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FORMATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN COOKED MEAT AND THE POSSIBILITY OF REDUCING THEIR FORMATION BY VITAMIN E

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Dedication

"*The family is the first essential cell of human society." – Pope John XXIII*

To my family, the only safe haven I know.

To my father Fatos, my guardian angel and lantern in the darkness. Hope your hand will hold mine as you have done always and forever.

To my mother Lavdi, my first and lifetime teacher. Grateful for every lesson learned and for the precious human heritage you are passing on every day.

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To my nephews Mattia and Nikolas, my lifeline.

To all people who brought out the best in me and let me be myself.

S.

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs), known as potential human carcinogens, have received an increased attention in the recent year, due to their source diversity. Human exposure to PAHs occurs primarily through dietary intake. As diet contributes to 88–98% of exposure, this rising concern results reasonable and of great importance. PAHs generation in food and in particular in meat is due to high-temperature cooking methods such as grilling. The European Union has established PAH4 (sum of benz[a]anthracene, chrysene, benzo[b]fluoranthene and benzo[a]pyrene) as the most appropriate indicator for the occurrence and carcinogenic potency of PAHs in food. In the light of the emerging issue health risk from exposure to PAHs and in order to provide useful information on the PAHs contamination of meat, different control measures aimed in inhibiting or reducing PAHs formation have been attempted and discussed worldwide. Among them mitigation strategies consisting in the use of antioxidants has gain particular attention. The present study, therefore, aimed to study the effect of vitamin E, potent natural antioxidant, in the formation of PAHs in cooked meat. For this purpose, two trials were carried out:

1. Beef and chicken meat model systems: vitamin E was added to the ground meat prior cooking. The aim of these two trials was to investigate how PAHs formation respond to different concentrations of vitamin E added before meat grilling;
2. Animal model: vitamin E was intramuscularly injected to broiler chickens in order to study how endogenous tissues levels of vitamin E could affect PAHs formation.

In the first two trials (beef and chicken model systems) different concentration of Vitamin E (*dl* α-tocopheryl acetate) were added to beef and chicken burgers prior grilling. For each meat type, the experimental groups (n=2 burgers for each group) consisted in the control group (C) and groups added with *dl* α-tocopheryl acetate: 2 μg/g (C1); 3 μg/g (C2); 4.5 μg/g (C3) and 6 μg/g (C4) respectively. Burgers of 80 g each were directly cooked in disposable barbecue obtaining well-done level of doneness. For the animal model trial, ten 43-d-old Ross 308 male chickens were divided into two equal weight groups. Each week, from the beginning of the study, 5 birds received intramuscular injections, in the left Pectoral muscle, of *dl*–α–tocopheryl acetate (50 mg/ mL) (Vitalene® E, Fatro, Bologna). The total doses for birds were as follows: 0 IU (Control group) and 100 IU (Group Vit E). Control group birds were given injections of physiological saline. Chicken burgers obtained from breast meat of control chickens (C, n =5) and vitamin E injected chickens (T, n =5) were prepared and

grilled following the same conditions as in the first trial. An acetonitrile based-extraction and high-performance liquid chromatography with fluorescence detection were used to assay 14 PAHs in meat samples. Data of both trials were analysed by one-way ANOVA.

The obtained results can be summarized as follows:

Trial with beef meat model system:

In relation to the total content of PAHs, no effect was observed in hamburgers added with 2 μg/g of vitamin E, while a significant reduction was found in those added with 3 and 4.5 μg/g compared to control samples. Higher vitamin levels reduced the formation of heavy and light PAHs. Benzo[a]pyrene (BaP) was detected only in beef burgers of control (0.06 ng/g) and in those added with 2 μg/g and 6 μg/g of vitamin E, while in other groups was below the LOQ (< 0.04 ng/g). Considering that several are the factors which can influence the formation of PAHs, such as the total lipid and fatty acid composition of meat, the same study was carried out on chicken meat, and the results are showed in the following trial.

Trial with chicken meat model system:

Raw chicken meat samples used presented an endogenous vitamin E content of 5.54 μg/g; total lipid content was 2.08 g/100g and the fatty acid profile was characterized by a higher proportion of MUFA (42.12%), followed by SFA (36.46%) and PUFA (21.42%).

Regarding the effect of vitamin on PAHs formation in grilled chicken, all vitamin E added groups were characterized by a significant lower total PAHs content as compared to C group. The control group was characterized by the highest PAH4 content (4.54 ng/g), while the addition of vitamin E to chicken patties reduced significantly the total PAH4 amount at all the concentration used. In all burgers added with vitamin E was observed a highly significant reduction of BaP content.

Animal model trial

As expected, meat from vitamin E treated group had higher content of tocopherol (5-fold) compared with the control one (2.93 μg/g). Total lipid content was similar between groups. Regarding the fatty acid profile, comparison of the experimental groups revealed no differences in MUFA and PUFA content; while a lower SFA content was found in treated chickens compared to control group

In grilled chicken, the concentrations of PAH4 were similar between groups (2.68 ng/g and 2.15 ng/g for control and vitamin E, respectively), values well below the maximum level of 30 ng/g established by the EU regulation. As well as, the concentration of BaP was similar between groups and lower (0.17 – 0.21 ng/g) than the maximum levels of 5 ng/g established by the EU regulation. Considering

these preliminary results, further research is needed with a higher number of samples and with a wider range of doses of vitamin E and a different timeline to better understand the pharmacokinetics of vitamin E in relation to its role on formation PAHs in grilled meat.

The results of this research could provide a theoretical basis to better understand the potential inhibitory role of vitamin E on the PAH formation and to gain insight into how the muscular endogenous levels of vitamin E could be a preventive agent for mutagen formation in grilled meat.

PART 1

INTRODUCTION

POLYCYCLIC AROMATIC HYDROCARBONS IN COOKED MEAT: PUBLIC CONCERN AND MITIGATION

1.1 Physical and chemical characteristic of polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs), over 200 organic compounds composed of two or more fused aromatic rings, constitute widespread ubiquitous pollutants (Zelinkova and Wenzl, 2015). Their aromatic/benzene rings are composed not solely of carbon and hydrogen but can contain heteroatoms, presenting different structural arrangements with varied relative physical and chemical characteristics. (Gupte et al., 2016). These aromatic rings can show in space a linear, angular, or in clusters molecular arrangement (Abdel-Shafy and Mansour, 2016; Figure 1).

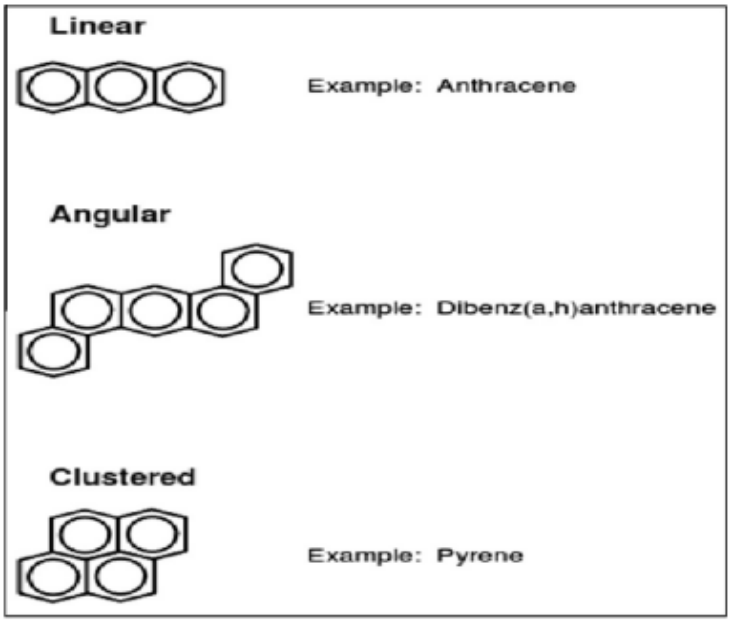


Figure 1. Molecular arrangement of PAHs. (*Source: Abdel-Shafy and Mansour, 2016)*

According to the molecular weight, PAHs are categorized in two groups: light-molecular weight PAHs formed by two or three benzene rings (LMW PAHs) and high-molecular weight PAHs having

four or more aromatic rings (HMW PAHs). Depending on this classification, LMW PAHs are generally emitted in gaseous phase, while HMW PAHs in the particulate form (Lee and Vu, 2010). The molecular weight generally varies from 166 to 328 g $mole^{-1}$ and depends on the position of fused rings and other compounds (Gupte et al., 2016). Considering instead the structure of rings they could be classified as alternant and non-alternant; the first contain only fusion of six carbon benzene rings, while the non-alternant PAHs contain fusion of six carbon benzene rings along with an additional ring of less than six carbon (Gupte et al., 2016).

Mostly colorless to white or pale-yellow-green, they are flat, crystalline solids and present high melting and boiling points but low water solubility, low vapor pressure. In particular, with the increasing of molecular weight is observed a decrease of water solubility and vapor pressure, while lipophilicity increases (Masih et al., 2012), making them very persistent and bioaccumulative compounds (Okere and Semple, 2011). All these characteristics justify the continuous study of their chemical structure, formation mechanisms, bioavailability, toxicity and health effects (EFSA 2008; Wenzl and Zelinkova, 2018). The table 1 presents the chemical and physical properties of most represented polycyclic aromatic hydrocarbons present in food.

Table 1. Chemical and physical properties of PAHs. *(Source: Sampaio et al., 2021)*

Compound name	Abbreviation	Chemical Formula	Molecular Weight (g/mol)	Melting Point (°C)	Boiling Point (°C)
5 -Methylchrysene	5MC	$C_{19}H_{14}$	242.3	118	458
Acenaphthene	ACE	$C_{12}H_{10}$	154.2	203	279
Acenaphthylene	ACY	$C_{12}H_{8}$	152.2	92-93	265-275
Anthracene	ANT	$C_{14}H_{10}$	178.2	218	340-342
Benz[a]anthracene	BaA	$C_{18}H_{12}$	228.3	158	438
Benzo[a]pyrene	BaP	$C_{20}H_{12}$	252.3	179	495
Benzo[b]fluoranthene	BbF	$C_{20}H_{12}$	252.3	168	481
Benzo[c]fluorene	BcF	$C_{17}H_{12}$	216.3	125-127	398
Benzo[e]pyrene	BeP	$C_{20}H_{12}$	252.3	177	492
Benzo[ghi]perylene	BPe	$C_{22}H_{12}$	276.3	273	550
Benzo[j]fluoranthene	BjF	$C_{20}H_{12}$	252.3	165-166	480

Compound name	Abbreviation	Chemical Formula	Molecular Weight (g/mol)	Melting Point (°C)	Boiling Point (°C)
Benzo[k]fluoranthene	BkF	$C_{20}H_{12}$	252.3	216	480
Chrysene	Chr	$C_{18}H_{12}$	228.3	254	448
Cyclopenta[cd]pyrene	CPP	$C_{18}H_{10}$	226.3	170	439
Dibenzo[a,e]pyrene	DeP	$C_{24}H_{14}$	302.4	233-244	592
Dibenz[a,h]anthracene	DBA	$C_{22}H_{14}$	278.3	262	535
Dibenzo[a,i]pyrene	DiP	$C_{24}H_{14}$	302.4	281-284	594
Dibenzo[a,l]pyrene	DlP	$C_{24}H_{14}$	302.4	162	595
Fluorene	FLR	$C_{13}H_{10}$	166.2	116-117	295
Fluoranthene	FLT	$C_{16}H_{10}$	202.3	111	375
Indeno[1,2,3-cd]pyrene	IcP	$C_{22}H_{12}$	276.3	164	530
Naphthalene	NAP	$C_{10}H_{8}$	128.2	80	218
Phenanthrene	PHN	$C_{14}H_{10}$	178.2	100	340
Pyrene	PYR	$C_{16}H_{10}$	202.3	156	393-404

1.2 Production of PAHs

It is generally accepted that PAHs are generated by the incomplete combustion of organic matter. The main types of PAHs sources, based on their origin of production, are classified as: ***pyrogenic, petrogenic and biogenic.***

When organic compounds are exposed to high temperatures under oxygen-deficient conditions ***pyrogenic PAHs*** are synthesized, through a pyrolysis process. Pyrogenic processes occur generally within ranges of temperature between around 350 °C to more than 1200°C. Pyrolytic processes could take place both intentionally or unintentionally (Abdel-Shafy and Mansour, 2016). By-products of coal such as coke and coal tar or the combustion of petroleum constitute some of the intentionally pyrolytic process, while unintentionally one occurs during the incomplete combustion of wood, fuel oils and motor fuels. Pyrogenic PAHs are mostly HMW PAHs and are generally found in urban areas. (Mojiri et al., 2019; Marris et al., 2020; Patel et al., 2020). Also, lower temperature (100-300°C) prolonged over time can cause generation of PAHs.

The widespread transportation, storage, use and leakage of crude oil and crude oil products, contributes to the ***petrogenic PAHs*** source. Oil spills in freshwater and oceans, storage tank leaks and

release of crude oils due to transportation are the major route of contamination (Abdel-Shafy and Mansour, 2016; Patel et al., 2020). They are predominated by LMW PAHs (Marris et al., 2020).
Not very well-known, ***biogenic PAHs*** are formed by microorganism, phytoplankton, plants and algae or during processes of conversion of vegetative materials (Mojiri et al., 2019; Patel et al., 2020).

1.3 Source of PAHs

The routes of environmental pollution by PAHs are numerous and can be divided in natural and anthropogenic sources (Mojiri et al., 2019; Figure 2). Natural sources include mostly natural wood forest fires, volcanic eruptions and hydrothermal processes (Srogi, 2007; Ravindra et al., 2008). In the atmosphere, PAHs undergo photochemical and chemical oxidation reactions with nitrogen oxides (NO_X), atmospheric oxygen (O_2), sulfur oxides (SO_X) and hydroxyl radical (OH) producing more toxic compounds (Naccari et al., 2011). Anthropogenic sources constitute the main determinants of PAHs pollution and are classified as: ***industrial, mobile, domestic,*** and ***agricultural emission sources*** (Ravindra et al., 2008).

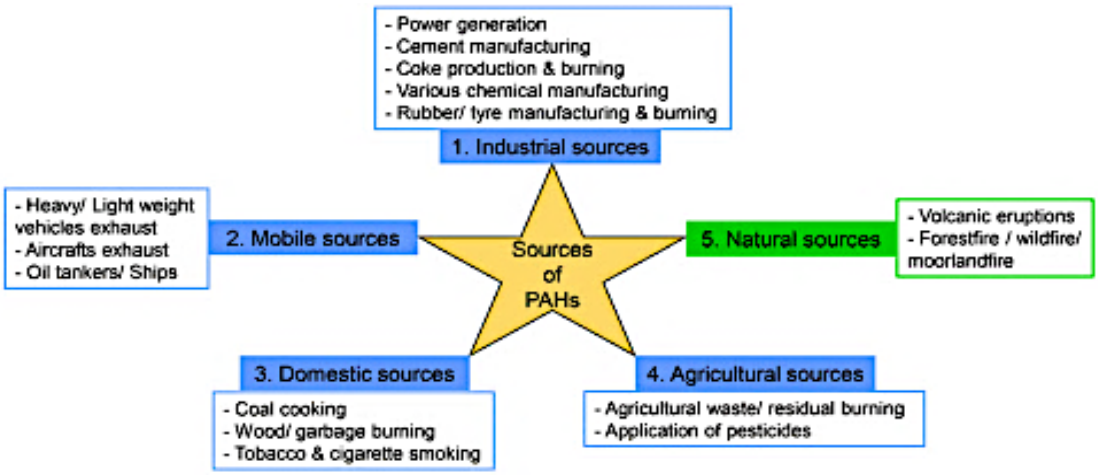


Figure 2. PAHs emission sources. *(Source: Patel et al., 2020)*

- Industrial activities include the production of iron and steel, aluminum, fungicide and insecticide, power and coal-tar pitch production, as well as coal gasification, manufacturing of cement, dye and rubber tire, asphalt industries, waste incineration, exhaust from refineries, electric arc and oxygen furnace, diesel engine, and gasoline-powered engines of large

machineries (Srogi, 2007; Ravindra et al., 2008; Abdel-Shafy and Mansour, 2016; Gupte et al., 2016; Mojiri et al., 2019).

- Among mobile emission sources are included: trains, aircrafts, ships and off-road heavy weight and light weight vehicles exhausts (Srogi, 2007; Patel et al., 2020). Factors such as the type of engine, load, fuel type and quality, influence mobile emissions of PAHs.
- Open biomass and agricultural waste burning, performed under sub-optimum combustion conditions, contribute to the agricultural emission sources of PAHs (Ravindra et al., 2008; Patel et al., 2020).
- Household activities such as garbage burning, coal coking, wood burning, cooking on oil/gas burners generating mostly from food processing (drying, smoking, roasting), cooking practices involving high temperatures (pan-frying, grilling and barbecuing) contribute to domestic sources (Johnsen and Karlson, 2007; Alomirah et al., 2011; Cereceda-Balic et al., 2012).

Industrial, mobile, and domestic sources are the main contributors of the high PAHs pollution in the urban areas, while domestic and agricultural ones in the rural areas. Regarding seasonality, PAHs concentration is highest in winter followed by spring, autumn, and summer. The high quantity of incomplete combustion of fossil fuel, elevated residential heating, lower photodegradation, and poor diffusion due to calm winds and low temperature justify the higher levels of PAHs in winter and spring (Miura et al., 2019).

Through dry/wet deposition processes, PAHs in gaseous phase (aerosols) are deposited in soil, water, and plants (Abdel-Shafy and Mansour, 2016). As a result, accumulation of PAHs in soil/sediment leads to the further transport of pollution to the groundwater, plants, and food. Taking into consideration the numerous sources of PAHs, it results very difficult to avoid PAHs exposure. The exposition of humans to PAHs results through different pathways, mainly through dietary (ingestion) and non-dietary sources (e.g., inhalation and dermal contact) in both occupational and non-occupational settings (Burchiel and Laster, 2001; Rengajaran et al., 2015). In some cases, the exposure may occur via more than one route simultaneously, influencing the total dose absorbed (e.g dermal and inhalation exposures from contaminated soil/ air) (Rengajaran et al., 2015; Abdel-Shafy and Mansour, 2016). The major route of exposure for non-smokers is the consumption of food (SFC 2002; EFSA 2008). It is estimated that over 70% of the PAHs exposure of non-smokers is associated with food consumption (Rengarajan et al., 2015) while smoking could significantly contribute for smokers.

