytosterols it emerges that β-sitosterol is the most present compound (83.15-141.17 mg/kg), followed by campesterol (2.21-3.59 mg/kg), by stigmasterol (0, 49-1.60 mg/kg) and finally Δ5-avenasterol (0.01 mg/kg). Based on the sterol content found in the analyzed olive paste (0.84-0.93%), approx. 200 g of olive paste per day to have a cholesterol-lowering effect. Finally, the olive paste in question was subjected to tasting by a panel of untrained consumers, together with 4 other commercial olive paste samples. From the Flash Profile carried out it was found that the sample formulated starting from the olive pomace and fermented is characterized by a bitter taste, dependent on the presence of polyphenols, acid, fermented and with a creamy appearance. The consensus of the panel demonstrated a strong presence of bitter and acid taste in these samples, so much so that the possibility of testing the olive paste with a Liking Test could be evaluated, to associate the sensory aspect with the emotional aspect to understand what moves the choice of the consumer in studies on the satisfaction of the product In conclusion, the present study has shown that the analyzed fermented olive paste is oxidative stable, and it would be interesting to carry out further studies*, in vitro* and *in vivo*, to understand and confirm the probiotic effect of its ingestion and the influence on the microbiota. In addition, it would be interesting to carry out a further sensory evaluation on the fermented olive paste with the addition of the two probiotics tested here for chemical-physical stability, in order to evaluate the existing differences with respect to the paste

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Chapter 8. Effect of *in vitro* digestion on phenols and antioxidant activity of three phenolic extracts of olive oil industry by-products

ABSTRACT

Living organisms are very complex systems in which a plethora of enzymatic reactions that require oxygen take place. To counteract such damages, organisms have developed several antioxidant defence systems. One of the most important areas of current research in nutrition lay in the search for compounds that can reduce the production or the reactivity of ROS. Besides, there is a general awareness for the quality and the sustainability in the nutritional products. All of this is causing both industry and basic research to focus on the search of natural and traditional products as supplements with a high antioxidant activity. In a clean label perspective of meat product formulation, synthetic food antioxidants could be replaced by natural extracts obtained from agri-food by-products and waste, which are rich in bioactive compounds (such as carotenoids, phenolic compounds, essential oils or β-glucans) that display different health properties, antioxidant and antimicrobial activities. In this work, three extracts rich in phenols obtained from purification of olive mill wastewaters (OMWW), were subjected to *in-vitro* digestion and characterized. The content and composition of phenols, condensed tannins and antioxidant activity was determined before and after *in-vitro* digestion.

KEYWORDS:

Phenolic extract, olive by-products, *in-vitro* digestion, antioxidant activity, DPPH, ORAC, ABTS, HPLC

8.1 INTRODUCTION

Living organisms are very complex systems in which a plethora of enzymatic reactions that require oxygen take place. Thus, molecular oxygen (O_2) is a main component of the metabolism and the production of energy, but it can also be present as very reactive short-lived derivatives (ROS, reactive oxygen species) such as superoxide ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (•OH). These molecules can cause cell damage, the most important ones affecting DNA and polyunsaturated fatty acids in the membrane (Wickens, 2001). To counteract such damages, organisms have developed several antioxidant defense systems. However, according to the ageing and the free radical theory (Harman, 1992) the efficiency of these protective systems decreases with age, and the accumulation of these species can favor the development of some diseases such as Alzheimer's and diabetes (Huang et al., 2016; Ullah et al., 2016).

One of the most important areas of current research in nutrition lay in the search for compounds that can reduce the production or the reactivity of ROS. Besides, there is a general awareness for the quality and the sustainability in the nutritional products. All of this is causing both industry and basic research to focus on the search of natural and traditional products as supplements with a high antioxidant activity (Krishnaiah et al., 2011; De Ciriano et al., 2011). In a clean label perspective of meat product formulation, synthetic food antioxidants could be replaced by natural extracts obtained from agri-food by-products and waste, which are rich in bioactive compounds (such as carotenoids, phenolic compounds, essential oils or β-glucans) (Carpentieri et al., 2021; Ramìres-Pulido et al., 2021) that display different health properties, antioxidant and antimicrobial activities. In this regard, one of the most interesting food by-products is olive mill wastewater (OMWW), which is generated during olive processing to produce olive oils and is characterized by a high content of organic compounds (sugars, tannins, pectins and phenolic substances) and mineral salts (Carrara et al., 2021). This composition, together with its high generation rate, makes OMWW a highly polluting by-product whose uncontrolled disposal such be avoided; in particular, large quantities of polyphenols can exhibit marked antimicrobial, phytotoxic and anti-nutritional properties, and resistance to degradation, thus leading to negative effects on ecosystems (Carrara et al., 2021). However, OMWW could be considered an exploitable source of hydrophilic phenols (mainly secoiridoids, which are found exclusively in the *Oleaceae* family) with antioxidant, antimicrobial and anti-inflammatory activity (Servili et al., 2014; Veneziani et al., 2017). Thus, phenols can be recovered from OMWW by using suitable membrane technology (Servili et al., 2011), for further applications in the food, pharmaceutical or cosmetic industries (Veneziani et al., 2017; Galanakis et al., 2018; Caporaso et al., 2018).