In fact, one cigarette causes the intake of 20-40 ng of benzo (a) pyrene (Skupinska et al., 2004). As the main exposure route, especially among non-smokers, food contamination can originate from: environmental pollution, during food preparation and processing, contact with contaminated packaging or non-food grade quality mineral oil (Purcaro et al., 2013; Bansal and Kim, 2015; Sampaio et al., 2021). The environmental route, particularly in industrial and traffic dense areas, constitute the commonest source pathways to raw food (fruits, vegetables, cereals etc) (Fähnrich et al., 2002; Bansal and Kim, 2015). On the other hand, accumulation of PAHs in products of animal origin and their derivatives can occur when contaminated vegetation is used to feed livestock. (Garcia et al., 2014; Zelinkova and Wenzl, 2015). Similarly, water contamination from PAHs significantly contribute to the accumulation of PAHs in aquatic organisms, such as fish and seafood (Ramalhosa et al., 2009; Dhananjayn and Muralidharan, 2012).

Man-made activities constitute the most relevant and efficient pathway of PAHs intake, majorly attributed to activities of food processing such as smoking or drying, as well as during preparations involving high temperatures and/or open flames, such as grilling, barbecuing, roasting, and frying (Purcaro et al., 2013; Garcia et al., 2014; Hamidi et al., 2016). The accumulation and concentration of PAHs, depends on food intrinsic properties and on various factors such as heat source, type of fuel, distance of heating, design of the food device, etc (Vyrand et al., 2007).

The main food matrices where PAHs can be formed and accumulated are fruits, vegetables and cereals, dairy products, meat and meat products, fish, beverages, infant formulations (Singh et al., 2016).

The determinants of PAHs accumulation and concentration, vary depending on each food matrix. In raw animal foods (such as meat and fish), the most important determinants for bioaccumulation are lipid deposits or tissues, skin tissue, metabolism, etc. Furthermore, levels of PAHs in animal products can be influenced by the capability to metabolically transformation PAHs to less toxic form and excrete the same out of the body system (Singh et al., 2016). On the other hand, some shellfish (e.g.mussels and oysters) are known to absorb and bioaccumulate PAHs from water, becoming a concerning matter for PAHs accumulation and via of exposure, especially in usually contaminated areas or after petroleum leakages and ship accidents (Wenzl and Zelinkova, 2018; Ireland, 2015).

1.4 Public concern: PAHs in meat

1.4.1 Worldwide and European meat consumption

Meat and meat products represent an important source to the human diet of high biological value proteins and essential micronutrients such as vitamins of group B, iron and zinc (Pereira et al., 2013; Milford et al., 2019). Consumption of animal products, in most countries, has significantly increased since the 1960's, particularly from the 80's to now days (Gonzales et al., 2020). Among all world regions was observed an increase in total meat consumption, particularly in Africa, Asia and Latin America. (Parlasca and Qaim, 2022; Figure 3).

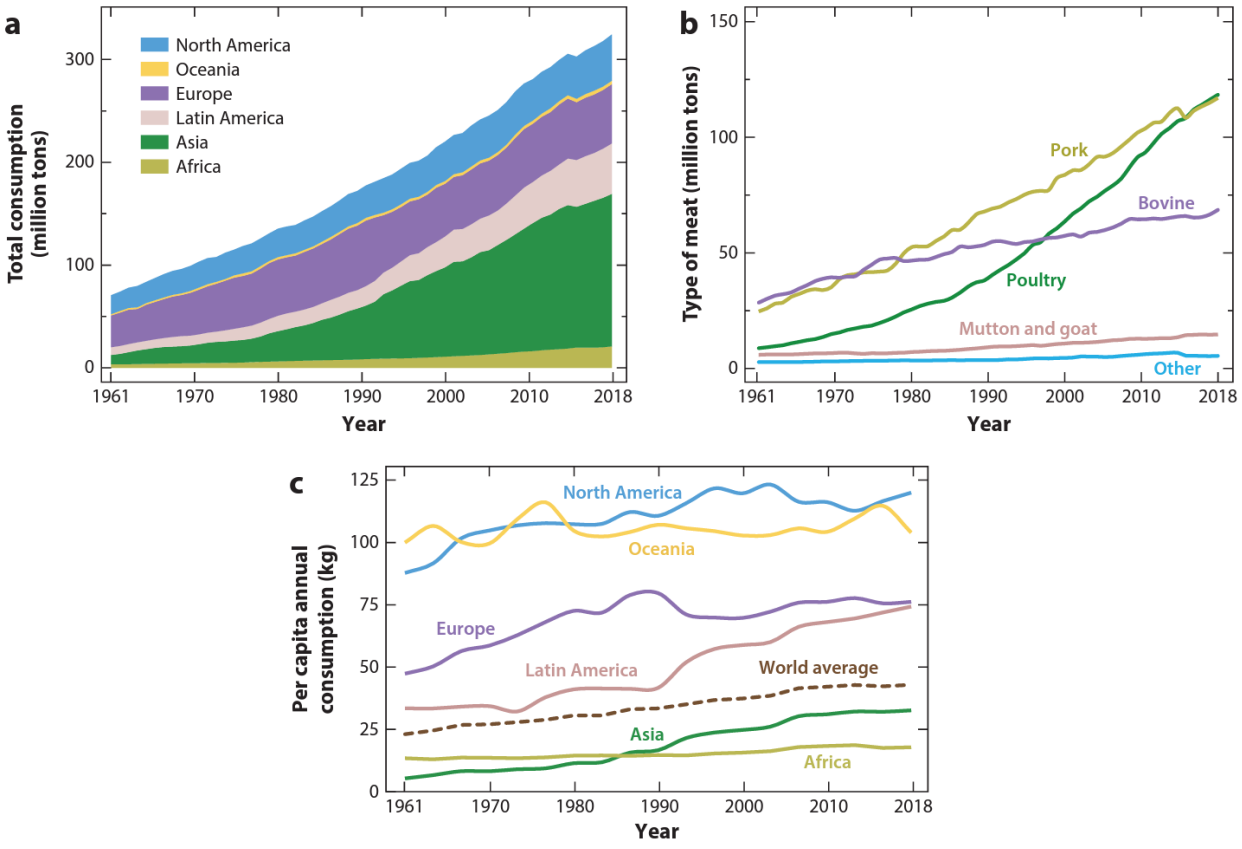


Figure 3. Global meat consumption. (a) Total annual consumption by region, (b) total annual consumption by type of meat, and (c) per capita annual consumption by region. Data from FAO Food Balance Sheets (https://www.fao.org/faostat/en/#data/FBS). *(Source: Parlasca and Qaim, 2022)*

Regarding the type of meat, pork and poultry meat consumption have registered a notable increment. China and some countries in Southeast Asia lead the increment in pork meat consumption. On the other hand, poultry meat being cheaper, less included in religious exemptions and considered healthier, has undergone a significant increment in consumption all over the world (Mottet et al., 2017). Over the years, has been observed also an increment of bovine, mutton and goat global meat consumption, although not at the extent of pork and poultry meat. During 2014-2016, worldwide total meat consumption per capita was 34.1 kg/ year, of which 60% was red meat (pork, sheep and beef) (Salter et al., 2018). Global population and income growth have contributed to the higher levels of meat consumption per capita.

The rising population and affluence in developing nations are predicted to contribute to the continued growth (+1.4% annually) of the global meat consumption by 2031. A substantial portion of the global need will be satisfied by domestic production, while the remaining part will be met by 3.4 million t of meat imports (particularly of beef and poultry), on top of the 37.3 million t currently. The EU will benefit only to a limited extent from the additional demand, particularly for poultry meat (EC 2021; Figure 4).

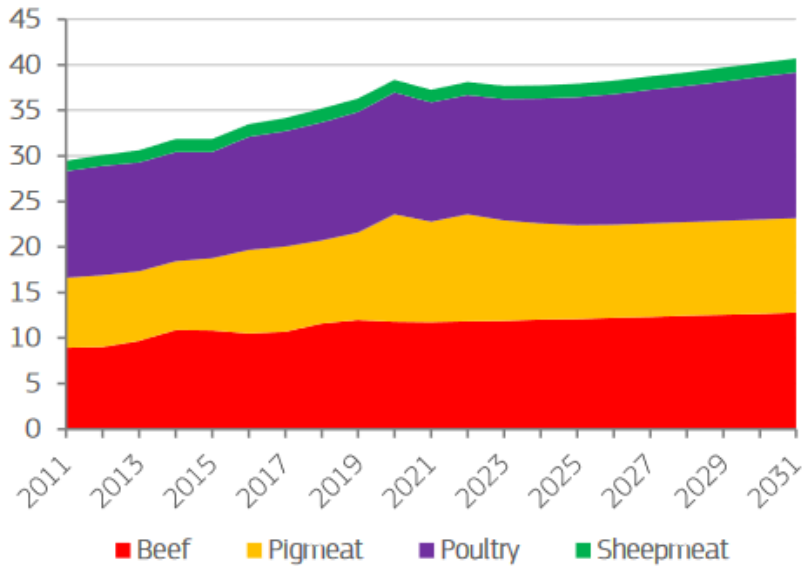


Figure 4. Global import meat demand (million t c.w.e). *(Source: EC 2021, EU agricultural outlook for markets, income and environment, 2021-2031)*

In the EU, meat consumption is expected to fall from 69.8 kg in 2018 to 67 kg retail weight per capita by 2031, bucking the global trend after a reduction brought on by COVID-19 and the meat crisis in China. The consumer basket will change in tandem with the general downturn. It is anticipated that beef consumption will continue to decline, while pig meat consumption will continue to be gradually replaced by poultry meat consumption in the interim. Due to changes in the EU population and the diversity of the meat diet, the consumption of sheep meat is expected to increase modestly (religious traditions and migration) (EC 2021; Figure 5).

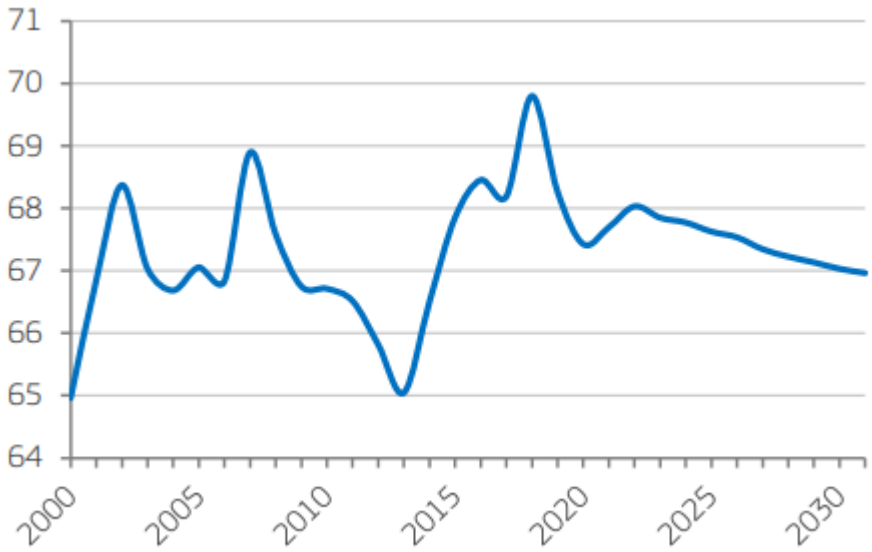


Figure 5. EU per capita meat consumption. *(Source: EC 2021, EU agricultural outlook for markets, income and environment, 2021-2031)*

Between 2021 and 2031, EU total beef production is expected to decrease by 0.6 million t (-8%) as it has been doing since 2018. The apparent consumption of beef in the EU, which was low in 2020 and 2021 as a result of COVID-19, will keep declining. It might decrease from 10.6 kg to 9.7 kilogram per person by 2031. (EC 2021; Figure 6).

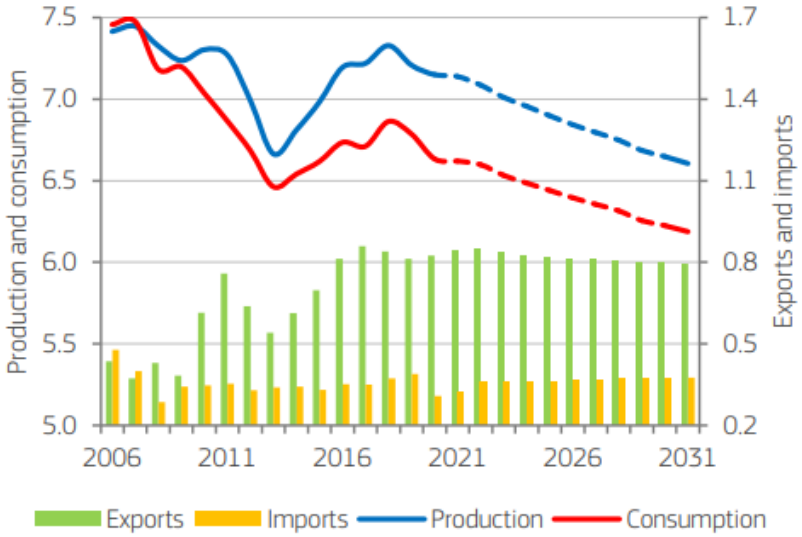


Figure 6. EU beef and veal market balance (million t). *(Source: EC 2021, EU agricultural outlook for markets, income and environment, 2021-2031)*

Regarding pig meat, concern about people's health, environment, and society should continue to influence consumer preferences and have a negative impact on how much pig meat is consumed in the EU. Hence, it is expected that apparent EU pigmeat consumption per capita will decline by 0.5% annually, from 32.5 kg in 2021 to 31 kg in 2031, following the pattern of the previous ten years. Pig meat production is projected to decline by 0.8% per year in 2021-2031. (EC 2021; Figure 7).

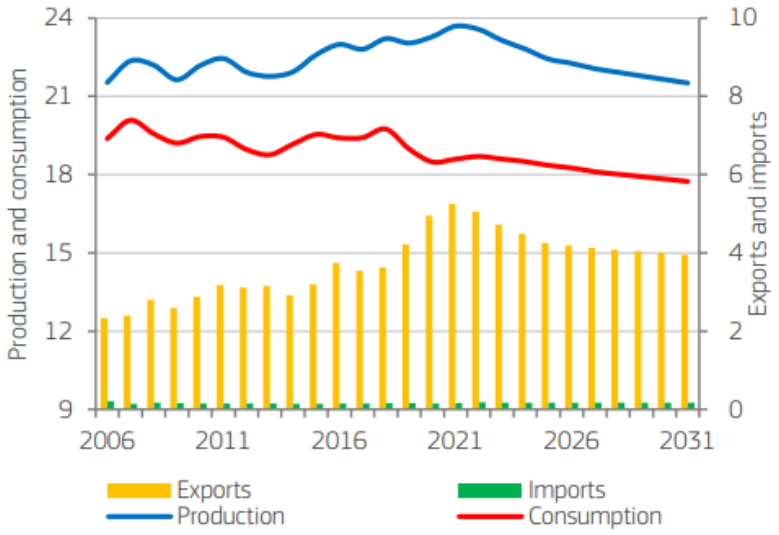


Figure 7. EU pig meat market balance (million t). (Source: EC 2021, EU agricultural outlook for markets, income and environment, 2021-2031)

The increase in poultry consumption in the EU may slow down, dropping from a 2% annual growth rate in 2011–2021 to a 0.5% annual growth rate in 2021–2031. The apparent consumption per person may rise from 23.5 kg in 2021 to 24.8 kg in 2031. It ought to be prompted by ongoing shifts in consumer preferences brought on by poultry's perceived health benefits compared to other meats, its convenient use and the lack of religious restrictions. EU poultry production is expected to continue growing by 0.4% year during the forecast period, adapting quickly to fluctuations in demand, though at a slower rate than the previous ten years (2.6%) (EC 2021; Figure 8).

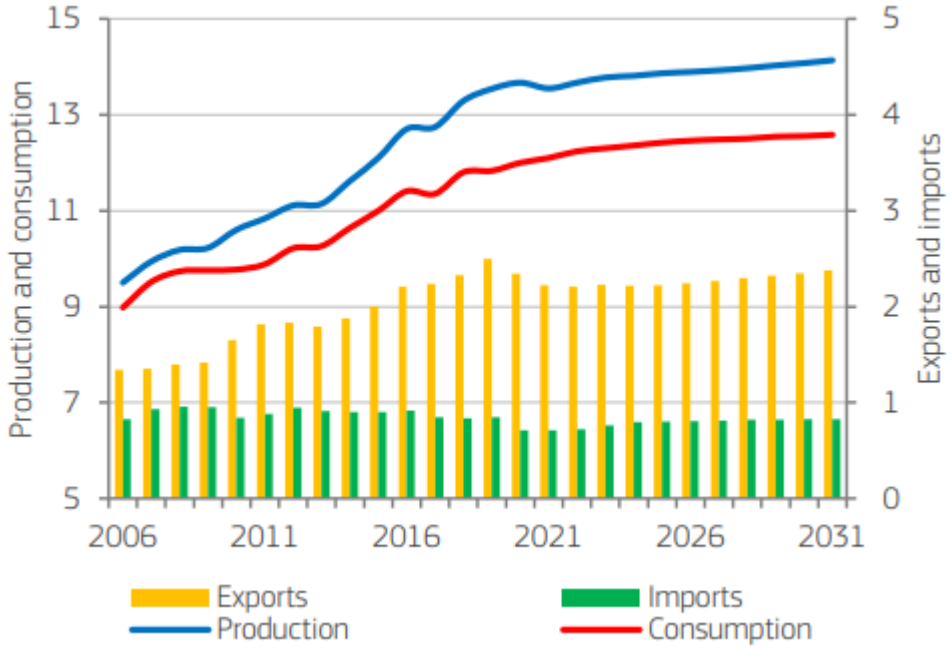


Figure 8. EU poultry meat market balance (million t). *(Source: EC 2021, EU agricultural outlook for markets, income and environment, 2021-2031)*

1.4.2 Meat cooking methods

Meat is mostly consumed cooked in European countries. The most common processing/cooking methods include boiling, steaming, frying, poaching, baking, microwaving, roasting, grilling, barbecuing, smoking, used daily to cook meat and fish (Sobral et al., 2018). More in detail:

- Boiling, cooking method widely used, consist in cooking meat in immersion in water that has been heated to near its boiling point ~100°C;
- Steaming, a moist-heat method of cooking employs hot steam resulting from boiling water. Meat is separated from this last one and only came in direct contact with steam;

- Frying, a rapid preparation technique, consists in submerging meat in hot fat, mostly oil. There are different types of frying. Pan- and deep-frying are the most common. Pan-frying is a form of frying food characterized by the use of minimal cooking oil or fat; while in deep-frying the food floats in the cooking oil.
- Poaching, a moist method of cooking, relies on placing food in a liquid which is brought and maintained at lower temperatures (70 to 80 °C). The cooking liquid may be water, milk, wine, or stock. Meat from poaching results tender and with less moisture loss.
- Oven baking uses dry heat at lower temperatures (170 °C) and food is cooked usually (Mottram, 2007).
- Microwaving, a cooking method of recent years, is generally used to reheat meals. Microwave, through electromagnetic waves, induces in the food the vibration of water molecules, resulting in heat producing. Non-uniform temperature distribution constitutes one of the main concerns related to this cooking method. (Vadivambal and Jayas., 2010).
- Roasting, a slow-cooking process, uses dry heat, at temperatures above 200 °C, which surrounds the food and generally takes more than 2 h in an open uncovered pan. It enhances the flavor of meat and fish via caramelization and Maillard browning occurring on the surface of the food.
- Grilling is a common method of cooking using a direct heat source, which depends on the type of grill. Meat is exposed to temperatures over 260 °C and shows characteristics (aroma and flavor) similar to roasting.
- Barbecuing relies on the use of smoke resulting from wood or charcoal burning as indirect heat source. Barbecued meat results tender and juicy.
- Smoking, ancestral cooking method, could be cold or hot-smoking. The first one is conducted at low temperatures (between 12 and 25 °C) followed by grilling, baking, or roasting of meat before eating. Generally, it is applied to enhance the flavor of meat. Hot smoking, instead, takes place at 40 and 100 °C, while the internal food temperature reaches 85 °C (Unlusayin et al., 2006).

Meat among European countries is cooked in different ways using different cooking methods, according to their culinary traditions, geographical position, religious practices etc. In 2002,

Rohrmann et al. conducted the first European Prospective Investigation into Cancer and Nutrition (EPIC) looking into the meat and fish cooking methods in different Western European countries. EPIC was conducted in 10 European countries. Among cooking methods, the most used were frying, boiling, baking, stewing, roasting, grilling, as well as breading and frying. They contributed to 62% of meat and fish preparation methods, showing variation between the countries: 45.2% of meat and fish in the EPIC cohort of The Netherlands were fried, while in Greece only 1.6%. Regarding three cooking methods such as barbecuing, grilling and frying, their frequency ranged from 11.5% in northern Italian centers to 46.5% in the Dutch cohort. Barbecuing was mostly used in Greece, where more than 8% of meat and fish were barbecued. Red meat was mainly consumed fried, baked and grilled.

In comparison to the north of Europe, the French, Greek, Italian, and British EPIC cohorts consumed more grilled meat and fish while eating less fried meat and fish. In the Spanish cohorts of the EPIC study, no grilled meat was consumed. The largest consumption of red meat cooked at high temperatures was found in The Netherlands (women, 39.4 g/day; men, 59.7 g/day), whereas the intake of fried and barbecued meat and fish was highest in northern Spain for men. (Rohrmann et al., 2002; Figure 9).

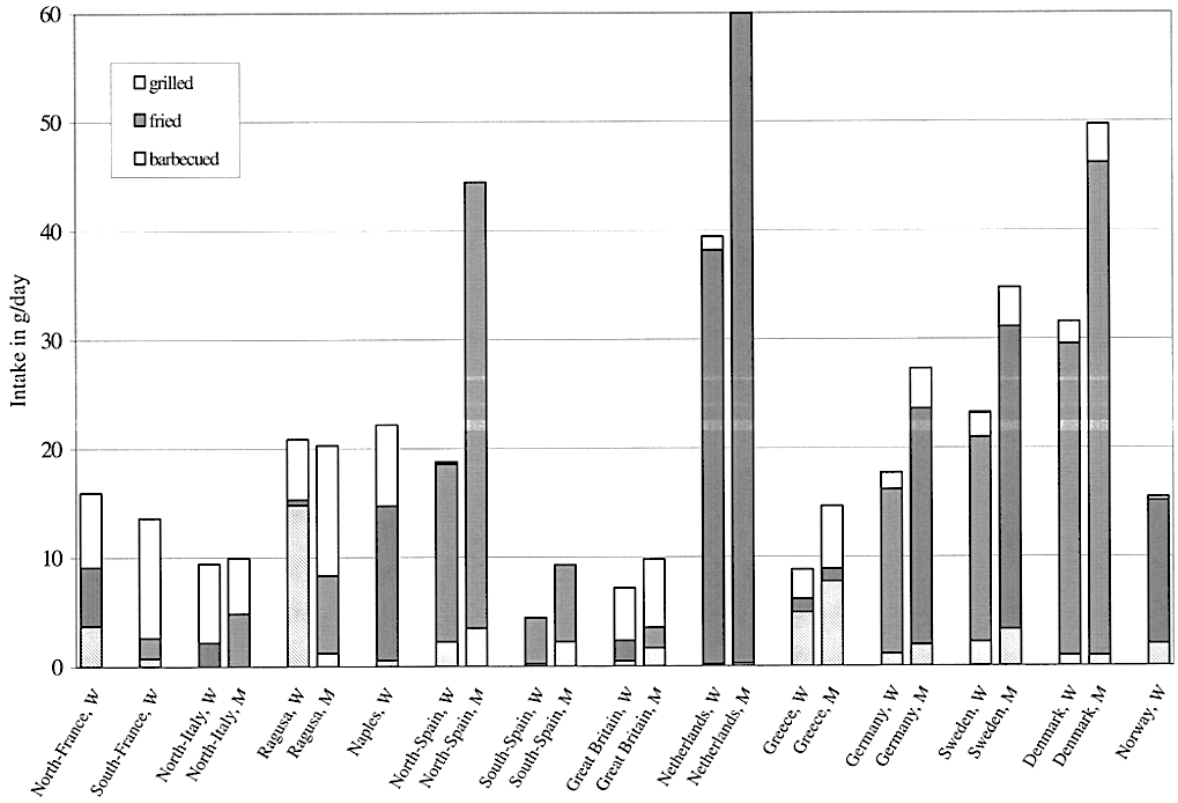


Figure 9. Mean intake of barbecued, fried and grilled red meat by region/country in EPIC assessed by 24 h recalls in 22 727 women and 12 917 men. Regions/countries include: northern France-northeast, northwest; southern France-south, south coast; northern Italy-Varese, Turin, Florence; northern Spain-Oviedo, Pamplona, San Sebastian; southern Spain-Granada, Murcia; Great Britain-general population; Netherlands- Bilthoven, Utrecht; Germany-Heidelberg, Potsdam; Sweden - Malmo, Umea; Denmark- Copenhagen, Aarhus; Norway-northwest, southeast. W ¼ women; M ¼ men. The cooking method 'fried' does not include deep fried and stir fried. Mean adjusted for age, season, and week day. *(Source: Rohrmann et al., 2002)*

The increasing consumption of meat in recent years and the possible relationship, observed by epidemiological studies, between, particularly red meat and its methods of preparation and risk of cancer development, has pointed out the attention to the study of PAHs, considered potential carcinogenic to humans. PAHs are more likely formed during high temperature cooking methods as in smoking, barbecuing and grilling, which are accordingly known to be the main contributors to PAHs formation, especially when subjecting meat directly over charcoal or open flames (Sobral et al., 2018; Pouzou et al., 2018).

1.4.3 Classification and legislation

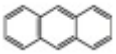
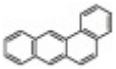
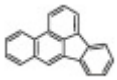
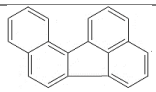
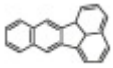
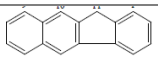
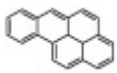
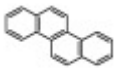
Numerous organizations have evaluated the occurrence and toxicity of PAHs and expressed recommendations and/or reference values such as: United States Environmental Protection Agency (U.S. EPA); the Joint FAO/WHO Expert Committee on Food Additives (JECFA); the International Agency for Research on Cancer (IARC); the Scientific Committee on Food (SCF), the European Food Safety Authority (EFSA); the International Programme on Chemical Safety (IPCS) (Zelinkova and Wenzl, 2015).

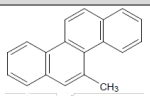
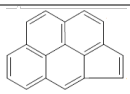
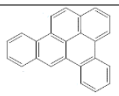
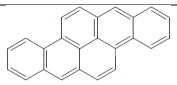
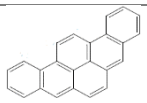
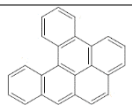
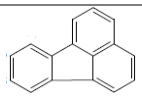
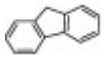
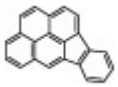
In order to categorize the substances most commonly found at facilities on the National Priorities List (NPL), that present a potential threat to human health due to their known or suspected toxicity, US-EPA were required to prepare a "priority pollutants" list (Zelinkova and Wenzl, 2015). This list did not constitute a list of "most toxic" substances, but a list of "priority-contaminants" based on their frequency of occurrence, toxicity and potential for human exposure. (US agency, 2013; Keith, 2015). In 1976, 16 EPA PAHs were identified namely acenaphthene, acenaphthylene, anthracene, fluoranthene, fluorene, naphthalene, phenanthrene, pyrene, benz[a]anthracene, benzo[b] fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, benzo[a]pyrene, chrysene, dibenz[a,h]anthracene, and indeno [1,2,3-cd] pyrene. (Mumtaz and George, 1995). These 16 PAHs are commonly studied and extensively monitored, becoming a de facto standard globally. (Andersson and Achten, 2015; Table 2).

In 2002, the European Commission Scientific Committee on Food (SCF), conducted a risk assessment on 33 PAHs, previously evaluated by the International Program on Chemical Safety (IPCS). In 1988 the SFC recommended the monitoring of 15 PAHs out of the 33 initially evaluated based mainly on their genotoxic and carcinogenic potential, namely: benz[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k] fluoranthene, benzo[ghi]perylene, benzo[a]pyrene, chrysene, cyclopenta[cd] pyrene, dibenzo[a,h]anthracene, dibenzo[a,e]pyrene, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene, dibenzo[a,l]pyrene, indeno[1,2,3-cd]pyrene, and 5- methylchrysene, including eight high molecular weight PAHs from the US-EPA list (Bansal and Kim, 2015, Zelinkova and Wenzl, 2015). Benzo[a]pyrene (BaP) was placed as a marker of occurrence of carcinogenic PAHs in food, however suggesting the need for additional data regarding PAHs profile. In 2005, after the SCF opinion, the European Commission introduced the Regulation No. 208/2005 (Reg 208, 2005) and the following Regulation No. 1881/2006 (Reg 1881, 2006), establishing maximum levels for BaP only in

different class of foods, including smoked meat and meat products, smoked fish and fish products, oils and fats. In the same year, JEFCA re-evaluated PAHs, concluding that 13 PAHs were genotoxic and carcinogenic. These PAHs were the same as those identified by SFC, except benzo[ghi]perylene and cyclopenta[cd]pyrene. Additionally, JECFA highlighted the need to include another compound such as benzo[c]fluorine, due to insufficient data regarding its occurrence in food (Additives 2005, EFSA 2008).

Table 2. Environmental Protection Agency PAH16 and European Union PAH15+1*(Adapted from Wongmaneepratip et. al, 2017)*

COMPOUND	CHEMICAL STRUCTURE	US-EPA [16]	EU [17]	IARC [7]
Acenaphthene (Ac)		✓	---	3
Acenaphthylene (Ace)		✓	---	-
Anthracene (A)		✓	---	3
Benzo[a]Anthracene (BaA)		✓	✓	2B
Benzo[b]Fluoranthene (BbF)		✓	✓	2B
Benzo[j]fluoranthene (BjF)		---	---	2B
Benzo[k]Fluoranthene (BkF)		---	---	2B
Benzo[c]fluorene (BcF)		---	✓	3
Benzo[g,h,i]Perylene (BgP)		✓	✓	3
Benzo[a]Pyrene (BaP)		✓	✓	1
Chrysene (Chr)		✓	✓	2B

COMPOUND	Chemical structure	US-EPA [16]	EU [17]	IARC [7]
5-Methylchrysene (5MC)		---	✓	2B
Cyclopenta[c,d]pyrene (CPP)		---	✓	2A
Dibenzo[a,h]Anthracene (DhA)		✓	✓	2A
Dibenzo[a,e]pyrene (DeP)		---	✓	3
Dibenzo[a,h]pyrene (DhP)		---	✓	2B
Dibenzo[a,i]pyrene (DiP)		---	✓	2B
Dibenzo[a,l]pyrene (DlP)		---	✓	2A
Fluoranthene (Fl)		✓	---	3
Fluorene (Flr)		✓	---	3
Indeno[1,2,3-cd]Pyrene (Ip)		✓	✓	2B
Naphthalene (Na)		✓	---	2B
Phenanthrene (Pa)		✓	---	3
Pyrene (P)		✓	---	3

US-EPA: 16 PAHs recognized as priority contaminants by the United States Environmental Protection Agency. EU: 15+1: 15 PAHs plus 5-methylchrysene recognized as priority PAHs by the European Union. IARC classification of PAHs in Group 1: Carcinogenic to humans; Group 2A: Probably carcinogenic to humans; Group 2B: Possibly carcinogenic to humans Group 3: Not classifiable as to its carcinogenicity to humans.

Based on the Recommendation 2005/108/EC (Reg 108, 2005) the EU recommended the investigation of the levels of the 15 PAHs identified by SCF and the one PAH (benzo[c]fluoreneBcF-) pointed out by the JECFA (NHMRC 1999), including 8 heavy PAHs (BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA), from which BaP was chosen as a marker of occurrence and carcinogenic/genotoxic potency of the entire class and for which analytical performance criteria was established in 2007, in the Commission Regulation (EC) No 333/2007 (Reg 333, 2007). On june 2007, a report published by EFSA representing data collected from the Member states, raised doubts about the suitability of BaP as a marker for PAH occurrence in food (EFSA 2007). In the light of this in 2008, the EFSA CONTAM Panel, the EU risk assessment authority for food and feed safety, published an Opinion on PAHs and stated that BaP alone was not a suitable marker for these contaminants' occurrence. Instead, the sum of four PAHs – PAH4 (BaP, BaA, Ch, BbF) – or the sum of eight PAHs – PAH8 (BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA) – were better indicators of carcinogenic PAHs contamination and should be considered (EFSA 2008). The Panel also concluded that PAH8 did not provide additional information compared to PAH4. Consequently, in 2011, the Commission Regulation (EU) 835/2011 (Reg 835, 2011) amended the Commission Regulation (EC) No 1881/2006 (Reg 1881, 2006), acknowledging the importance of determining PAH4 instead of solely relying on BaP, therefore establishing maximum levels for the sum of the four PAHs – BaP, BaA, BbF and Ch, while maintaining separate maximum level for BaP (Reg 835, 2011; Table 3). In the same year, the Commission Regulation (EU) No 836/2011 (Reg 836, 2011) was published, amending the Regulation (EC) No 337/2007 (Reg 337,2007), extending the analytical performance criteria to the new three PAHs – BaA, BbF and Ch. (Reg 836, 2011).

Since the established maximum values for smoked meat/fish and smoked meat/fishery products are hard to achieve despite the application of good practices, some traditionally smoked products from several small and medium size enterprises would disappear from the market, thus the Commission Regulation (EU) Regulation No 1327/2014 (Reg 1327, 2014) amended the Regulation (EC) No 1881/2006 (Reg 1881, 2006) about maximum levels of PAHs, excluding some requirements for traditionally smoked products (meat and meat products, fish and fishery products). Consequently, the maximum levels are currently 5.0 μg/kg for BaP and 30.0 μg/kg for PAH4, since 2014, as established in the Commission Regulation (EU) No 1327/2014. (Table 3).

Table 3. Maximum levels of PAHs in some foodstuffs established in the Regulations (EU) No 1881/2006, (EU) No 835/201 and (EU) No 1327/2014

Food Product	**Maximum Levels (µg kg^{-1})**	
	BaP	**PAH4**
Oils and fats (excluding cocoa butter) for human consumption	2.0	10.0
Smoked meat and smoked meat products	2.0	12.0
Traditionally smoked meat and smoked meat products [a]	*5.0*	*30.0*
Heat treated meat and heat-treated meat products (grilled or barbecued)	5.0	30.0
Smoked fish and smoked fishery products	2.0	12.0
Traditionally smoked fish and smoked fishery products [b]	*5.0*	*30.0*
Bivalve mollusks (Smoked)	6.0	35.0
Processed cereal-based foods and baby foods for infants and young children	1.0	1.0
Infant formulae and follow-on formulae, including infant milk and follow-on milk	1.0	1.0

BaP = Benzo(a)Pyrene. PAH4 = benzo[a]pyrene, chrysene, benz[a]anthracene, benzo[b]fluoranthene. [a]These levels apply to the following countries: Ireland, Latvia, Romania, Finland, Sweden and the United Kingdom; [b]These levels apply to the following countries: Ireland, Spain, Croatia, Cyprus, Latvia, Poland, Portugal, Romania, Slovak Republic, Finland, Sweden and the United Kingdom.

Since the Commission Regulation 1327/2014 (Reg 1327, 2014), the European Union Member States have continuously monitored PAHs levels in traditionally smoked products, applying strategies to ensure good smoking practices. Based on a new assessment conducted in 2018, despite the mitigation strategies related to the smoking practices, the European Commission concluded that they were not sufficient to ensure that smoked food comply with the maximum levels set for PAHs. In this regard, through the latest Commission Regulation 1255/2020 (Reg 1255, 2020), maximum levels of PAHs

were reviewed in traditionally smoked meat and fish and in derived products. Based on the considerations obtained, a derogation for local production and consumption was granted without a time limit for certain traditionally smoked meat, fish and derived products, allowing BaP and PAH4 levels above 5.0 and 30.0 µg/kg, respectively as those established for heat treated meat and heat-treated meat products (grilled or barbecued).