In this work, three extracts rich in phenols obtained from purification of olive mill wastewaters (OMWW), were subjected to *in-vitro* digestion and characterized. The content and composition of phenols, condensed tannins and antioxidant activity was determined before and after *in-vitro* digestion.

8.2 MATERIAL AND METHODS

8.2.1 Phenolic extract

Three samples of phenolic extracts (Spray-Dried, A20 and A21) obtained from the purification of olive mill wastewater (OMWW), an olive oil industry by-product, supplied by University of Perugia, were analyzed.

For Spray-Dried extract preparation a crude phenolic extract (PE) was obtained by a three-step membrane filtration of fresh OMWWs, as previously reported (Barbieri et al., 2021). The OMWWs derived from processing of olives from Moraiolo cultivar, which were harvested in Umbria (Central Italy). The PE was added with maltodextrin (1:1, d.w.) as a support, and then spray-dried to get a powder formulation of the PE.

To produce A20 and A21 phenolic extract, a mixture or fresh OMWWs of different olives cultivar obtained from different harvest years were filtered by a membrane system (microfiltration, ultrafiltration, and reverse osmosis) after 12 h enzymatic hydrolysis at 20 °C, to obtain a crude phenolic concentrate that was treated as follow: 100 mL of CPC were homogenized with 50 mL of ethyl acetate for 3 min and the organic phase was recovered (the process was repeated twice), added with anhydrous sodium and then filtered with a fluted filter to remove residual water. A rotavapor was employed to evaporate ethyl acetate at 35 °C, followed by dissolution with 5 mL of ethanol which was evaporated by nitrogen flow.

8.2.2 *In-vitro* digestion

The 3 samples were subjected to an *in-vitro* digestion process using the procedure described by Gayoso et al. (2018). Samples (200 mg for A20 and A21 and 500 mg for spray dried sample) were placed in three different Falcon tubes (Sigma) and dissolved in 12 mL of distilled water. Tubes were maintained at 37 °C in a water bath with continuous stirring. The pH of the solution was adjusted to 6.5 with 1 M sodium bicarbonate and oral digestion started in the three tubes by adding 125 µL α-amylase (1.3 mg/mL in 1 mM $CaCl_2$) for 2 min. At the end of this step pH was adjusted to 2.5 using 3 M HCl, and a tube was immediately frozen (-20°C). Pepsin (165 µL, 160 mg/mL in 0.1 M HCl) was added to the other two tubes to simulate the gastric digestion for 2 h, then, pH was brought up to 6.5 using 1 M sodium bicarbonate to inactivate pepsin and another tube was immediately frozen. The intestinal digestion was initiated in the remaining tube by adding 1250 µL of a 1:1 mixture of pancreatin (4 mg/mL) and bile extract (25 mg/mL) for 2 h, and pH was then adjusted to 7.5 using 1 M sodium bicarbonate. Then the tubes were stored at -20 °C, for a maximum of 2 days. The three tubes were thawed (4 °C) then, centrifuged at 4000 g (A-4-62 Rotor, Model 5810R centrifuge, Eppendorf, Barcelona, Spain) for 40 min at 4 °C and the supernatant, which is considered as the bioaccessible fraction, was collected and lyophilized for further analysis. The supernatant from the first tube represents the bioaccessible fraction from oral digestion (OD), and that from the second tube represents the bio-accessible fraction from gastric digestion (OD and GD) while the

supernatant from the third tube represents the bioaccessible fraction from intestinal digestion (ID, OD and GD). In parallel, non-digested samples were subjected to the same procedures in the absence of digestive enzymes. For each digestion three independent triplicates were done.

8.2.3. Total polyphenols content (TPC)

The TPC content was estimated using the Folin-Ciocalteu assay using the method of Singleton and Rossi (1965). Briefly, 15 µL of sample were mixed with 1185 µL of distilled water and 75 µL of Folin-Ciocalteu reagent. After incubation (2 min), 225 µL of sodium carbonate was added. After 2 h incubation, the absorbance was measured at 765 nm using a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). GA was used to plot a calibration curve and results were expressed as mg GA equivalents (E)/100 g dw phenolic extract.

8.2.4. Condensed tannins content (CTC)

CTC was estimated using the optimizations of Sun et al. (1998). Briefly, 250 µL extract was mixed with 625 µL vanillin (1% in methanol) and the same volume of sulphuric acid (10% in methanol), the mixture was incubated for 15 min and the absorbance was measured at 500 nm using the microplate reader. A calibration curve was plotted using C as the standard and results were expressed as mg CE/100 g dw of phenolic extract.