1.4.4 Human exposure

As mentioned, PAHs exposure occurs through three possible routes: inhalation, skin contact and ingestion (consumption of contaminated food) (Hamidi et al., 2016; Table 4).

Table 4. Routes of human exposure to PAHs. *(Source: Hamidi et al., 2016)*

Route of exposure	Occurrences
Inhalation	Inhalation of tobacco smoke, wood smoke and ambient air Inhalation of engine exhaust People working in industries such as mining. Oil refining and metal working
Skin contact	Breathing in or touching PAHs for a long time were suggested to cause lung or skin cancer
Consumption of contaminated food	Eating food containing PAHs Fruits and vegetables grow in contaminated soil Processed food (charbroiling and grilling at high temperatures)

As 70% of PAHs exposure to smokers and non-occupationally exposed people may be diet-related, PAHs presence in food requires constant monitoring (Domingo and Nadal, 2015; Hussain et al., 2018). Environmental PAHs amounts are increasing in non-industrialized and developing countries, causing higher levels of PAHs in foodstuffs (Yebra-Pimentel et al., 2015). In 2008, EFSA assessed the dietary exposure to PAHs across European countries, estimating that the median dietary exposure for mean and high dietary consumers varied from 1168 ng/day to 2068 ng/day respectively, for PAH4, and 1729 ng/day to 3078 ng/day respectively, for PAH8 (EFSA 2008; Table 5).

Table 5. Dietary exposure to BaP, PAH2, PAH4 and PAH8 (ng/day) for average and high consumers across Europe. *(Source: EFSA 2008)*

	Average exposure Whole population				**High exposure Sum of P97.5 for cereals and seafood+average exposure for whole population**			
Country	BaP	PAH2	PAH4	PAH8	BaP	PAH2	PAH4	PAH8
Belgium	232	637	1158	1732	393	1101	2108	3138
Bulgaria	209	560	1020	1526	385	1053	2027	3018
Czech Republic	239	654	1196	1777	426	1207	2328	3449
Denmark	223	617	1135	1690	288	818	1545	2300
Finland	185	535	978	1422	231	623	1155	1693
France	245	655	1220	1814	380	1021	1966	2921
Germany	255	682	1258	1888	422	1194	2311	3439
Hungary	231	647	1168	1716	314	877	1636	2410
Iceland	205	558	1039	1522	694	2232	4486	6568
Ireland	238	646	1188	1793	370	1049	2013	3009
Italy	255	719	1332	1962	487	1502	2943	4322
Netherland	239	658	1197	1785	535	1687	3318	4886
Norway	252	765	1449	2136	461	1470	2900	4262
Slovakia	244	626	1158	1727	709	1905	3769	5601
Sweden	230	621	1168	1719	364	1003	1949	2876
United Kingdom	188	499	936	1415	315	854	1661	2489
Median EU	235	641	1168	1729	389	1077	2068	3078

The major contributors to dietary exposure were cereals and cereal products, most likely due to the frequently high daily consumption, followed by seafood (mainly shellfish) and seafood products (EFSA 2008; Table 6).

Table 6. Consumer exposure to BaP, PAH2, PAH4, PAH4 and PAH8 for each food category for which occurrence data are available. *(Source: EFSA 2008)*

Category	Consumption	Exposure			
	Median g/day	BaP ng/day	PAH2 ng/day	PAH4 ng/day	PAH8 ng/day
Cereals and cereal products	257	67	129	257	393
Sugar and sugar products including chocolate	43	5	13	25	39
Fats (vegetable and animal)	38	26	112	177	239
Vegetables, nuts and pulses	194	50	124	221	378
Fruits	153	5	40	75	87
Coffee, tea, cocoa (expressed as liquid)	601	21	55	106	156
Alcoholic beverages	413	4	12	25	74
Meat and meat products and substitutes	132	42	107	195	279
Seafood and seafood products	27	36	140	289	421
Fish and fishery products	1	21	84	170	210
Cheese	42	6	12	20	30

In addition to the food groups above reported, PAHs are also found in the milk of some ruminants, although at low levels (Wenzl and Zelinkova, 2018). Although in the EFSA report (2008) was mentioned the importance of high consumption of barbecued foods, little data was available regarding consumption of foods with higher PAHs content, such as smoked/grilled/barbecued/roasted muscle foods. In 2010, IARC determined that BaP was the only PAH compound classified as carcinogenic to humans (group 1), due to its ability to form DNA adducts possibly leading to mutagenic/carcinogenic effects (IARC 2010). Later in 2012, IARC stated that in urban areas the average concentrations of individual PAHs in ambient air could range from 1 to 30 ng/m^3 (excluding inside road tunnels and cities which use coal as the residential heating fuel). Concerning food, they stated that the intake varied widely, going from a few nanograms to a few micrograms per person, per day (IARC 2012).

Regarding in particular children, it has been suggested that the diet is a relevant route of exposure, since infant foods such as cereals and powdered milk, as well as some baby food, meat or fish are likely to contain PAHs and hydroxylated metabolites (Rey-Salgueiro et al., 2009; Domingo et al., 2015; Wenzl and Zelinkova, 2018). Other relevant ingredients of infant formula such as milk powder and vegetable fats, have been shown to present significant PAHs levels. (Santonicola et al., 2017). The EU has hence established some limits for PAHs in baby foods and cereal-based foods for infants and young children as well as for infant and follow-on formulae and milk (Reg 835, 2011).

In 2015, Domingo et al. summarized the existing publications reporting dietary intake of total PAHs and BaP in European countries, highlighting the importance of the agro-food sector for the European continent. The need for information on the amounts of PAHs in foods and dietary intake by the general population was also pointed out, noting that variations occur between countries due to the respective food habits. Spain, Italy, the Netherlands and the United Kingdom showed more information available compared to Azerbaijan, Estonia, France, Greece and Sweden. No other countries showed information, which highlights the importance of determining PAHs levels in foods estimating the population's intake. PAHs levels should be continuously monitored also because epidemiological studies have linked, at least partly, some human cancers to the dietary exposure of PAHs (Domingo et al., 2015).

Lodovici et al. (1995) reported the first data on estimated dietary intake of PAHs by the Italian population, with 3μg/day total dietary PAH intake, while estimated one was 1.4 μg/day. Contaminated cereal products were the main route of exposure. In fact, the highest amounts of PAHs were found in pizza baked in wood-burning oven, followed by other food groups such as meat, milk products, vegetables and fruits. In 1996, Turrio-Baldassarri et al. assayed eight PAHs in four mean Italian diet samples, namely national, southern, north-eastern and north-western. The study reported benzo[a]pyrene intakes of 0.17, 0.32, 0.19, 0.16 μg/day, respectively, for the four samples. Primary school children (7–9 years old) of Naples were investigated on their dietary habits and on 16 PAHs exposure (Cirillo et al., 2010). Median concentrations in foods of benzo[a]pyrene varied from 0.06 to 0.33 μg/kg. Amounts of PAHs in various Italian food items are reported by other studies, such as Menichini et al., 1991 (olive oil), Storelli et al., 2003 (smoked seafood), Amodio-Cocchieri et al., 2003 (fish and mussels), Perugini et al., 2007 (marine organisms), Purcaro et al., 2006 (frying oils and snacks). These studies reported only PAH levels in specific food items.

Dietary exposure assessment combines data from the concentrations of a contaminant in food, e.g. PAHs, with the amounts of those foods that are consumed, therefore estimating the likelihood that the consumer will be exposed to a contaminant and the extent of such exposure. The health impact is then the combination of the health hazard effect and exposure. Data concerning the concentration of a contaminant in foods can be obtained from: experimental data; monitoring and surveillance programs; legislated Maximum Permitted Levels (MPLs), Maximum Residue Levels (MRLs) or usage levels reported by manufacturers; estimation from mathematical modeling; a Total Diet Study (TDS) method. The TDS method is highly endorsed by WHO and FAO, since it includes a representativeness of the sampling and the preparation of each sample as consumed; it involves the selection, collection and analysis of usually consumed food purchased at retail, processing the food as if for consumption, separating the prepared food in representative food groups, homogenizing the samples and analyzing them for hazardous chemicals such as PAHs. They are projected to cover the average diet based on dietary surveys. This method should be the most accurate to measure the average quantity of a contaminant consumed through food by the chosen population, although its accuracy strongly depends on the sampling design and sample size. Also, it might not be adequate to assess acute dietary exposures when a high level of composition of samples is used, because high contamination of less consumed foods is easily camouflaged by the dilution effect (EFSA 2011). Unfortunately, despite some efforts such as the creation of guidance documents (e.g. the "Towards a harmonized total diet study approach" elaborated by WHO, FAO and EFSA), there is still no standard or coordinated method to assess dietary PAH exposure which is usually decided to fit a specific purpose, depending on a number of factors (target contamination, population group, degree of accuracy needed, availability of information, budget, etc.), making it difficult to compare or harmonize data (EFSA 2011).

1.4.5 Effect on human health

The effect of PAHs on human health relies on various factors such as: route and duration of exposure, the amount and concentration of PAHs as well as their innate characteristics and toxicity (ACGIH 2005; Valavanidis et al., 2011). In addition, other factors such as age, pre-existing health status can influence PAHs impact on human health (Abdel-Shafy and Mansour, 2016). PAHs are not toxic *per*

se, but are activated in the human body and exposure is never to single PAHs, highlighting the need to understand the mechanism of modifications occurring in PAHs mixtures to obtain an accurate assessment of their hazard. Being lipophilic, the bioavailability of PAHs is significant after ingestion and inhalation, where organs particularly rich in adipose tissue can serve as storage points from which PAHs are gradually released (Abdel-Shafy and Mansour, 2016). Once released in the organism, in attempting to eliminate them, it generally increases their toxicity via addition of polar groups (Foods ECSC 2002; Tarantini et al., 2011). PAHs mixture capacity to cause short term health effects in humans is yet to be clarified, resulting in acute effects such as skin irritation and inflammation, possibly eye irritation, nausea, diarrhea and vomits (Rengajaran, 2015). However, it is not known which component of the mixture is responsible for such effects (ACGIH 2005; Bansal and Kim et al., 2015). On the other hand, regarding long-term health effects, continuous assessing of health risk after contact/ingestion of PAHs is essential as they are considered mutagenic, genotoxic and carcinogenic contaminants, besides being linked to cytotoxicity and immunosuppression (affecting the host resistance, immune system development and humoral immunity), epigenetic mechanisms and possibly being neurotoxic (EFSA 2008, IARC 2012, Crepeaux et al., 2013; Bansal and Kim, 2015; Abdel-Shafy and Mansour, 2016; Hussain et al., 2018). The gastrointestinal absorption of dietary PAHs is determined by the size and hydrophobicity of the PAH molecules and the fat content in food. As mentioned, once ingested and absorbed, PAHs circulating in blood are distributed to several tissues, especially to those with a high lipid content. Generally, PAHs are metabolized by phase I enzymes and peroxidases, which produce DNA reactive metabolites, and phase II enzymes, which form polar conjugates. Metabolic activation of PAHs seems to occur primarily in the liver but also in other tissues, including the epithelial barriers (IARC 2010). A scheme of the main biotransformation pathways is provided in the figure 10. Briefly, PAHs are quickly transformed into their hydroxylated metabolites, thought to occur via cytochrome P450 oxidase system, resulting in activation to diol-epoxides (reactive metabolites), which adhere to macromolecules such as genetic material DNA and other tissues, inducing mutations which may lead to carcinogenic processes. Their toxicity is due to their interference with cellular membranes functioning, as well as with enzyme systems. (Philips, 1999; Samanta et al., 2002; Pussa, 2013; Bansal and Kim, 2015). Then, the formed epoxides or phenols might be further metabolized producing glucuronides, sulfates or glutathione conjugates (unmetabolized) increasing the polarity to facilitate their excretion (Samanta et al., 2002; Chauhan et al., 2015). PAHs are also potent ligands for the aryl hydrocarbon receptor (AhR), a ligand-dependent

transcription factor; through this interaction PAHs activate the transcription of various genes encoding phase I and II xenobiotic metabolizing enzymes, which include CYP1A1, CYP1A2, CYP1B1, and also GST, UGT1A1 and NQO1, resulting in adverse effects such as bioactivation of procarcinogens or alteration of normal homeostasis (Ramadoss et al., 2005; Dietrich and Kaina, 2010). The transcription of Phase II genes is also regulated by Nrf2 (Nuclear factor erythroid 2 related factor 2) transcription factor which binds to the cis-acting element named the antioxidant response element (ARE). The Nrf2 regulates the genes that produce enzymes to detoxify electrophiles and reactive oxygen species, as well as the removal or repair of some of their damage products, which includes GSTs, UGTs and NQO1 (Viegas et al., 2019).

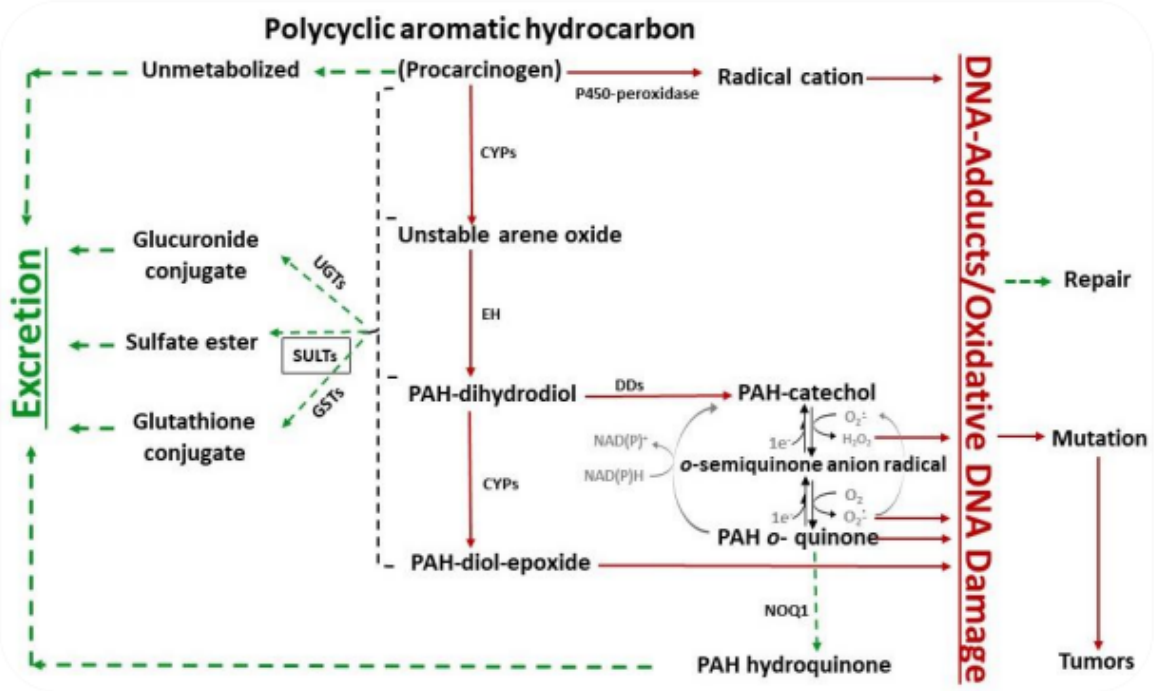


Figure 10. Major metabolic transformations of PAHs. Activation (green arrows) and detoxification (red arrows) routes. Abbreviations: CYPs, cytochromes P450 1A1, 1A2, 1B1, others; EH, epoxide hydrolase; NQO1, NAD(P)H:quinoneoxireductase 1; UGTs, UDP-glucoronosyltransferases; SULTs, sulfotransferases; GSTs, glutathione S-transferases; DDs, dihydrodiol dehydrogenases. *(Adapted from Viegas et al., 2019).*

Since PAHs have different molecular structures and inherent characteristics, each compound presents different *in vivo* carcinogenicity (Pussa, 2013). Although the unmetabolized PAHs could have toxic

effects, the reactive ones (e.g epoxides and dihydrodiols) are of major concern, due to their ability to bind cellular proteins and DNA (Armstrong et al., 2004). As a consequence, cell damage and biochemical alterations leads to mutations, tumors, developmental malformations and cancer (Bach et al., 2003). In this regard, evidence shows that mixtures of PAHs may be more carcinogenic to humans than individual PAHs. This evidence comes mainly from long-term occupational studies on workers exposed to mixtures containing PAHs, showing an increased risk of skin, lung, gastrointestinal and bladder cancers (Lippman and Hawk, 2009). However, it remains unclear if the association: exposure to PAHs and related carcinogenicity is due exclusivity to PAHs or could be related also to cancer-causing agents (e.g., aromatic amines) to which these workers had been simultaneously exposed. All 15 PAHs considered by the SCF showed mutagenic/genotoxic activity in somatic cells, and excepting benzo[*g,h,i*]perylene, they also showed carcinogenic activity in experimental cells as well (EFSA 2008). Since these substances occur in complex mixtures that present infinite possible combinations, interactions between PAHs in mixtures, as well as their effects and toxicity in humans, may be complex and unpredictable. (Choi et al., 2010; Tarantini et al., 2011). Laboratory animal studies have well established the carcinogenicity of certain PAHs (Samanta et al., 2002). These studies reported that animals exposed for a long period of time to high levels of PAHs suffered from lung cancer from inhalation, skin cancer from skin contact and stomach cancer from ingesting PAHs in food (Hecht, 1999; Latif et al., 2010; Lynch and Rebbeck, 2013). BaP was the first PAHs to be discovered as a chemical carcinogen and the most common to cause cancer in animals (Latif et al., 2010). Regarding teratogenicity, effects of PAHs at embryonal level have been described in animal models exposed to PAHs such as benzo(a)anthracene, BaP, and naphthalene. In fact, laboratory trials on mice have shown that the ingestion of high levels of BaP during pregnancy resulted in birth defects and a decreased body weight in the offspring (Ng et al., 2009). Although it is not known whether these effects can occur in humans, some studies demonstrate that low birth weight, premature delivery, and heart malformations are reported in children from mothers exposed to PAHs during pregnancy. This exposure is also related at age three to a lower Intelligence Quotient (IQ) at ages six and eight to increased behavioral problems, and childhood asthma (Perera et al., 2012).