8.2.5. DPPH• inhibition

Antioxidant activity can be monitored using the scavenging effect of radicals on DPPH• (cat. no. D9132, Sigma-Aldrich Co., St. Louis, MO, USA) (Pinacho et al., 2015). To do that, 150 µL of the sample at different 10 concentration (A20 and A21 at the initial concentration of 25 ug/ml and spray dried powder at initial concentration of 2.5 mg/ml) was mixed with 150 µL of DPPH• 0.04 mg/mL (cat. no. G5767-25G, Sigma-Aldrich Co., St. Louis, MO, USA). The reaction was monitored every 15 min up to 90 min. Absorbance at 517 nm (spectrophotometer UV PowerWave XS, BioTek Instruments, Inc., Winooski, VT, USA) was used to calculate radical scavenging activity (% of inhibition) with the formula:

$$Inhibition\ (\%) = 1-(Abs_{sample}-Abs_{blank}/Abs_{control}-Abs_{blank})\times 100$$

where Abs_{sample} was the absorbance of the reaction in presence of sample (sample dilution+DPPH solution), Abs_{blank} was the absorbance of the blank for each sample dilution (sample dilution+DPPH solvent) and $Abs_{control}$ was the absorbance of control reaction (sample solvent+DPPH solution).

Then, this value obtained for every concentration was plotted to obtain EC_{50} values (concentration in which the 50% of the free radical DPPH is reduced) in each time point.

8.2.6. ABTS•+ inhibition

In a 96 well microplate, 18 µL of sample was mixed with 182 µL of ABTS•+ solution and incubated. After 6 min, the absorbance was measured at 734 nm using the microplate reader. T was used as standard compound to plot a calibration curve and results were expressed as mM TE/100 g dw phenolic powder (Re et al., 1999).

8.2.7. Oxygen radical absorbance capacity (ORAC)

The antiradical activity was further tested against the peroxyl radical AAPH using the ORAC method as described by Prior et al. (2003). The assay was done using the microplate reader in a 96 well microplate, where 40 µL of the sample were mixed with 120 µL of sodium fluorescein (132.5 nM in 10 mM potassium phosphate buffer) and fluorescence was immediately measured at T0 (excitation wavelength of 485 nm and emission wavelength of 520 nm). AAPH solution (40 µL, 0.3 M in 10 mM potassium phosphate buffer) was added and measurements were taken every 45 s for 1 h. The net area under the curve (AUC) of the standard (T) and the samples was calculated. The standard curve was obtained by plotting T concentrations (4–250 µM) against the average net AUC of the three measurements for each concentration. Final ORAC values were calculated using the regression equation between T concentrations and the net AUC and were expressed as mM TE/100 g dw phenolic extract.

8.2.8. High-performance liquid chromatography (HPLC)

Individual polyphenols were measured using a reversed phase HPLC- DAD using a Waters 2695 (Milford, MA, USA) 600 E multi-solvent de-livery system, a Waters U6K sampler and a Waters 2996 photodiode-array detector. Samples were dissolved in methanol at 20 mg/mL and filtered through 0.45 µm filters (Sigma). Injection volume was 20 µL. The separation was done on a C18 column (Nova-Pak, 150 ×3.9 mm, 4 µm, Waters) maintained at 25 °C. The detection wavelengths ranged from 210 to 550 nm. A mixture of acetonitrile (A) and acidified distilled water (pH 2 with orthophosphoric acid) (B) was used as the mobile phase, in the following proportions: 1–15 min, 10–20% A; 15–20 min, 20–40% A, 20–25 min 40-10% A, then it was maintained at 10% A until the end of the analysis. The flow rate was 0.8 mL/min. The amount of each compound was expressed as mAUC. It was calculated before and after digestion (Pinacho et al., 2015).

8.2.9. Statistical analysis

All experiments were done in triplicate and data were analyzed using XL-STAT. The results were expressed as mean values and standard deviation (SD). The differences between the non-digested and digested samples were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's significant difference post hoc test with p=0.05. For DPPH•, EC_{50} values were generated with GraphPad Prism v6.01 (GraphPad Software, La Jolla, CA, USA).

8.3. RESULTS AND DISCUSSION

8.3.1 Phenolic content

Total phenolic (TP) and condensed tannins (CT) content of the three extract was measured before (ND) and after *in-vitro* digestion (OD, GD and ID). As shown in **Figure 8.1** samples A20 and A21 show a significantly higher (p<0.05) TP content than Spray-Dried (SP) sample, both before and after *in-vitro* digestion. However, it can be noted that for samples A20 and A21 the phenol content tends to decrease by more than half, while for SP sample it remains constant, probably thanks to the presence of maltodextrins that protect the phenols during digestion.