Immunosuppression in rodents has also been reported for PAHs, although the precise mechanisms of PAH-induced immunotoxicity are still not clear. However, it seems that may be involved in the mechanisms by which PAHs induce cancer (Gao and Burchiel, 2014).

In humans, a high incidence of esophageal and colorectal cancers development has been associated with exposure to high levels of PAHs, as well as endocrine disruption-related infertility (Ramesh et al., 2004). The relationship between consumption of well-done cooked meat and risk of cancer has been evaluated by several epidemiological studies (Marchand et al., 2002; Jinap et al., 2013). Different studies reported a positive correlation between well-done cooked meat intake with cancer incidences, especially for colorectal and breast cancer (Sinha et al.,1999; Afzaninawati et al., 2013; Jinap et al., 2013, White et al., 2016). According to Boldo et al. (2022), among consumers of well-done white or red meat was observed an increased risk of gastric and intestinal tumors. Griddled/barbecued/ stewed red and white meats could have an important role in gastric cancer pathogenesis. Although liver is the main site of PAHs metabolism, extrahepatic organs such as the gastrointestinal tract may also have an important role. It is supposed that the cause of gastrointestinal cancers relies in the fact that cells of esophagus, duodenum and colon can metabolize PAHs and that microsomes of gastric mucosa metabolize BaP (Diggs et al., 2011; Bulanda and Janoszka, 2022). As reported by John et al. (2011) an increased risk of prostate cancer was associated with a frequent consumption of grilled or well-done red meat, highlighting that the cooking methods and level of doneness more than the fat content itself, determine the risk of this kind of cancer. A small number of cases are reported concerning the association between meat-derived mutagens and kidney cancer (Chow et al., 2010). Daniel et al. (2011) reported that PAHs, including BaP, in grilled meat was associated with a higher risk of kidney cancer, which risk increased with the increasing consumption. With the increasing of BaP consumption, the risk of renal cell carcinoma was twice higher.

1.4.6 Risk/Safety assessment

Toxic contaminants may enter the food chain at different points, meaning that successful understanding of the risk of potentially toxic compounds in food and food products requires continuous assessment throughout the chain, “from farm to fork” (Pussa, 2013) and posterior comprehension of digestion and metabolization (Engel et al., 2015). Chemical risks are usually assessed through the content of ingested contaminants such as PAHs. Only a fraction of the ingested amount is **bioaccessible:** *namely fraction of a compound released from the food matrix in the digestive tract during digestion, available for absorption in the intestinal epithelium* (Guerra et al., 2012) and **bioavailable**: *namely fraction of a bioavailable substance that is transferred to the blood circulation,*

hence capable to reach target organs and have a toxic effect (Cardoso et al., 2015) due to the modulatory effect of digestion, therefore suggesting that only a fraction of the ingested contaminants is assimilated by the consumer's organism (Cardoso et al., 2015; Engel et al., 2015). Bioaccessible fraction of contaminants vary widely even among the same family of contaminants, which may be explained by different factors: chemical properties of the contaminant; matrix composition (content of lipids or percentage of water,); particle size of meat ingested/mastication (as the particle size decreases, the surface area increases, so bioaccessibility may as well increase); food processing prior consumption (before cooking and cooking methods themselves); different protocols used to simulate digestion *in vitro* (static or dynamic models; incubation time with gastrointestinal solutions; pH values) (Engel et al., 2015).It is important to note that the referred assays of bioaccessibility focuses on the upper digestive system; however, the digestive environment including microbial ecosystems (particularly intestinal microbiota) should also be considered.

Not all PAHs lead to health hazards but they may act as synergists, antagonists or present an additive effect (Tarantini et al., 2011). This is important when interpreting risk assessment, since many risk assessments use toxic equivalent factors (TEF) for PAHs (as well as for dioxins, furans and polychlorinated biphenyl (PCBs)). TEF is based on the sum of each PAH concentration modulated by a factor reflecting its toxicity compared to a standard substance (which is usually established based on toxicity data using total contamination concentration instead of considering factors such as bioaccessibility and bioavailability) (Nisbet and LaGoy,1992; Tarantini et al., 2011). Consequently, it presumes that the chemicals have the same mechanism of action when in reality there is no evidence that all PAHs are activated through the same metabolic pathway, bind to DNA in the same positions or induce the same type of mutations in the same organs. Based on this, SCF, JEFCA and EFSA concluded that using the TEF approach was not suitable for assessing the risk of PAHs in food. (Nisbet and LaGoy,1992; EFSA 2008). Another approach is based on benchmark doses (BMD) and Margin of Exposure (MoE). The benchmark dose (BMD) estimates the dose able to cause a low but measurable response and it is based on a mathematical model fitted to the experimental data within the observable range (Yebra-Pimentel et al., 2015). The MoE is the ratio of the no-observed-adverse effect level (NOAEL) or benchmark dose lower confidence limit (BMDL) for the critical effect to the theoretical, predicted, or estimated exposure dose or concentration. So far, it seems to be the most credible approach since it considers both the dietary exposure and available data on the dose-response relationship (Yebra-Pimentel et al., 2015). The magnitude of the MoE indicates a level of concern,

despite not being a precise quantification of risk; the larger the MoE, the smaller the potential risk, but the comparison between the MoE of two substances cannot be directly done. A carcinogen which MoE is of 100 does not necessarily increase 10 times the cancer risk of a different substance with a MoE of 1000.

In 2006, WHO/JECFA chose to use the MoE approach to assess the risk from PAHs in food and concluded that estimate intakes of PAHs from food based on the available exposure data were of low concern for humans (WHO 2006). With the same goal in 2008, the EFSA Panel used a similar approach on Contaminants in the Food Chain (CONTAM), reaching similar results to WHO/JEFCA, despite considering that consumers of high amounts of PAHs contaminated food could be at risk (EFSA 2008). Another approach to assess cancer risk for PAHs is the relative potency factor (RPF), which estimates the risk of individual PAHs relative to BaP. This approach is currently used by EPA to assess cancer risk (Yebra-Pimentel et al., 2015). In 2010, the External Review draft of EPA (EPA 2010) collected studies in which one or more PAHs was tested at the same time as benzo[a]pyrene, concerning their carcinogenicity bioassay. Based on this, a database of primary literature relevant to the RPF approach was developed by EPA's Office of Research and Development (ORD) by carrying out a comprehensive review of the scientific literature from the 1950s through 2009 on the carcinogenicity and genotoxicity of PAHs (EPA 2010). 74 PAHs for the RPF analysis were evaluated from which final RPFs based on tumor bioassay data was established for 27 PAHs.

1.4.7 Analytical methods for PAHs determination

PAHs are usually present at low concentrations in food (in ppb concentrations). Different techniques have been developed to extract PAHs from foodstuffs, depending on the type and complexity of the food matrix (Bansal and Kim, 2015; Zelinkova and Wenzl, 2015). The analytical methods to determine PAHs had been developed long prior to the publication of the US EPA's priority PAHs list. Their number increased significantly afterwards and there are a wide number of relative published methods although no standardized procedure is published (Wenzl and Zelinkova, 2018). The main factors on which analytical methods are depended are the type of product, fat composition, extraction methods, instrumentations. The solvent used to extract PAHs, along with the robustness of the method and potential for automatization are important as well (Singh et al., 2017; Wenzl and Zelinkova, 2018). Since PAHs are sensitive to light and may decompose by photoirradiation and oxidation, protecting

the extracts from light can be critical to obtain reliable results (Mottier et al., 2000; Diletti et al., 2005; Plaza-Bolanos et al., 2010). An important step to take into consideration to accurately quantify PAHs and separate them from the matrix is the sample collection and preparation. The sampling usually requires long extraction and/or preparation steps, depending on the nature and complexity of the food matrices. Sampling methods are established according to the type and weight of the sample and many precautions such as protecting the sample from tobacco smoke, light and high temperatures should be taken to avoid variations in the composition of the sample (EFSA 2008; Yebra-Pimentel et al., 2015). Being high hydrophobic compounds, PAHs are associated with the lipid constituents when extracted, so they usually need to be removed by cleaning steps (Purcaro et al., 2013). This generally requires a sample cleanup and pre-concentration technique to extract analytes from different matrices, constituting a significant time-consuming step, especially in fat rich matrices (Gu et al., 2009; Yebra-Pimentel et al., 2015). Several approaches have been proposed within the lipid extraction methods. The most used methods to isolate PAHs from food include saponification of lipids with KOH-methanol, followed by extraction methods such as Soxhlet or sonication extraction, liquid-liquid extraction (LLE), solid phase extraction (SPE), solid phase microextraction (SPME), supercritical-fluid extraction (SFE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), ultrasound-assisted emulsification-microextraction (USAEME) (Wang et al., 1999; Farhadian et al., 2010; Viegas et al., 2012a; Purcaro et al.2013;Yebra-Pimentel et al., 2013). Limitations and advantages are presented by each technique. Several methods have been published to analyze both PAHs and their metabolites. Both liquid and gas chromatography have been demonstrated to be suitable to analyze PAHs, with no difference in accuracy of results therefore roughly equivalent in quality criteria (Sykes et al., 2013). Different detection systems can be used, most commonly, LC coupled to fluorescence (FLD) and ultraviolet-visible (UV) detection, and GC coupled to mass spectrometry (MS) (Plaza-Bolanos et al., 2010; Purcaro et al., 2013). The most frequently used analytical method to determine carcinogenic priority PAHs in food samples, is HPLC with fluorescence detection, although acenaphthene is not detected by fluorescence therefore determined otherwise by LC with UV detection, or by GC-MS. (Plaza-Bolanos et al., 2010; Benson et al, 2017). The most used methods are single quadrupole GC-MS and HPLC-FD. Only a few laboratories apply gas chromatography high resolution magnetic sector mass spectrometry to determine PAHs in food (EFSA 2007; Wenzl and Zelinkova, 2018). The increasing affordability of instruments and advances of mass spectrometry has led to the large application of mass spectrometers combined with both GC

(GC-MS/MS) and ultra-performance LC (UPLC- MS/MS) (Vyrand et al., 2007). GC time of flight MS (GC-TOF) and GC-quadrupole TOF (GC-QTOF) mass spectrometers were used for PAH analysis only in special cases, since detection systems of quadrupole mass spectrometers are more sensitive than those of TOF mass spectrometers (Drabrova et al., 2012; Wenzl and Zelinkova, 2018). Fast non-chromatographic methodologies for PAHs analysis in food have not yet been described (Wenzl and Zelinkova, 2018). Being of such interest within the public concern and scientist's needs, there is an ongoing demand to develop techniques that are less time-consuming, environmentally friendly, less solvent-consuming, as well as techniques presenting high sensitivity/low detection limits (LODs), since PAHs maximum levels allowed in foodstuffs established by the current legislation are low (Reg 835/2011; Wenzl and Zelinkova, 2018). In the quantification methods, external calibration curve and standard addition have been used. Viegas et al. (2012a) showed that standard addition method was the most adequate method to quantify PAHs levels in processed meat and fish.

1.4.8 PAHs in cooked meat

PAHs formation in cooked muscle foods strongly depend on the: chemical composition of food, cooking method (grilling, smoking, roasting, frying, baking) (EFSA 2008; Farhadian et al., 2010; Farhadian et al., 2011; Ledesma et al., 2014; Min et al., 2018), heating source (type of fuel and reaction conditions) (Stumpe-Viksna et al., 2008; Viegas et al., 2012a; Ledesma et al., 2014; Singh et el., 2016; Wenzl and Zelinkova, 2018). It is generally accepted that PAHs generate from the incomplete combustion of organic matter when subjected to a wide range of high temperatures (125°C to 900 °C) (Lee et al., 2016; Min et al., 2018). After that, PAHs migrate to lipophilic food compartments that make fatty food items more prone to accumulate PAHs (Luzardo et al., 2013). Although the precise mechanism of PAHs formation in food is still unclear (Alomirah et el., 2011; Hamidi et al., 2016; Min et al., 2018) three possible of them in smoked/grilled food have been proposed (Figure 11):

- Pyrolysis of organic matter (mostly fat and protein) at temperatures above 200 °C. Pyrolysis is the chemical decomposition of organic matter through heat. Temperature ranging from 500 to 900°C represent favourable conditions for PAHs formation (Bartle, 1991; Knize et al., 1999). Pyrolysis of fat has been shown to lead to the highest concentrations of PAHs (Bartle, 1991).

- Lipids dripping directly over the flame at intense heat can generate volatile PAHs, which in their turn could be deposited on the surface of the food as the smoke rises (Phillips, 1999; Farhadian et al., 2010).
- Incomplete combustion of the heat source. The incomplete combustion of charcoal can generate PAHs, which are then transported by smoke and adhered to the surface of the food. (Wu et al., 1997; Alomirah et al., 2011).

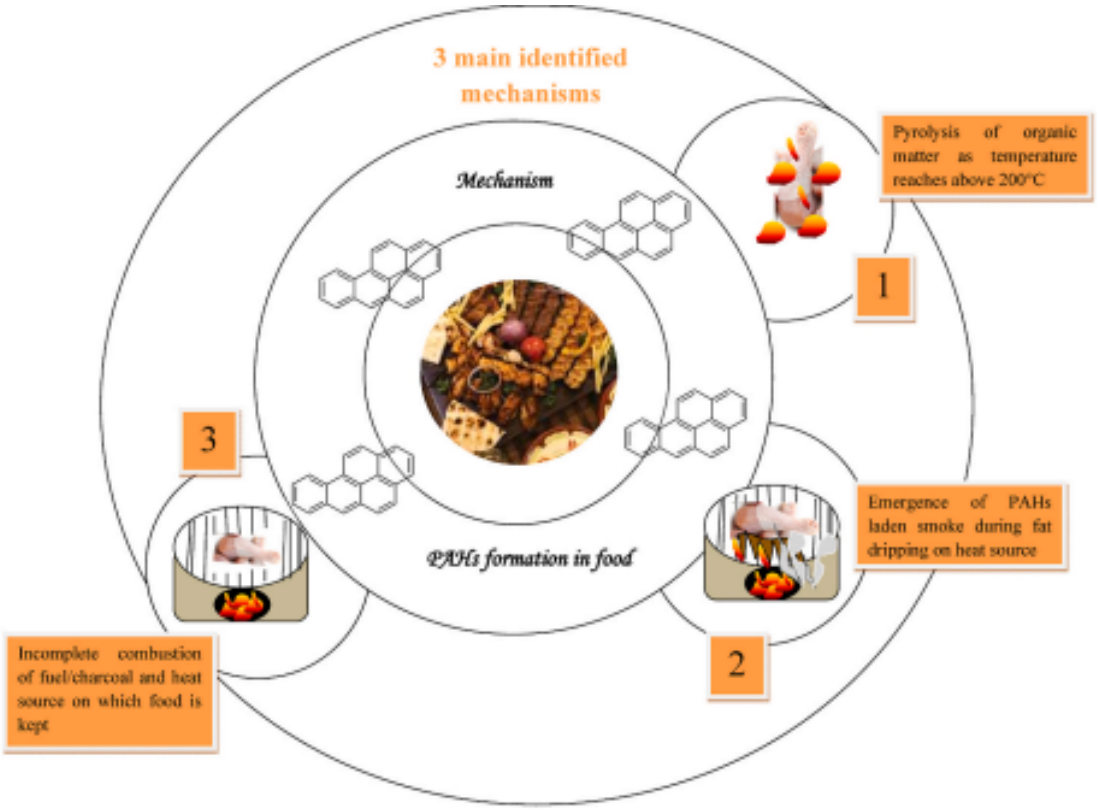


Figure 11. The three-mechanism proposed for PAHs formation. *(Source: Singh et al., 2022)*

In processed and cooked meat, PAHs are generated from pyrolysis of organic matter as well as from direct deposition/ adherence of PAHs arising from smoke produced by the incomplete combustion of different heat sources (Farhadian et al., 2011; 2012). In addition, meat and fish juices released during grilling, dripping into the heat source may undergo pyrolysis and lead to formation of PAHs which in their turn, conveyed by the smoke, could be deposited on the surface of the food. In addition, fat dripping causes flame formation, increasing the temperature near the surface during the flashing and the release of smoke that carries PAHs (Viegas et al., 2012b).

1.4.9 Factors affecting the formation/contamination of PAHs in muscle foods

Several factors can influence the formation of PAHs in cooked meat. Therefore, it is crucial to understand the elements that encourage greater levels of PAHs generation in order to eventually regulate and minimize their formation. Model system studies appear to be of utmost importance for understanding the mechanisms of PAHs formation and subsequently implementing measures for their mitigation.

Food contamination with PAHs can happen at various phases, from farming to processing and cooking (Jakszyn et al., 2004). In this regard, as stressed by Domingo and Nadal (2017), it is crucial to be aware of the amount of these toxicants in raw and unprocessed foods before evaluating the impact of cooking processes as contamination can also happen during earlier stages. Despite the main factors as type of food, cooking methods and type of heat source, other factors are known to contribute to PAHs formation such as: distance between the food and heating source (Chen and Chen, 2001; Sharma et al. 2006; Alomirah et al.2011, Viegas et al., 2014) temperatures and duration of the heating process (Min et al.2018, Haritash and Kaushik, 2009); fat content and fat profile (Singh et al. 2016, Ledesma et al. 2014) water content, pre-cooking procedures and marinades etc (Viegas et al.2012b).

1.4.9.1 Cooking method

Since meat is frequently processed or cooked using temperature-related techniques such boiling, steaming, poaching, baking, microwaving, roasting, grilling, barbecuing, smoking, etc it has been investigated how these techniques affect the levels of PAHs in food (Sobral et al., 2018). Despite the fact that all cooking techniques appear to raise PAH levels in comparison to unprocessed foods, each one has unique properties that directly affect the formation of these pollutants (Singh et al., 2016; Pouzou et al., 2018). High temperature cooking techniques (150 to 300° C), specifically roasting, frying, grilling, smoking, and barbecuing, are recognized to be the principal contributors to PAH generation, especially when meat is cooked over charcoal or open flames (Chung et al., 2011; Garcia-Lomillo et al., 2017; Sobral et al., 2018). In their 1997 study on the effects of processing on duck breast steak, Chen and Lin found that, when cooked for 0.5 to 1.5 hours, smoking produced the highest quantities of PAHs, followed by charcoal grilling, roasting, steaming, and liquid smoke flavoring. In detail, smoking had the highest BaP concentration and total EPA 16 PAHs (BaP/sum EPA PAHs

=14/154 μg/kg) followed by charcoal grilling (8.5/151 μg/kg), roasting (nd/88.5 μg/kg), and steaming (nd/4.4 μg/kg). In 2001, Kazerouni and colleagues found that well-done barbecued chicken with skin produced the greatest BaP concentrations (5.9 μg/kg), compared to oven broiled, pan-fried, roasted, stewed, baked, microwaved, and grilled meat preparations. Electric heating, oven broiling, or pan-frying typically produced BaP concentrations far below 0.5 μg/kg (Kazerouni et al., 2001; El Badry, 2010; Farhadian et al., 2010; Rose et al., 2015), showing that these heating modalities are not pertinent when concerns are raised about concentration of PAHs.

The quantities and profiles of 16 PAHs in various grilled and smoked foods were assessed by Alomirah et al. (2011). They found eight genotoxic PAHs, the highest mean values were found for Chr (4.88μ g/kg) and BaA (2.27 μg/kg). The authors claim that the type of heat source, grilling time, grill geometry, and fat content were the elements that had the most impact on PAH development. In several samples of beef and salmon cooked using various techniques, such as grilling with coal and wood, deep-frying, roasting in a natural gas oven, and roasting in an electric oven, Rose et al. (2015) analyzed the generation process of 15 different PAHs, including PAH4. The highest PAH contents were found in the samples of salmon and beef that were cooked over mineral charcoal and wood, respectively, with a substantial BaP concentration after cooking times of more than 8 and 10 minutes, suggesting that these animal products are vulnerable to PAH production and contamination. According to Park et al. (2017), the BaP levels in pork meat prepared using various methods, such as charcoal grilling, frying, and electric heating, were significantly higher in grilling. Additionally, meat cooked directly over a charcoal fire showed a higher level of BaP than the one cooked using a modified charcoal grill in terms of charcoal position and air circulation. However, studies comparing the total amount of EPA 16 PAHs in raw and grilled meat showed a considerable rise in almost all grilled food products, reporting a total risk of PAHs increased by a factor of five (Cheng et al., 2019). Regarding the PAH profile, it varies according to the type of food. While in grilled vegetables this profile is represented by light-molecular weight PAHs, in grilled chicken wings and mutton meat high-molecular weight PAHs are much more common and consequently more likely to be linked to negative health impacts (SCF 2002; Perello et al., 2009; Cheng et al., 2019). Meats that have been broiled, smoked, or barbecued present the higher concentration of PAHs. Each method's utilization of fuel and heat source, as well as the duration and temperature attained, have an impact on PAHs formation (Singh et al., 2016).

1.4.9.1.1. Grilling/Barbecuing

The process of grilling involves heating food by exposing it to temperatures up to 260°C directly to a heat source, which varies depending on the type of grill. Barbecuing, on the other hand, is a method of slow cooking that uses smoke and heat from wood or charcoal that has been burned, as an indirect heat source (Sobral et al., 2018). Grilling may be performed directly or indirectly, with a variety of grilling implements, including the traditional charcoal barbecue, a modified smokeless charcoal grill, and even without the use of charcoal by using other grills like electric one (Viegas et al., 2012a). Grilling over an open flame or by direct contact between the food and the heat source (grill or flame) encourages PAHs formation (Chen and Lin, 1997; Zelinkova and Wenzl, 2015). When food is grilled over an open flame in a barbeque, fat's drips over the charcoal flames, promotes the formation and volatilization of PAHs, which adhere to the surface of food as the smoke particles rise (Farhadian et al., 2010; Lee at al., 2016). A preliminary investigation on the charcoal grilling of 15 beef steaks, followed by an analysis of 15 PAHs in the outer 1 cm of the steak, represents one of the earliest studies on the generation of PAHs during grilling (Lijinsky and Shubik, 1964). In comparison to broiled beef, charcoal grilling produced BaP concentrations of up to 8 μg/kg. Their research provided the first proof that melted fat may drip onto heat surfaces, causing pyrolysis and the production of PAHs at high temperatures. Regrettably, minute smoke particles would then carry these PAHs back to the surface of the meat product being barbecued. They validated the link between fat dripping, fat concentration, and pyrolysis in a follow-up investigation conducted in 1967 by Lijinsky and Ross. They evaluated three types of meat (beef, pork, and chicken), five different cooking techniques (electric and gas broiling, charcoal, charcoal with no drip, and charcoal with a limited amount of charcoal), and the distance to the heat source (7, 12, 25 cm). According to the study's findings, factors influencing the BaP concentration included meat fat concentration, flame contact, time and temperature combination, indicated by heat source distance. Unlike Lijinsky and Shubik (1964) today's analysis of barbecued meat looks at the entire meat rather than just the outer surface. Typically, consumers consume the entire piece of barbecued meat, not just the top layer. However, if the meat is severely burnt, the removal of the outer covering might be suggested as a mitigation strategy.

1.4.9.2 Type of heat source/fuel

A crucial factor regarding PAH meat contamination, consists in the type of fuel used during the grilling cooking methods. The relative contamination varies according to them (Lijinsky, 1991; CAC 2009; Farhadian et al., 2010). The PAHs released during combustion of charcoal are influenced by the charcoal's composition, which is a significant contributor to the PAHs contamination of grilled food. Charcoal may release PAHs before grilling as it is typically prepared to be used through a carbonization, or a pre-heating for a brief period of time at 600–650°C. Chaemsai et al. (2016), investigating three different coal carbonization temperatures (500°C, 750°C, 1000 °C) and three burning duration (5 min, 20 min, 5 h) found the higher level of PAHs at 500 °C: 19.86 μg/kg, 1.23 μg/kg and 0.69μg/kg of PAHs released at 5 min, 20 min and 5 h respectively. At 700 °C and 1000°C the values were lower (0.95 μg/kg, 1.25 μg/g, 0.28 μg/g at 700°C and 1.38 μg/kg, 0.82 μg/kg, 0.15 μg/g at 1000°C). This is most likely because high carbonization temperatures result in the development of charcoal with high quantities of carbon, facilitating lower formation of volatile matter and PAHs upon combustion. Charcoal should also burn completely to lower its amount of PAHs. The importance of pre-heating charcoal before starting barbecue was reported by Lee et al. (2016). Monitoring the formation of PAH4 in meat barbecued over 4 periods of 12 minutes each, authors observed higher levels of PAHs in the first 12 minutes of heating, characterized by incomplete combustion of the charcoal with visible flames. No significant differences in PAHs concentration were observed for the other periods. According to the findings of numerous studies below-reported (Table 7), charcoal- grilled meat presents greater PAH amounts than gas-grilled meat (Kazerouni et al., 2001; Farhadian et al., 2010; Alomirah et al., 2011; Viegas et al., 2012a; Duedahl-Olesen et al., 2015; Rose et al., 2015; Gorji et al., 2016).

Table 7. BaP concentrations in different charcoal-grilled meat versus gas-grilled. Direct heat refers to meat directly cooked over the heat source, while indirect heat refers to cooking adjacent to the heat source. *(Adapted from Duedahl-Olesen et al., 2021)*

MEAT PRODUCT	Heat source				
	Gas (µg/kg)	Charcoal (direct heat) (µg/kg)	Charcoal (indirect heat) (µg/kg)	Charcoal (not specified) (µg/kg)	Reference
Beef	-	-	-	0-24	Aaslyng et al., 2013
	-	ND-0.92	-	-	Chung et al., 2011
	-	-	-	ND-17.5	Duedahl-Olesen et al., 2015
	ND-0.84	-	-	7.34-12.5	Farhadian et al., 2010
	-	ND-3.16	-	-	Farhadian et al., 2011
	-	-	-	0.11-4.78	Farhadian et al., 2012
	-	0.17-0.39	-	-	Garcia-Lomillo et al., 2017
	0.35-4.02	0.36-5.81	-	-	Gorji et al., 2016
	-	0.27-0.29	-	-	Haiba et al., 2019
	-	-	-	0.09-4.86	Kazerouni et al., 2001
	-	2.20-5.07	0.78-1.13	-	Lee et al., 2016
	-	ND-42	-	-	Maga, 1986
	-	-	-	ND-0.16	Olatunji et al., 2013
	-	-	-	ND-0.29	Oz and Yuzer, 2016
	0.07-16.6	0.23-29.1	-	-	Rose et al., 2015
	-	0.41-0.5	-	-	Viegas et al., 2012b
Pork	-	-	-	-	Kazerouni et al., 2001
	-	0.68-5.99	0.56-0.66	-	Lee et al., 2016
	-	-	-	0.13-0.19	Olatunji et al., 2013
	-	5.51-8.04	1.28	-	Park et al., 2017
	-	0.7-1.8	-	-	Reinik et al., 2007

	Gas (µg/kg)	Charcoal (direct heat) (µg/kg)	Charcoal (indirect heat) (µg/kg)	Charcoal (not specified) (µg/kg)	Reference
Pork	ND-0.98	0.36-1.9	-	-	Rose et al., 2015
	-	-	-	-	Tkacz et al., 2012
	-	-	-	1.07-2.71	Viegas et al., 2014
	-	ND-30.2	-	-	Wongmaneepratip et al., 2019
Pork sausages	-	-	-	ND-1.9	Duedahl-Olesen et al., 2015
	-	-	-	0.01-0.11	Kazerouni et al., 2001
	-	ND-1.0	-	-	Larsson et al., 1983
	-	ND-1.2	-	-	Reinik et al., 2007
	ND-1.88	0.27-3.55	-	-	Rose et al., 2015
Chicken (no skin)	-	-	-	0-1.2	Aaslyng et al., 2013
	-	-	-	ND-0.6	Duedahl-Olesen et al., 2015
	ND-0.6	-	-	1.61-6.46	Farhadian et al., 2010
	0.28-5.02	0.28-5.23	-	-	Gorji et al., 2016
	-	0.97-1.34	-	-	Haiba et al., 2019
	-	-	-	ND-0.04	Olatunji et al., 2013
	0.96-5.30 (direct) ND-1.01 (indirect)				El Badry, 2010
	-	-	-	ND-3.96	El Hussein et al., 2018
	-	ND-2.44	-	-	Farhadian et al., 2011
	-	3.14-8.73	-	-	Viegas et al., 2012b
Chicken with and without skin	0-0.93	ND-4.63	0.12-0.64	-	Alomirah et al., 2011
	-	ND-4	-	-	Kao et al., 2012
	-	-	-	0.39-4.57	Kazerouni et al., 2001

MEAT PRODUCT	Gas (µg/kg)	Charcoal (direct heat) (µg/kg)	Charcoal (indirect heat) (µg/kg)	Charcoal (not specified) (µg/kg)	Reference
Chicken (not specified)	-	-	-	ND-1.0	Reinik et al., 2007
	ND-0.48	0.26-0.57	-	-	Rose et al., 2015
	-	ND-3.68	-	-	Wang et al., 2018
	-	0.44-1.51	-	-	Wang et al., 2019a
	-	0.51-2.81	-	-	Wang et al., 2019b
Duck with and without skin	-	3.7-9.2	-	-	Chen and Lin, 1997
Duck (not specified)	-	2.6-3.1	-	-	Kao et al., 2012
Goat	-	-	-	0.09-1.52	Ahmad et al., 2017
Lamb	ND-4.45	ND-4.28	ND-0.21	-	Alomirah et al., 2011
	-	-	-	ND-3.9	Duedahl-Olesen et al., 2015
	-	ND-5.8	-	-	Kao et al., 2012
	-	0.32-2.81	-	-	Mottier et al., 2000
	0.18-0.59	0.54-2.39	-	-	Rose et al., 2015
Mutton	0.3-2.1 (horizontal) <0.1 (vertical)	-	-	-	Saint-Aubert et al., 1992

Only a single study by Alomirah et al. (2011), showed that barbecued lamb cooked over gas had greater BaP concentrations than the one cooked over direct charcoal, nevertheless with substantially lower BaP in indirect charcoal-grilled lamb meat. Direct charcoal grilling of chicken, beef, and pig meat was compared to indirect charcoal and BaP concentrations and other PAH were shown to rise by a factor of five or more when direct charcoal grilling was used (Alomirah et al., 2011; Lee et al., 2016; Anjum et al., 2019). The same trend was observed by Rose et al. (2015) within an in-depth barbecue study develop in the UK taking into consideration different type of meat (chicken, beef, lamb, pork, fish, and sausage), as well as various heating methods and fuels (gas, charcoal, charcoal

with wood chips, and briquettes), The use of charcoal with wood chips was linked to the highest concentrations for all grilled meat products. When compared to gas grilling of the same products, the use of briquettes generated equivalent or reduced BaP, PAH4 and sum PAH27 concentrations for meats except of beef burgers. Besides the food itself, the strongest determinant seemed to be the closeness to the heat source (where closer translated into the highest levels of PAHs). For gas-fueled barbecues the main source of PAHs was not the pyrolysis of fat drips but the gas flames themselves or the contact between the surface of the food and the gas flames (Table 8). However, the differences in PAHs amounts generated by gas and charcoal as heat source is probably caused by the fact that charcoal burns more slowly than gas does, which causes more soot to accumulate and higher levels of PAHs (Duedahl-Olesen et al., 2021).

Table 8. Concentrations of BaP, PAH4 and sum of PAH in different type of barbecued meat and using different heat sources. The results reported by Rose et al. (2015) refer the shortest distance to the heat source. *(Adapted from Duedahl-Olesen et al., 2021)*

Type of meat	Heat source	BaP (µg/kg)	Sum PAH4 (µg/kg)	Sum PAH_{total} (µg/kg)	Reference
SAUSAGE	Charcoal	3.29	15.7	PAH_{27}= 117	Rose et al., 2015
	Charcoal+wood chips	22.4	22.4	PAH_{27}= 410	
	Briquettes	0.31	3.65	PAH_{27}= 62.3	
	gas	1.50	4.48	PAH_{27}= 43.7	
BEEF PATTIES (30% FAT)	Mesquite wood	42.0	144	PAH_{31}= 549	Maga, 1986
	Hickory sawdust	1.0	6.0	PAH_{31}= 68.0	
BEEF BURGER	Charcoal	17.3	66.6	PAH_{27}= 563	Rose et al., 2015
	Charcoal+wood chips	20.0	70.6	PAH_{27}= 790	
	Briquettes	10.4	38.7	PAH_{27}= 449	
	gas	14.8	35.2	PAH_{27}= 547	
BEEF	Coconut shells	0.50	3.49	PAH_{15}= 43.8	Viegas et al., 2012b
	Wood charcoal	0.41	2.33	PAH_{15}= 48.1	

BEEF	Charcoal	0.27	3.86	PAH_{27}= 37.0	Rose et al., 2015
	Charcoal+wood chips	1.20	7.77	PAH_{27}= 82.8	
	Briquettes	0.18	1.74	PAH_{27}= 30.3	
	gas	0.25	0.86	PAH_{27}= 21.1	
PORK CHOP	Charcoal	1.90	8.07	PAH_{27}= 84.4	Rose et al., 2015
	Charcoal+wood chips	3.38	13.5	PAH_{27}= 148	
	Briquettes	0.23	1.84	PAH_{27}= 43.0	
	gas	0.98	3.03	PAH_{27}= 39.3	
CHICKEN	Charcoal	0. 41	7.64	PAH_{27}= 73.6	Rose et al., 2015
	Charcoal+wood chips	0.73	7.07	PAH_{27}= 102	
	Briquettes	0.15	1.66	PAH_{27}= 23.0	
	gas	0.18	0.59	PAH_{27}= 4.76	
	Charcoal+wood chips	3.24	18.2	PAH_{27}= 171	
	Briquettes	0.29	2.73	PAH_{27}= 36.4	
	gas	1.12	3.49	PAH_{27}= 41.6	
LAMB	Charcoal	2.39	11.0	PAH_{27}= 150	Rose et al., 2015
	Charcoal+wood chips	3.03	11.0	PAH_{27}= 130	
	Briquettes	0.71	3.79	PAH_{27}= 71.3	
	gas	0.59	1.88	PAH_{27}= 22.8	

Viegas et al. (2012b) showed that different types of charcoal lead to different PAHs formation. They reported that meat grilled with coconut shell charcoal, labeled as flameless and smokeless, led to less formation of PAHs compared to wood charcoal. These results were more prone in fattier foods. Despite that, different types of charcoal can differently generate PAHs and coconut shell charcoal was suggested as a safer charcoal to human health, considering the reduction of PAHs formation in muscle

foods, especially in fatty ones in comparison with wood charcoal. Authors justified that coconut charcoal was smokeless and flameless and absorbed the fat that drips from the cooking food (Viegas et al., 2012b). On the other hand, Kim et al. (2020), evaluating the formation of 16 EPA PAHs during barbecuing of beef, pork and chicken meat using three charcoal types (white, black and extruded) found that extruded charcoal from coconut shell had the greatest quantities of these PAHs, followed by black charcoal from oak. The higher levels of PAHs in extruded charcoal may be due to barium nitrate and sodium nitrate content. No matter the type of meat used, white charcoal from broadleaf trees generated the lowest PAH concentrations. Concerning wood as a heat source, wood pyrolysis is linked to PAHs formation and different woods may significantly influence the PAH levels in foods (EFSA 2008, Ledesma et al., 2014). Studies report that the amount of BaP in apple-tree and alder-tree smoked meats ranges from 6.0 to 35 μg/kg in spruce smoked meat (Stumpe-Viksna et al., 2008.) Investigations regarding the PAH profiles in the smoke composition of softwood (pine) and hardwood (beech) have not outlined any differences while others have noted a small variation in the concentrations of high molecular PAHs in the finished barbecued products (Larsson et al., 1983; Toth and Pothast, 1984). According to Maga et al. (1986), grilling beef patties presenting 30% of fat and cooked using mesquite wood (64% lignin) and hardwood hickory sawdust (54% cellulose) produced noticeably different BaP, PAH4 and total PAH31 concentration. They also showed different PAHs profiles: 24 PAHs and 16 PAHs in wood grilled and hickory grilled beef respectively.