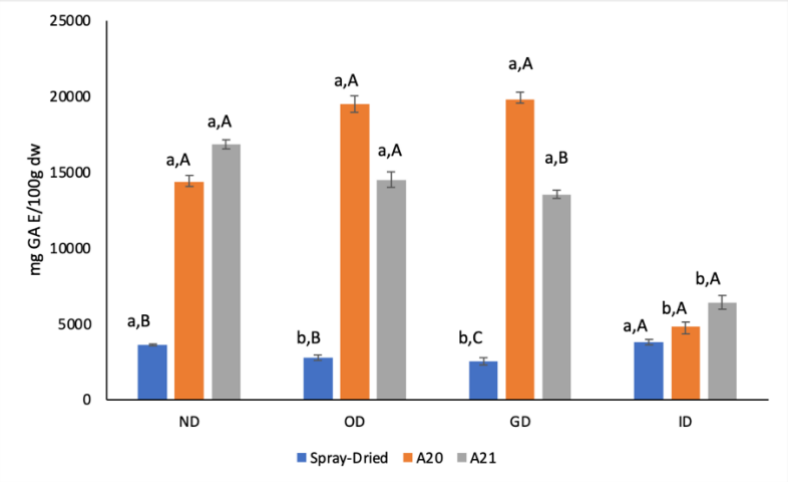

Figure 8.1 Effect of *in vitro* digestion on the phenolic classes of the three phenolic extracts. ND: non-digested; OD: oral digestion; GD: gastric digestion; ID: intestinal digestion. Values are means of triplicates ± standard deviation (SD), a-c, statistically different means during digestion (p≤0.05), A-C, statistically different means between samples (p≤0.05).

In **Figure 8.2** CT content was shown. The ND samples shown a significantly lower content of CT compared to samples after *in-vitro* digestion. CT content was 2.3, 3.9 and 7.1 times higher after OD, 4.9, 4.2 and 5.5 times higher after GD and 9.6, 6.2, 10.1 times higher after ID in SP, A20 and A21 samples respectively. Campos-Vega et al. (2015) reported the release of condensed tannins during intestinal digestion, in addition, condensed tannins are oligomeric or polymeric catechins, flavan or

flavan-3-ols; they are more subjected to degradation by digestive enzymes and alkaline pH, in fact, during the digestive process, these molecules are split into monomeric and dimeric units (Adarkwah-Yiadom & Duodu, 2017). The vanillin assay, method used in the present study to determine CT content, is sensitive to monomers and dimers formed during the digestion process this may lead to an increase in their reactivity, giving higher values in the spectrophotometric analytical determination of CT (Sun et al., 1998).

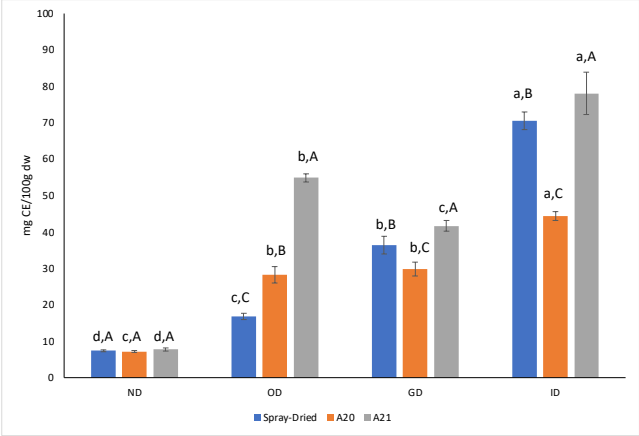

Figure 8.2 Effect of *in vitro* digestion on the condensed tannins of the three phenolic extracts. ND: non-digested; OD: oral digestion; GD: gastric digestion; ID: intestinal digestion. Values are means of triplicates ± standard deviation (SD), a-d, statistically different means during digestion ($p \leq 0.05$), A-D, statistically different means between samples ($p \leq 0.05$).

8.3.2 Antioxidant Activity

The antioxidant activity of SP, A20 and A21 extract was measured against DPPH•, ABTS+• and the peroxyl radical AAPH in the ORAC assay. The effect of *in-vitro* digestion on the antioxidant activity of the three extract is shown in **Figures 8.3** and **8.4** and in **Table 8.1**.

ABTS (**Figure 8.3**) and ORAC (**Figure 8.4**) show how in the A20 and A21 samples this tends to decrease during in vitro digestion, while for the spray dried sample it remains almost constant throughout the in vitro digestion process.

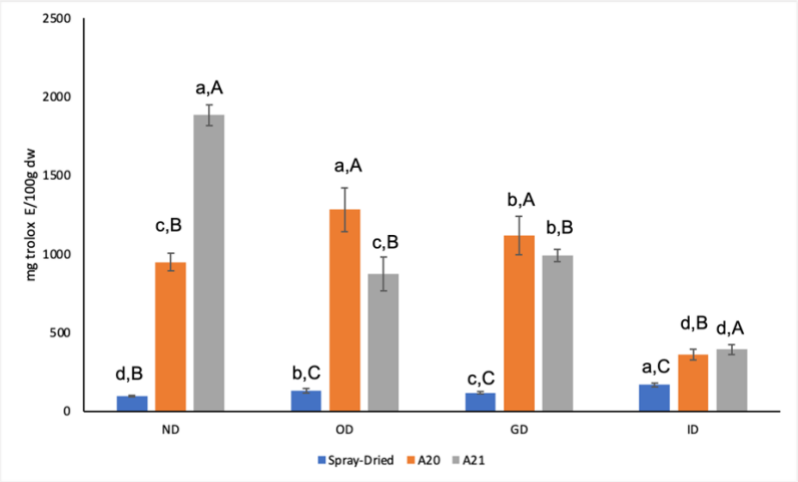

Figure 8.3 Effect of *in vitro* digestion on ABTS inhibition of the three phenolic extracts. ND: non-digested; OD: oral digestion; GD: gastric digestion; ID: intestinal digestion. Values are means of triplicates ± standard deviation (SD), a-d, statistically different means during digestion ($p \leq 0.05$), A-C, statistically different means between samples ($p \leq 0.05$).

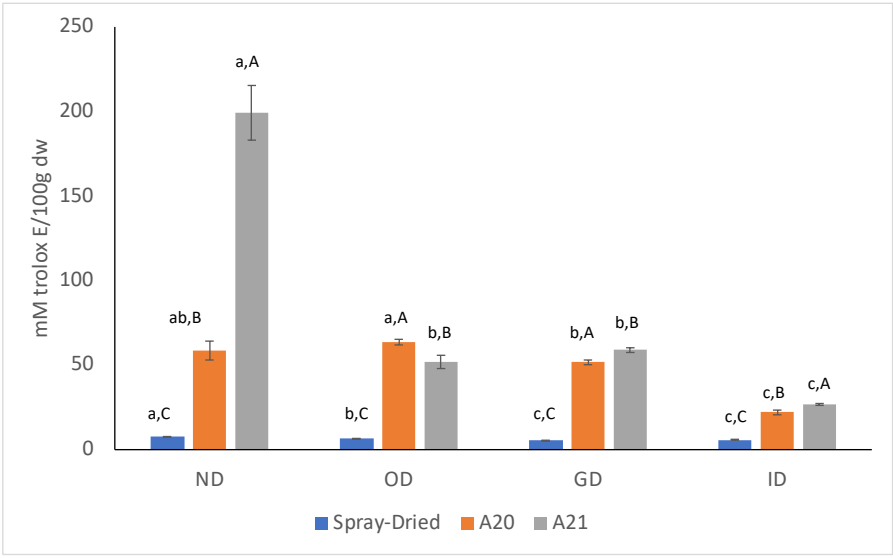

Figure 8.4 Effect of *in vitro* digestion on ORAC assay of the three phenolic extracts. ND: non-digested; OD: oral digestion; GD: gastric digestion; ID: intestinal digestion. Values are means of triplicates ± standard deviation (SD), a-c, statistically different means during digestion ($p \leq 0.05$), A-C, statistically different means between samples ($p \leq 0.05$).

A different speech must be made for the DPPH (**Table 8.1**), the value of IC_{50} tends to increase in the A20 samples, while the A21 sample seems to lose its antioxidant capacity during the intestinal phase of digestion. The SP sample, on the other hand, shows to increase its antioxidant capacity, the IC_{50} value becomes lower, probably thanks to the maltodextrins that help protect the phenolic compounds during digestion.

Table 8.1 Effect of *in vitro* digestion on DPPH inhibition of the 3 phenolic extracts calculated as IC_{50}.

			15	30	45	60	75	90
			min					
Spray-Dried	IC_{50} µg Trolox E/mg	ND	169.08±20.31^{a,A}	156.06±15.91^{a,A}	151.85±13.08^{a,A}	147.62±10.71^{a,A}	159.11±10.29^{a,A}	159.59±12.05^{a,A}
		OD	102.24±14.99^{c,A}	96.90±15.85^{c,A}	95.73±16.74^{c,A}	94.48±16.89^{c,A}	96.66±16.95^{c,A}	99.39±15.70^{c,A}
		GD	143.29±10.12^{b,A}	131.03±8.24^{b,A}	128.86±7.87^{b,A}	131.68±7.35^{b,A}	133.94±7.71^{b,A}	134.77±7.06^{b,A}
		ID	53.36±1.69^{d,A}	49.48±1.70^{d,A}	49.87±2.01^{d,A}	52.52±2.11^{d,A}	53.99±2.78^{d,A}	75.34±12.14^{d,A}
A20		ND	2.64±0.21^{c,C}	2.14±0.16^{d,C}	2.41±0.23^{c,C}	2.25±0.23^{d,C}	2.18±0.23^{c,C}	2.11±0.22^{c,C}
		OD	17.75±1.64^{b,C}	17.67±2.14^{c,B}	19.14±2.95^{b,B}	17.09±2.95^{c,B}	15.41±1.55^{b,B}	15.17±1.03^{b,B}
		GD	22.47±0.87^{a,C}	21.47±0.80^{b,B}	21.27±1.20^{b,B}	21.33±1.20^{b,B}	21.23±1.41^{a,B}	21.40±1.43^{a,B}
		ID	0^{d,B}	36.74±0.00^{a,B}	31.52±1.70^{a,B}	27.81±1.70^{a,B}	0^{d,B}	0^{d,B}
A21		ND	7.36±0.54^{b,B}	6.91±0.40^{c,B}	6.56±0.29^{c,B}	6.21±0.26^{c,B}	5.93±0.28^{c,B}	5.64±0.29^{c,B}
		OD	25.35±2.14^{a,B}	15.53±1.73^{b,B}	15.47±2.09^{b,C}	14.17±1.29^{bC,}	14.43±1.65^{b,C}	13.70±0.96^{b,C}
		GD	22.47±1.57^{a,C}	21.47±0.85^{a,B}	21.27±1.27^{a,B}	21.33±1.25^{a,B}	21.23±1.26^{a,B}	21.40±1.38^{a,B}
		ID	0^{c,B}	0^{d,C}	0^{d,C}	0^{d,C}	0^{d,B}	0^{d,B}