1.4.9.3 Distance to the heat source and equipment geometry

In order to avoid the generation of PAHs, direct contact between the meat and the heat source should be avoided (Chen and Lin, 1997; Farhadian et al., 2010). In addition, cooking equipment geometry and design are very important and might prevent PAHs formation, which is mainly influenced by the formation of smoke and pyrolysis after fat dripping contact with the heating source (EFSA 2008). Regardless the type of meat, a study of Noll and de Figueredo (1997), comparing the BaP concentrations in charcoal-broiled beef, pork, and chicken cooked over a rack placed 40 cm above the heat source and a portable grill placed 15 cm above the charcoal, found higher BaP concentrations in meat cooked in the portable grill. According to Reinik et al. (2007), home-grilled pork and chicken meats presented greater BaP and PAHs concentrations, with disposable (portable) grills producing up

to a 1.6-fold higher PAH concentration than conventional wood-burning barbecues. Nevertheless, barbecued sausages did not reveal PAH concentrations influenced by the geometry of the barbeque and, consequently, by the proximity to the heat source. In contrast to these results, Larsson et al. (1983) reported that PAH levels in flame-grilled frankfurters cooked at well-done state in 20 cm flames and at different distance to heat source (1, 6, 16, and 26 cm), were strongly dependent on the samples positions over the flame. Samples cooked 1 cm above the fuel presented moderate levels of BaP (~5 μg/kg), while those cooked in a 6-7 cm zone showed considerably higher PAHs levels (BaP ~80 μg/kg). BaP concentrations at extended distances from the fuel decreased with distance, with values about 25 μg/kg and 15 μg/kg at 16 cm and 26 cm of distance, respectively. Rose et al. (2015) analyzed different barbeque conditions in different samples, including the distance to the heat source, finding that in general, PAHs levels were higher when food was cooked closer to the heat source (4 and 7 cm versus 9 and 11.5 cm) regardless of the heat source. According to Larsson et al. (1983), Rose et al. (2015) found that the concentration of PAHs was lower when the sausage cooked over briquettes and beef burgers, beef, and salmon cooked over charcoal food were closer to the heat source. Regarding equipment geometry, when the heat source is vertical or above the food, no PAHs are detected in food, most likely because no fat drips onto the heat source (Lijinsky and Ross, 1967; Saint-Aubert et al., 1992; Rose et al., 2015). Already in 1967, Lijinsky and Ross illustrated the importance of a good equipment design, finding that when meat was cooked above an electric cooker or a gas flame (even when the flame is in direct contact with the surface), and the fat drops were collected to a pan below the burner, the PAH levels dropped 10-fold (despite fat pyrolysis on the meat surface). Also Saint-Aubert et al. in 1992, showed the importance of the geometry of the barbecue by changing the position of the heat source from horizontal to vertical, preventing the formation of fat drips. In vertical barbecue, PAHs levels in samples significantly decreased or were not detected. As reported by Park et al. (2017), BaP concentrations were shown to differ significantly when pork belly was barbecued on a modified charcoal grill (1.3 μg/kg), which prevented flowing fat into the heat source, compared to a standard charcoal grill (8.0 μg/kg). In the modified charcoal grill, the charcoal was placed in the corner of the grill and meat was not in direct contact with the heat source. All of the aforementioned findings support the initial results by Lijinsky and Shubik (1964), which found that smoke produced by incomplete combustion of fat dripped onto the heat source is one of the most important factors contributing to the generation of PAHs during barbecue.

1.4.9.4 Time-temperature combination

The binomial time/temperature constitute another crucial factor in PAHs generation, as different times and temperatures of combustion lead to different PAHs contents in meat. Consequently, it should be monitored and regulated (Pouzou et al., 2018). This binomial seems to be more important than temperature or time alone. However, temperature remains the main influencer with more pronounced effect since PAHs can be formed at both high temperatures (500-800 °C) and low as 100 °C, if the organic matter is subjected to it for a long time (Haritash and Kaushik, 2009; Farhadian et al., 2011; Singh et al., 2016; Min et al., 2018). The degree to which a food is cooked – cooking intensity – translates into different doneness levels: rare, medium, well done and very well done, and is essential to estimate PAHs exposure (Pouzou et al., 2018). The term "doneness" describes how completely a piece of meat has been cooked; it normally ranges as mentioned from rare (soft and red center) to well-done (firm and gray-brown). However, “doneness” is a limited measure since it is quite subjective and can induce misclassification. For example, Sinha et al. (1999), defined beef doneness level in relation to the internal temperature: 60 °C as rare, 70 °C as medium, 80 °C as well done and 90 °C as very well done, but the classification is not uniform among studies. In the light of this, the quantitative binomial time and temperature itself is more objective and rigorous to assess the degree to which a food is cooked, and can be achieved using predictive models based on quantitative time and temperature as demonstrated by Pouzou et al. (2018). As a general rule, the concentration of PAHs tends to be higher the longer and more rigorously the meat is grilled (Saint-Aubert et al.,1992; Kazeoruni et al., 2001; Haiba et al., 2019). In 1983, Larsson et al. reported that no matter the heat source distance respectively of 1, 6, 16, or 26 cm, barbecued frankfurters to a well-done state resulted from the highest heating time (~4.5 min) at the greatest distance (6 cm) and the shortest time (1.2 min), with highest final BaP concentration of 83 µg/kg. In addition, different level of doneness and relative concentration of BaP were obtained when barbecuing frankfurters over a log fire for 3.5 minutes in 20 cm flames: well-done level at 7 cm distance (BaP~ 27 µg/kg), medium at 13 cm distance (BaP~ 22 µg/kg), light (BaP~ 6 µg/kg) and a burnt product at 1 cm distance (BaP~ 12 µg/kg). According to Kazerouni et al. (2001), well-done steaks, hamburgers, and chicken with skin barbecued for long time, are inclined to present higher amounts of BaP respectively of 4.9, 1.5, and 4.6 µg/kg, if compared to medium-done steak (BaP 4.2 µg/kg) and hamburger (BaP 0.1 µg/kg). These BaP level tendencies suggest a link between the entity of doneness and PAHs concentration. Chen and Chen (2001), by grilling duck breast without skin for 0, 1, and 1.5 hours, reported variation of total EPA

16PAH concentrations of 151 μg/kg, 200 μg/kg, and 300 μg/kg respectively. After 1 hour, the BaP concentration did, however, reach a maximum of 5 μg/kg while duck breast with skin presented final BaP concentrations of 8.4 μg/kg after 1 h of grilling and overall EPA 16PAH concentrations of 182 (0,5h); 276 (1h) and 319 μg/kg (1.5h). In 2016, Lee et al. tested different times and combustive temperatures using the same fuel (charcoal). After heating the charcoal (temperature increased from 127 °C to 320°C), the meat samples were cooked in 4 periods for 12 minutes. Grill temperature decreased for the next 12 minutes from 285 to 250 °C and from 250 to 200 °C for the 24 minutes after the third period. Authors found that higher levels of PAH4 were present during the first period of grilling than the subsequent times, most likely due to the changes in the combustion of the burning charcoal over time. In the first period, more PAHs were formed and deposited into the surface of the food, potentially because at first there are flames and the combustion is predominantly incomplete and then, after the flames subside, more complete combustion occurs. Similarly, Rose et al. (2015) found that PAHs levels in beef burgers cooked directly over charcoal and wood chips, surprisingly decreased with longer cooking period. In fact, doubling the cooking time, independently from the cooking distance, a decrease in final PAHs concentration was observed. This may suggest that PAHs may be formed in the first period with the hot charcoal, being subsequently re-volatilised or degraded during prolonged cooking time. Compared to beef meat, it is common knowledge that chicken meat may need shorter barbecuing time. In this regard, Gorji et al. (2016) findings showed that chicken samples had significantly lower total PAH contents than beef samples. Comparing different types of meat (chicken, pork, lamb) including seafood, cooked in two barbecuing time (doubling time), Kao et al. (2012) reported that increased heating time was linked to increased total PAHs concentrations, except for seafood and pork meat, which didn't show any variation. Viegas et al. (2012b) studied the effects of continuous barbecuing with the same charcoal, on PAHs levels in grilled chicken, by measuring two different periods: the first period corresponds to the chicken grilled in the beginning of charcoal combustion, when all the flames had subsided, and the second period corresponds to the chicken grilled a half hour later, before adding new charcoal to the processing bed. The authors observed a doubling of BaP concentration (from 3.1 to 8.7 μg/kg) and of the sum of PAH8 (from 25 to 61 μg/kg), increasing trend caused by the accumulated high molecular weight PAHs being released by reheating. When examining barbeque type and doneness, none of the PAH 8 were found by Oz et al. (2016) in beef samples cooked to rare and medium on the wire grill, while the total sum PAH 8 increased with cooking time to 0.8 and 0.9 μg/kg, for well-done and very well-done cooked samples, respectively.

The rare cooked samples on the stone barbeque did not contain PAH 8, whereas the medium ones presented the highest levels (2.6 μg/kg). In the light of the inconsistent results in the literature and the diversity in experimental conditions, it is difficult to make generalizations based solely on time and temperature given. According to authors, in order to prevent charring and overcooking, the barbecuing temperature should be decreased with a corresponding increase in cooking time (Kazerouni et al., 2001; Perello et al., 2009; Farhadian et al., 2010; Oz et al., 2016; Anjum et al., 2019).

1.4.9.5 Meat type

According to the results of numerous studies on barbecue, the PAH concentrations vary depending on the type of meat (Saint-Aubert et al., 1992; Perello et al., 2009; Dost and Ideli, 2012). Nevertheless, because of the high number of parameters taken into consideration, no obvious trends for particular meat varieties and PAH concentrations have been found. However, the presence of PAHs in meat seems to be strongly affected by its chemical composition, in particular by the fat content (Mottier et al., 2000; Kazerouni et al., 2001; Viegas et al., 2012b; Lee et al., 2016; Oz et al., 2016; El Husseini et al.,2018; Kafouris et al., 2020; Kim et al., 2020). PAHs formation appears to be more related to fat content than to protein or carbohydrates (Farhadian et al., 2011; Singh et al., 2016; Lee at al., 2016). Additionally, PAH formations more likely occur in presence of higher content of unsaturated fat (Chen and Chen, 2001). Besides composition, determinant factors to the final concentration of PAHs are: the edible surface area and surface texture implied in cooking, the ability of losing fat and water content (Rose at al., 2015). Some authors reported the highest BaP concentrations in barbecued pork and salmon (Chung et al., 2011; Viegas et al., 2012a; Kim et al., 2020), while in other studies highest concentrations in barbecued beef compared to pork, chicken, or lamb were reported (Larsson et al., 1983; Aaslyng et al., 2013; Rose et al., 2015). In particular, Rose et al. (2015) observed that no matter the heat source, when comparing BaP, PAH4, and the sum of 27 PAH concentrations for beef, beef burger, pork chops, sausage, lamb, chicken and salmon, beef burger showed a concentration up to twenty times higher than for the lowest concentration of chicken. Compared to lean meats, grilled fatty meats present higher PAHs levels (Lijinsky and Ross, 1967; Maga, 1986; Olatunji et al., 2013). Mottier et al. (2000) found substantially higher levels of PAHs in high fat lamb sausages compared to other less fattier meat products, in accordance with the study of Lee et al. (2016) which found that grilled beef loin presenting 31,7% of fat showed higher amounts of PAHs than beef ribs (24,4 % of

fat). The same relation was observed for grilled pork belly (26.4% of fat) and pork neck lean (9,5 % of fat). Also, lean beefs form less PAHs compared to pork, chicken and even salmon as described by Viegas et al. (2014). Lu et al. (2017a) also found that PAH levels were positively correlated to fat content, and negatively correlated to moisture content. Regarding chicken meat, according to numerous studies that report the lowest BaP concentrations in barbecued chicken filets (Larsson et al., 1983; Duedahl-Olesen et al., 2015; Rose et al., 2015), it has been suggested that chicken compared to others meat types tends to be linked to a lower PAHs concentration. However, according to Gorji et al. (2016) charcoal-grilled chicken wings with skin have shown the highest BaP concentrations while gas-grilled chicken the lowest. This confirms previous research that found higher PAH concentrations in chicken with skin compared to without (Chen and Lin, 1997; Duedahl-Olesen et al., 2015; Kafouris et al., 2020). Contrarily, Haiba et al. (2019) reported higher PAHs and BaP concentrations in skinless chicken. Fat factor was taken into consideration, also by Babaoglu et al. (2017) who observed PAH concentration trends in Turkish lamb and beef kukurec, a traditional food produced from fresh and washed lamb and calf small intestines, obtained in combination with lamb tallow, lamb subcutaneous or lamb tail fat and cooked on charcoal-grill. The beef kukurec and tail fat group showed the highest levels of PAHs compared to lamb meat. Furthermore, Karsioglu and Kolsarici (2022), studying the effect of different cooking methods, fat content and degrees of doneness on PAHs formation in beef doner kebabs, found the highest PAH concentration in well-done charcoal grilling high fat donner samples. As already mentioned, the surface exposed to cooking represents another factor besides the meat composition. El Hussein et al. (2018) carried out a study on traditional Lebanese barbecued beef and chicken products observing that PAH4 content doubled with the increased surface areas. Additionally, a strong association between the surface-to-mass ratio and the PAHs concentration was discovered. In fact, large meat pieces (steak and breast) showed lower levels of PAHs than minced meat and cubed meat suggesting that PAH levels rose when surface exposure to heat increased and meat size decreased. In addition, Rose et al. (2015) came to the conclusion, studying BaP concentrations in barbecued beef burgers obtained from minced beef meat, that fat content and a large surface to volume ratio constitute important factors influencing the PAHs formation. Considering meat products, barbecued frankfurters and sausages have shown a lower PAH concentration than other whole meat products (Larsson et al., 1983; Rose et al., 2015). In sausages, fat is incorporate in stable emulsions, therefore during barbecue, small amounts of fat will drop onto the heat source. Moreover,

the liquid derived from broken sausage casing during grilling, going in different directions, will not reach the heat source in its entirety.

PAHs formation seems to be related to the saturation degree of lipids as well, since the more unsaturated a lipid is, the more formation increases, most likely because the unsaturated fatty acids are more susceptible to oxidation during thermal processes, among other factors such as the possibility of polymerization of polyunsaturated fatty acids to form cyclic monomers or dimers (Min et al., 2018). Li et al. (2021) observed a positive correlation between unsaturated fatty acids and PAH4 content.

Among the other components of meat, protein and amino acids are considered possible factors in PAHs formation, although the relationship between protein content and PAHs formation is not largely studied. According to Nie et al. (2018), when amino acids (0.5 g/100 g) were added to grilled pork sausage, more PAHs were produced in grilled sausages added with basic amino acids compared to acidic amino acids. In comparison to other amino acids, the inclusion of the amino acid with a benzene ring reduced the amount of PAHs as it seems that stable benzene rings restricted their fraction in small molecules. However, despite being a major component of animal food products, most investigations on high-protein foods came to the conclusion that fat has the greater impact on PAH generation than protein does. The association between protein and PAHs generation, however, calls for more research due to the paucity of studies on the effect of different protein types (Saito et al., 2014; Liu et al., 2019).

Other factors influencing the generation of PAHs in food is the water content and pH. Regarding water, studies (Min et al., 2018; Lee et al., 2020) revealed that raw foods that present a higher content of water, once cooked show less PAHs concentration. According to Min et al. (2018), by adding an equal weight of water to ground beef that had been heated to 80°C, for instance, the amount of PAHs in the meat could be reduced by up to 62%, and when the temperature was raised to 200°C, the decrease rate rose to 72%. It seems that water, acting as a conduit for oxygen, minimizes the incomplete combustion of organic compounds during the cooking. Concerning pH factor, Wongmaneepratip et al. (2017) found a higher PAHs content in grilled chicken marinated with 30 g/kg of sodium bicarbonate (pH = 7.51) than the sample marinated with 0.21 g/kg of citric acid (pH = 3.62). This is because the PAHs were produced more quickly as a result of the Maillard reaction being amplified by the higher pH (Wongmaneepratip et al., 2017; Nie et al., 2018). However, also in the case of pH, studies on its effect in PAHs formation are limited.

1.4.10 Strategies to reduce PAHs contamination in meat

1.4.10.1 Cooking methods and processing parameters

Alternative cooking methods have been demonstrated to minimize the development of PAHs in foods (Bansal and Kim, 2015). In terms of the heat source, non-smoke formation heating such as electric heating and infra-ray are suggested to reduce PAHs levels in food (Wang et al., 2022). Considering open-flame heating sources, to minimize PAHs generation, gas is widely suggested as the better option than charcoal and wood. Flame-free areas created between each nozzle (gas flame) reduce the possibility of flame contact with fat drops compared to an open flame generated by charcoal or wood (Ghorbani et al., 2020). In fact, substituting charcoal with gas the PAHs content of grilled Turkish kebab and Iranian kebab decreased by 76% and 25%, respectively (Gorji et al., 2016). From the most to least preferred, the fuels to be used to minimize PAHs contamination of foods are: non flame cooking fuel (infrared and electric heating), gas fuels, charcoal, and low resin wood (Wang et al., 2022).

Regarding processing parameters, PAHs contamination could be reduced by intervening on processing time and temperature, the extent of exposure of food etc. Similarly, to the findings reported by Rose et al. (2015) in increased level of PAHs in various fried, grilled, barbecued and roasted animal products when cooking time was extended by 50%, Min et al. (2018) found in beef heated in aluminum heating block, an increased levels of PAHs with the prolongation of cooking time. Considering processing temperature, Min et al. (2018), by decreasing heating temperature from 120 °C to 80 °C for ground beef, reported the maximal reduction of PAHs levels in meat cooked for 25 min, compared to 15, 20 and 30 min. As the correlation between temperature and time is not completely clear, additional research on the combined effect of temperature and time on PAHs development in processed foods are needed. New insight of this binomium will help to determine the best temperatures and processing times to reduce the development of PAHs in processed foods.