GD, gastric digestion; ID, intestinal digestion; ND, non-digested; OD, oral digestion. Values are means of triplicates ± standard deviation (SD), a-d, statistically different means during digestion ($p \leq 0.05$), A-C, statistically different means between samples ($p \leq 0.05$).

Burgos-Edwards et al. (2017) reported that the effect of simulated GI digestion on the antioxidant activity depended on the assay being used showing higher or lower values. Pavan et al. (2014) furthermore, it points out how the different methods used may not detect the structural transformation in polyphenols and their related activity the same way.

8.3.3 Extracts characterization

The compounds identified in SP, A20 and A21 extracts using HPLC-DAD are shown in **Table 8.2** along with their bioaccessibility. The analysis showed the presence of 5 compounds, 3,4-DHPEA, hydroxytyrosol; 3,4-DHPEA-EDA, oleacein; *p*-HPEA-EDA, ligstroside-aglycone di-aldehyde; *p*-HPEA, tyrosol and VB, verbascoside.

The composition and concentrations of phenolic compounds in virgin olive oil (VOO) and its by-products depend on several factors, mainly environmental (soil and climate), agronomic (irrigation, fertilization), cultivation (harvesting and ripeness), and technological questions (post-harvest storage and extraction system). It is also important to point out how the different varieties of olive fruits are mainly responsible for the different phenolic profiles of VOO and its by-products (Reboredo-Rodríguez et al., 2021).

Table 8.1 Effect of *in vitro* digestion on the phenolic content (AUx10^6) of three phenolic extracts.

		3,4 DHPEA	*p*-HPEA	3,4 DHPEA-EDA	*p*-HPEA-EDA	VB
Spray-Dried	ND	6.64±0.67^{c,C}	0.20±0.01^{c,C}	1.07±0.06^{a,C}	*n.d.*^{C}	1.10±28.99^{a,B}
	OD	7.18±0.50^{b,C}	0.21±0.06^{c,C}	*n.d.*^{c,C}	*n.d.*^{C}	0.91±15.69^{b,B}
	GD	5.18±0.01^{d,B}	0.30±0.01^{b,C}	0.39±0.01^{b,C}	*n.d.*^{C}	0.82±12.19^{c,B}
	ID	8.03±0.07^{a,B}	0.40±0.07^{a,B}	*n.d.*^{c,C}	*n.d.*^{B}	0.78±14.88^{d,A}
A20	ND	12.56±0.38^{b,B}	3.81±0.21^{b,A}	72.40±0.48^{a,A}	9.50±0.19^{a,A}	2.52±0.06^{a,A}
	OD	17.89±0.83^{a,B}	7.32±0.40^{a,A}	46.35±0.47^{b,A}	7.25±0.20^{b,A}	1.39±0.22^{c,A}
	GD	5.69±0.26^{c,B}	2.47±0.39^{c,A}	49.20±0.57^{b,A}	6.97±0.24^{c,A}	1.95±0.05^{b,A}
	ID	5.50±0.13^{c,C}	*n.d.*^{d,C}	3.89±0.07^{c,B}	*n.d.*^{d,B}	*n.d.*^{d,C}
A21	ND	14.76±1.78^{b,A}	2.16±0.07^{a,B}	53.15±5.64^{a,B}	3.58±0.11^{a,B}	0.63±0.03^{a,C}
	OD	31.10±1.15^{a,A}	2.41±0.12^{a,B}	33.60±1.36^{b,B}	2.30±0.13^{b,B}	0.50±0.01^{b,C}
	GD	8.87±0.41^{d,A}	0.88±0.02^{b,B}	12.32±1.11^{c,B}	1.04±0.02^{c,B}	0.37±0.01^{c,C}
	ID	9.15±0.16^{c,A}	0.96±0.02^{b,A}	6.22±0.74^{d,A}	0.73±0.01^{d,A}	0.05±0.00^{d,B}

3,4-DHPEA, hydroxytyrosol; 3,4-DHPEA-EDA, oleacein; *p*-HPEA, tyrosol; *p*-HPEA-EDA, ligstroside-aglycone di-aldehyde; GD, gastric digestion; ID, intestinal digestion; ND, non-digested; OD, oral digestion; VB, verbascoside. Values are means of triplicates ± standard deviation (SD). a-d, statistically different means during digestion ($p \leq 0.05$), A-C, statistically different means between samples ($p \leq 0.05$).

Samples A20 and A21 appear to have a higher phenol content than the SP sample, especially before undergoing *in-vitro* digestion. It is also possible to note that the *p*-HPEA-EDA compound was not found in the SP sample. This could be due to the fact that the A20 and A21 samples were obtained starting from the purification of a mix of OMWW obtained from different cultivars and in several years of collection, while the SP sample was obtained from the purification of the OMWW obtained from the Moraiolo cultivar. By analyzing the data obtained, it is possible to note how the pH during the various phases of digestion influences the behavior of each phenolic compound. The 3,4 DHPEA-EDA in the presence of the pH of 6.5 of the OD is not detected in the SP sample or decreases in the samples A20 and A21, with a consequent increase of the 3,4 DHPEA. During GD, however, the pH drops to values of 2.5 and there is a decrease in the content of 3,4 DHPEA, with an increase of 3,4 DHPEA-EDA. Finally in the ID the pH rises to values of 6.5 and there is a decrease of 3,4 DHPEA-EDA and an increase of 3,4 DHPEA. A similar behavior was found in samples A20 and A21 as regards *p*-HPEA and *p*-HPEA-EDA. Finally, as regards VB, this compound tends to decrease during all phases of digestion, until it disappears completely in sample A20 during ID. It has also been shown that the enzymes used during the various phases of in vitro digestion also influence the behavior of the various molecules and their destruction-formation (Reboredo-Rodríguez et al., 2021). Although samples A20 and A21 were those with a higher content of phenols before in-vitro digestion, it was seen that the maltodextrin encapsulation used in the SP sample was able to protect the phenolic compounds and allow them to exert their action. antioxidant during all stages of digestion.

8.4. CONCLUSIONS

From the chemical characterization of undigested and *in-vitro* digested extracts obtained from purification of olive mill wastewaters, it emerged that the most promising extract to be used in the food field is the spray-dried one. Thanks to its formulation with maltodextrins, it manages to maintain its antioxidant capacity even after undergoing *in-vitro* digestion. In fact, thanks to its ease of use, its stability, and the fact that it can be stored at room temperature, unlike the A20 and A21 extracts which must be stored at -20°C, the SP extract could be an excellent ingredient with antioxidant function to use in the formulation of innovative meat foods with a reduced content of additives.

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Chapter 9. Conclusions and future outcomes

The aim of this thesis work was the valorization of the main by-products obtained from olive oil industry (wastewater and pomace) and their utilization in innovative food formulation.

Specifically, an olive mill wastewater (OMWW) extract rich in phenols (PE) was used in the formulation of 3 innovative meat products (beef hamburgers, cooked ham and würstels). Another experiment was focused on the analysis of the lipid composition and oxidative stability of a spreadable product obtained from the olive pomace during its shelf-life. Finally, 3 extracts obtained from the purification of olive mill wastewater were subjected to *in-vitro* digestion and their antioxidant composition and capacity were characterized

- The main conclusions of the different studies are summarized as follows: The first study demonstrated the efficacy of the powder formulation of a phenolic extract from olive vegetation water (PE) at improving the overall oxidative stability and sensory quality of raw and grilled beef hamburgers, which had been previously subjected to cold storage for 9 days. Both PE concentrations tested (87.5 and 175 mg of phenols/kg meat) proved to effectively reduce primary and secondary lipid oxidation, as well as cholesterol oxides, during the burgers' shelf-life study and after cooking. In particular, PV, TBARs and total COPs were up to 1.4-, 4.5- and 8.8-fold lower in PE raw hamburgers, respectively, than in control samples; a similar trend was also noted in cooked hamburgers (1,3-, 5.7- and 4-fold lower). Sensory analysis also confirmed the effectiveness of PE addition in beef hamburgers, having a positive effect especially on the intensity of the red color (raw product) as it resulted in a reduction of browning during storage. Furthermore, the presence of phenols was not perceived by panelists, so they did not negatively influence the organoleptic characteristics of the products. However, the discriminant test evidenced a qualitative decay of all products during storage, which was more relevant in the control and the phenol-enriched burgers at the highest PE dose.
- The second study demonstrated the efficacy of the PE to limit the formation of COPs in grilled beef hamburgers, which had been previously subjected to cold storage for 9 days. Very low values of HCAs (<0.5 ng/g of meat) were detected in all grilled samples, therefore it was not possible to evaluate the effect of PE on this parameter. A genotoxic effect was observed in the meat extracts without phenols (C); the damage was about twice as high as that of DMSO and it did not vary according to the storage time. Inclusion of the phenols in the meat during cooking significantly reduced the DNA damage and this effect was more evident at the lower

phenol dose (L1). For the mutagenicity, the different meat extracts did not induce a significant increment of revertants compared to the control. Although the inclusion of phenols slightly reduced the revertants number, this effect was not statistically significant.

- The third study demonstrated the efficacy of PE at improving the overall oxidative stability of cooked ham, which has been previously subjected to cold storage for 30 days. During the shelf-life study, sample S1 (PE 200 mg/kg and NO_2 150 mg/kg) showed the best oxidative stability, with a TBARs value close to 1.0 mg MDA/kg of meat (reference value for rancidity development in cooked pork meat), while the control sample had significantly higher TBARs values (< 3.80 mg MDA/kg of meat). S2 (PE 200 mg/kg and NO_2 35 mg/kg) and S3 (PE 200 mg/kg without NO_2) displayed a similar oxidative trend, with TBARs values below 1.45 mg MDA/kg of meat; therefore, it is possible to hypothesize that the antioxidant activity in both S2 and S3 was mainly due to phenols, while nitrites in S1 were more involved in the development of color and in the microbial stabilization of the product. Regarding COPs and COR%, no significative differences were found between PE and control cooked ham samples.
- The fourth study demonstrated the efficacy of PE at improving the overall oxidative stability and sensory quality of steam cooked and grilled würstels, which had been previously subjected to cold storage for 30 days. In general, the steam-cooked control sample showed an increase in both conjugated dienes and trienes during storage, while these parameters remained almost constant in ungrilled PE samples. After grilling, all samples exhibited the classical bell-shape behavior of primary oxidation products. Regarding secondary lipid oxidation, grilled samples had TBARs values that were about 2 times higher than those of steam-cooked würstels, confirming the pro-oxidant effect of grilling. However, surprisingly, TBARs in both control steam-cooked and grilled würstels were lower than those of the phenol-enriched samples during the shelf-life. This behavior might be partly due to the thermolysis and hydrolysis of phenols during the cooking treatments; moreover, during grilling at 220°C, phenols might have been involved in the Strecker reaction, preventing them from exerting their antioxidant role. Regarding COPs and COR%, no significative differences were found between PE and control steam-cooked würstel samples. However, no PE concentration-dependent effect was found. Discriminant sensory analysis confirmed the excellent stability of the products along the shelf-life study and no taste anomalies were detected for any product. The key attribute for distinguishing among samples was color, even though a certain difficulty was found for differentiating between samples C (NO_2 150

mg/kg + maltodextrins 0.35 g/100 g) and W2 (PE 200 mg/kg of phenols and 150 mg/kg of NO_2).

- The purpose of the fifth study was to evaluate the lipid composition and oxidative stability of an innovative product obtained from a fermented and biologically de-bittered olive pomace In terms of total phenols, there was a loss of 24% and 47% of phenolic compounds in C samples after 5 and 10 days of storage at 2°C, respectively, whereas a minor loss was detected in P1 (added with probiotic strain of human origin *Lpb. plantarum* IMC513) (40 and 36%, respectively) and P2 (added with a mixture of two *Lpb plantarum* strain, isolated from fermented foods) (32 and 35%) samples, respectively. A decrease in TAG was found in all samples, C, P1 and P2, with a consequent increase in DAG and FFA, which could be attributed to an hydrolytic phenomena induced by probiotic strains. As for the composition in total fatty acid classes, its distribution was similar to that of virgin olive oil; a significant presence of butyric acid (5.27-8.93 %) was detected, which could derive from the metabolism of heterofermentative lactic bacteria that generate this fatty acid from lactic acid. All products showed a high stability along the shelf-life, but their color tends to shift to browner tones during storage, which could be attributed to both chlorophylls' conversion into the corresponding derivatives free of Mg (mostly pheophytins) and the oxidation of the polyphenolic compounds. Furthermore, the pasteurization treatment to which the samples were subjected before being inoculated with probiotics could also have influenced the specific extinction values and the color change over the shelf-life. Finally, the Flash Profile evidenced that the product C, formulated starting from the olive pomace and fermented, is characterized by a bitter, acid and fermented taste, with a creamy appearance.
- Finally, from the chemical characterization of undigested and *in-vitro* digested extracts obtained from purification of olive mill wastewaters, it emerged that the most promising extract to be used in the food field is the spray-dried one. Thanks to its formulation with maltodextrins, it manages to maintain its antioxidant capacity even after undergoing *in-vitro* digestion.

This thesis work demonstrated how the main by-products from the olive oil production chain (wastewater and pomace) represent an excellent source of bioactive molecules, which can be used in the food sector for the production of clean label and innovative food products. In the future, it would be interesting to test these phenolic extracts in other types of food and non-food products

and to perform more in-depth studies on their impact on human health with a combined *in vitro* and *in vivo* approach.