Regarding the extent of exposure of food to heating source, Lee et al. (2016) designed an equipment to remove meat juice drippings during grilling, collecting them in a beaker placed in an ice bucket, finding a significant decrease up to 89% in the sum of PAH4 in barbecued pork and beef meat. In addition, Lee and colleagues, reported how the reduction of smoke by a ventilated barbecue (causing a minor contact between meat and smoke particles) was reflected in a significant reduction in PAH4 concentrations of grilled food (41–74% reduction). In the light of this, to minimize the exposure of

meat to heating sources a greater distance and use of indirect heating are recommended (Wang et al., 2022). Indirect heating entails creating a barrier between the food items and heating source (Farhadian et al., 2011; Gomes et al., 2013). In fact, wrapping meat in banana leaves and aluminum foil before charcoal barbecuing have been found to reduce PAHs levels in grilled beef and chicken: 40% and 80% respectively when using banana leaves, and 46% and 80% using the common aluminum wrapping (Farhadian et al., 2011). Wrapping meat prevents fat dripping into the heat source, reducing the possibilities of PAHs generation. It has been shown that heating of meat before grilling can affect PAHs formation. In this regard, Farhadian et al. (2011) investigated the effect of non-flame methods such as microwave and steaming on the PAHs levels in charcoal grilled beef and chicken. B(a)A and B(b)F levels were reduced by 100% in both chicken and beef samples, while fluoranthene (Fl) concentration was reduced by ~54% and ~60% in charcoal grilled beef and by ~50 % and ~ 39 % in charcoal grilled chicken with steam and microwave pretreatment respectively. Charcoal grilling with heating meat before cooking lasted less than charcoal grilling process without suggesting lower pyrolysis duration, lower depositions of smoke generated by fat drippings. Moreover, in order to shorten the cooking time of high-temperature processing of meat, pre-boiling has been suggested as pre-treatment processing. As boiling can enhance texture properties and conserve sensorial characteristics, the combination of boiling and common cooking methods could be an effective reduction strategy (Wang et al., 2022).

Another reduction strategy related to the charcoal-grilling process consists in preheating charcoal to avoid the exposure of meat to the initial wave of smoke. As reported by Chaemsai et al. (2016) a longer preheating time to a higher temperature resulted in lower PAHs concentration, suggesting that coal carbonized at high temperature contains more carbon causing limited release of volatiles and PAHs upon combustion. Some of the aforementioned strategies to reduce PAHs concentration in meat by changing processing parameters are summarized in the table below (Table 9).

Table 9. Reduction PAHs strategies by changing processing parameters (*Adapted from Wang et al.,2022)*

Strategy	Meat type/cooking method	Parameters	PAHs	PAHs reduction (%)	References
Reduce processing time	Heated beef system	15 min reduced (30-15 min)	PAH8	Up to 40% it wet condition; 56% in dry condition	Min et al., 2018
Reduce processing temperature	Heated beef system	40, 80, and 120^0 C decreased	PAH8	In wet condition, up to 36%, 46% and 62% reduction, when 40, 80, and 120^0 C decreased respectively	Min et al., 2018
				In dry condition, up to 45%, 61% and 74% reduction, when 40, 80, and 120^0 C decreased respectively	
Collect fat drops	Grilled meats	Fat droplets were collected under the grilling grid	PAH4	85%, 76%, 48% and 89% reduction in beef loin, beef rib, pork neck lean and pork belly, respectively	Lee et al., 2016
Remove smoke	Grilled meats	A ventilation duct was placed between flame and meat		73%, 71%, 41% and 74% reduction in beef loin, beef rib, pork neck lean and pork belly respectively	

Indirect processing	Grilled beef and chicken	Wrapped by aluminum and banana leaf; Steam and microwave preheating	FA, Bb Fa, and BaP	46% and 40% reduction in beef sample wrapped by aluminum and banana, respectively	Farhadian et al., 2011
				74% and 80% reduction in chicken sample wrapped by aluminum and banana respectively	
				49% and 44% reduction in beef sample preheated by steam and microwave, respectively.	
Preheating of charcoal	Smoke of charcoal	Charcoal was preheated for 5, 20 min, and 5 h and at 500, 750 and 1000° C.	EPA 16PAH	99% reduction in PAH4 preheated for 5 h at 1000 °C compared with the one preheated for 5 min at 500° C.	Chaemsai et al., 2016
Type of fuels	Grilled pork belly	Charcoal infrared-ray and electric grilling	BaP	100% (8.04 µg/kg in charcoal grilled sample, and BaP was not detected in infrared-ray nor electric grilled sample)	Park et al., 2017
	Grilled Iranian kebab	Gas and charcoal	Most EPA 16PAH (excluding NA)	25%, 29%, 18% and 24% reduction by using gas in juje kabab, juje kabab bal, kabab barg, and kabab koobideh, respectively	Gorji et al., 2016

	Grilled pork sausage, and chicken	Wood and disposable charcoal	PAH8+ 5-MCH, BjFA, DalP, and DaeP	35%, 9% and 34% reduction by using wood in pork, sausage, and chicken, respectively	Reinik et al., 2007
	Grilled beef, pork, and chicken	White, black, and extruded charcoal	EPA 16PAH	60,87% and 75% reduction by using white charcoal rather to extruded charcoal in beef, pork, and chicken respectively	Kim et al., 2020

1.4.10.2 Ingredient-base strategies

The oxidative status of food, namely meat, may have an impact on the formation of carcinogenic compounds as PAHs, since their formation involves radical reactions (Garcia-Lomillo et al., 2017). Antioxidant agents of synthetic or natural origin, found especially in herbs, plant extracts, spices, seasonings, or others interfere with radical reactions and may trap free radicals, including intermediates of PAHs, preventing their formation (Janoszka et al., 2011). Therefore, the use of antioxidants particularly at the pre-treatment stage is becoming a strategy to reduce the formation of PAHs in foods (Alomirah et al., 2011). Ingredients containing antioxidants may have an individual or a synergic effect, therefore many times the combination defines the strength of the final effect; the type of antioxidants may have different activities in different meat systems as well (Lu et al., 2018). The scavenging ability of each antioxidant agent might depend on each specific molecular structure and especially the strong reactivity of phenol or polyphenol moieties with free radicals (Wang et al., 2019a).

1.4.10.2.1 Marination

A common practice before cooking meat, in order to enhance flavor, aroma and texture relies on marinating meat using a mixture of condiments (Farhadian et al., 2012). Depending on the ingredients used, several studies have shown that this pretreatment can affect the generation of PAHs. While some

of the components in these condiments can hinder PAHs formation, others can accelerate this process (Sampaio et al., 2021). Among marinades, especially the use of vegetable additives rich in antioxidants is related with the reduction/inhibition of PAHs formation (Sampaio et al., 2021; Duedahl-Olesen et al., 2021). On the other hand, grilling chicken pre-treated with oil or alkaline substances significantly raised PAHs levels. Meat that had been marinated for one hour in either pam or sunflower oil or alkaline components such as sodium bicarbonate, showed an increased 16 PAHs and BaP levels of approximately 70% and 80%, respectively. In particular, a significant increase was observed in the concentrations of heavy PAHs, particularly BbF (146.5 μg/kg) and BaP (84.6 μg/kg) (Wongmaneepratip et al., 2017).

Ingredient- combined marinades: Farhadian et al. (2012) studied the effect of seven marinades applied in four times (0,4, 8, 12 h) on PAHs formation (BaP, BbF, Fl) in charcoal-grilled beef. The different marinades analyzed were classified based on their ingredient composition: they were defined as "commercial" a marinade bought in stores, and "basic" a marinade containing the most common ingredients. While the duration of marinating seems not to affect PAHs formation, the below different combination had a reductive effect. Their composition was:

-Basic marinade: sugar, water, onion, garlic, turmeric, lemon grass, salt, coriander, cinnamon.

- Basic oil marinade: basic marinade, cooking oil.

- Commercial marinade: spices, onion, garlic, salt, sugar, water, cooking oil.

- Basic lemon juice marinade: basic marinade, lemon juice.

- Basic oil lemon juice: basic marinade, cooking oil, lemon juice.

- Basic oil tamarind: basic marinade, cooking oil, tamarind.

- Commercial tamarind: commercial marinade, tamarind.

Basic marinade with lemon juice resulted in the most effective one to inhibit (~70% reduction) PAHs formation in beef. Moreover, the antioxidant properties of the sulfhydryl compounds contained in added spices (garlic and onion) may be responsible for the decrease in PAHs levels (Farhadian et al., 2012). By trapping electrophilic molecules, these compounds are known to act as scavengers of free radicals, inhibiting in this way the formation of PAHs. On the other hand, marinating with cooking oil as an ingredient leads to the highest concentration of PAHs formation and should therefore be used

with an acidic ingredient (especially lemon juice) or avoided at all. El-Badry et al. (2010) observed the effect of another marinade (containing tomato juice, garlic paste, onion, sodium chloride, cumin, coriander, and black pepper) on the formation of 16 EPA PAHs in chicken, cooked by different methods: indirect-flame butane gas, pan-fried, microwave oven, and direct-flame butane gas, not including any charcoal grilled or barbecued products, finding a high reduction of PAHs formation (84 to 92%). The authors suggested that this result may be due to the synergistic action of the antioxidant components in the marinating mixture.

Tea marinades has also been investigated, being tea one of the most consumed beverages in the world. Tea can be divided into six groups according to their way of processing and characteristic aroma, color and taste: green (GT), yellow (YT), black (BT), oolong (OT), white (WT) and dark (DT). Tea is rich in catechins (polyphenols), especially epigallocatechin gallate (RGCG). The different teas go under different fermentation, in which partial catechins are oxidized or aggregated to macromolecules such as the flavins (TF) and thearubigins (TBs): GT and WT are not fermented; YT, OT and DT are semi-fermented; BT is fully fermented (Wang et al., 2018). Wang et al. (2018) tested the effect of marinating chicken wings with these 6 types of tea on PAH8 formation. Total and individual phenolic content and phenolic profile, as well as the antioxidant activity, were assessed. The strongest scavenging activity was observed in GT, followed by WT, YT, OT, BT, DT and BT, before and after marinating. Both total phenolic content and radical scavenging activity diminished before marinating, most likely due to interactions between tea infusions and chicken wings (before processing). Radicals and phenolic compounds may have played a role in the PAH8 inhibition in tea-marinated charcoal-grilled chicken wings. The tea-layer may have intercepted the reaction between the heat source and the chicken wings. Additionally, the polyphenolic tea compounds primarily epigallocatechin gallate (EGCG), must have quenched or scavenged the free radicals produced during the grilling. Moreover, these antioxidant compounds must have protected the oxidative sites or species found on chicken wings, avoiding Maillard reaction. Additionally, Wang et al. (2019b), found that marinades containing 8 phenolic compounds from green tea decreased the PAH8 levels in charcoal-grilled chicken wings. Different marinades produced different PAHs profiles. Control samples showed PAHs concentration twice as high as that in the marinated samples with quinic acid, the substance within the 8 compounds that had the largest inhibitory effect on PAHs synthesis. Also, Park et al. (2017), studied the effect of tea marinades, observing that yerba mate tea marinade showed higher radical-scavenging activity and prevented benzo[a]pyrene formation more than the green tea

marinade. Contrarily to Farhadian et al. (2012) the highest BaP reduction was observed after 8h from marinating.

Beers have an antioxidant capacity, able to scavenge free radicals and destroy fatty acid hydroperoxides and have been demonstrated to inhibit PAHs formation in meat (Viegas et al., 2014). There are many different types of beer, therefore the impact they have on PAHs decrease/depletion will not be the same. Viegas et al. (2014) tested the effect of different beer marinades (Pilsner beer (PB), nonalcoholic Pilsner beer (NPB), and Black beer (BB)) on PAH8 formation in charcoal-grilled pork meat, finding that BB presented the highest percentage of inhibition (~53%), followed by NPB and PB with similar inhibitions (25 % and 13% respectively). They also found that the inhibitory effects of beer marinades grew as their radical-scavenging activities increased. The different effects obtained by different types of beer on the decrease of PAHs are ascribed to the various factors such as: fermentation process, the inclusion of sweeteners like glucose-fructose syrup, and the use of food coloring and other additives, which increased the antioxidant activity of beer. Additionally, the action of beer on the oxidative species of meat surface encouraged meat's resistance to the formation and accumulation of PAHs. Wang et al. (2019c) exploited the phenolic compounds in beers that can effectively inhibit the formation of PAH8 in charcoal-grilled chicken wings. Authors observed that phenolic acid presented a greater inhibitory effect than other compounds. From the six beers under study, Heineken was the most effective on inhibiting PAH8 formation (67%) and exhibited also the highest phenol content and excellent performance in total phenolic capacity and ability to scavenge free radicals. Additionally, Wang et al. (2019a) investigated the inhibitory effects of three phenolic acid (commonly found in commercial beers) marinade on PAHs formation in charcoal-grilled chicken wings. Protocatechuic acid marinade showed the highest inhibitory effect, followed by gallic acid and ferulic acid. Regarding antiradical activity, gallic acid showed the strongest one followed by protocatechuic acid and ferulic acid. However further investigations are needed to better understand the mechanism underlying antioxidant activity of phenolic compounds taking into consideration that themselves undergo processing becoming phenoxyl radicals and may act as donors for PAHs formation (Singh et al., 2022).

Spice application: Garlic and onion are two of the most used ingredients in meat marinades. They are both from the Allium family and contain many antioxidants, especially organosulfides (such as diallyl sulfide and diallyl disulfide) (Lu et al., 2018). Onion also contains quercetin, a potent

antioxidant and antibacterial agent. The organic sulphur compounds are found to be associated with PAHs reduction. As reported by El-Badry et al. (2010), garlic in combination with other spices had a great impact in PAHs reduction in grilled meat. The substitute–garlic essential oil (GEO) is often chosen to add garlic more conveniently in meat. GEO consists in a liquid mixture containing volatile compounds, obtained by supercritical carbon dioxide fluid extraction (SFE) or steam distillation from garlic. Hu et al. (2021) studied the effect of garlic and the substitute–garlic essential oil (GEO) in charcoal-grilled pork sausages, observing that the application of garlic and GEO significantly reduced the BaP content. Free radical scavenging is a factor in how sulfides influence the formation of BaP during the grilling of pork sausage. According to this study, marinating meat in GEO is an alternative approach that barbecue enthusiasts can use to reduce their exposure to BaP. However further investigation is warranted into the biochemical mechanism of GEO.

Also, sage and thyme, both used to marinate meat products providing characteristic flavor, have been studied about their effect on PAHs formation. According to Büyükkurt et al. (2017) they both significantly decreased the levels of PAHs in barbecued beef, lowering PAH4 the most. Therefore, the amount of PAH compounds generated in meat during cooking could be decreased by using the extracts of sage and thyme in commercial marinating preparations.

1.4.10.2.2 Seasoning

Seasoning derived from Red Wine Pomace (RWPS) are rich in phenolic compounds, especially flavonoids. Garca-Lomillo et al. (2017) examined the impact of RWPS and storage in high-oxygen environments on the development of PAHs in grilled beef patties. Despite being at low levels, PAHs were shown to rise in control patties over the course of 9 days of storage. This increase was discovered to be connected to a drop in antioxidant content. In fact, control patties showed a higher radical scavenger activity than the treated group. This reduction in the antioxidant activity was suggested as consequence of the interaction between the seasoning antioxidants and proteins of meat. However further studies are needed and result necessary to consider the antioxidant activity of the whole food rather than of the single ingredient added. Cordeiro et al. (2020) spraying on meat different vinegars (white wine vinegar, red wine vinegar, apple cider vinegar, elderberry vinegar and apple cider vinegar with raspberry juice) observed a reduction in PAH4 levels in the range from 55 % (apple cider vinegar) to 82% (elderberry vinegar). Nevertheless, the mechanism through which vinegars affect PAHs

formation probably relies on factors such as on the phenolic profiles, pH and the interactions that can occur between meat and vinegar components.

1.4.10.2.3 Treatment-based strategy

Treatment-based strategy: The effect of carbohydrates in PAHs formation has not been extensively studied. Although**,** Nie et al. (2018), observed that in a complex meat system, the aldehyde groups in reducing sugars such as d-glucose could contribute to the PAHs formation through complex reactions not yet fully studied. With the addition of 0.5 g/100 g d-glucose, the concentration of PAHs in grilled pork sausages significantly increased. In addition, the aldehyde and keto group seem to be more influencing parameters than molecular weight of carbohydrates. Therefore, concerning the use of carbohydrates, the production of PAHs during food processing would be more affected by the addition of simple sugars than by complex carbohydrates like starch. Lately, it is gaining interest among researchers and meat processors the replacement of saturated fats with unsaturated fatty acids. In fact, likely adding olive oil increases the content of monounsaturated fatty acids while sunflower and grape seed oils increases the percentage of polyunsaturated fatty acids in finished products. In this regard, Lu et al. (2017b) examined the impact of replacing pig back fat in pork patties with olive, sunflower and grape seed oil, on PAH content. Cooking temperature had no effect on BaP generation, while fat type had a great impact. While there was no effect when the patties were cooked at 180°C, olive oil and grapeseed oil prevented the development of BaP in the patties cooked at 220°C. Contrarily, sunflower oil prevented the synthesis of BaP at 180°C but promoted it at 220°C. Authors suggested that the production of PAHs was accelerated by the free radicals produced during the breakdown of oil. In addition, they conclude that high temperatures encouraged the pyrolysis of fat and that the variation in smoking points across the studied oils explained the variation in PAHs concentration. In fact, sunflower oil and grape seed oil contain PUFAs, which render them more susceptible to oxidation at a lower temperature (227 °C and 216° C, respectively) compared to olive oil (242°C) (Lu et al., 2017b).

Some of the aforementioned ingredient-based strategies to reduce PAHs concentration in meat are represented in the table below (Table 10).

Table 10. Ingredient-based PAHS reduction strategies. (*Adapted from Wang et al., 2022; Singh et al., 2022)*

Strategy	Meat type/cooking method	Parameters	PAHs	PAHs reduction (%)	References
Marination	Grilled beef meat	Seven marinade 1) basic marinade, which include sugar, water, onion, turmenic, lemon grass, salt, garlic, coriander and cinnamon, 2) basic-oil, 3) Commercial marinade, 4) basic-oillemonjuice, 5) basic-lemon juise, 6) basicoil-tamarind and 7) commercial-tamarind at four time intervals (0,4,8 and 12)	3PAHs	Temperature of grilling 150^0 C. 47%, 48%, 52%, 57% and 70% reduction in BaP concentrations for basic-oil-tamarind, commercial-tamarind, basic, basic-oil-lemon juice and basic-lemon juice marinades respectively.	Farhadian et al., 2012
	Charcoal grilled chicken wings	Tea marination (green tea (GT), white tea (WT), yellow tea (YT), oolong tea (OT), dark tea (DT), and black tea (BT)	8PAHs	38.2%, 40.6%, 45.1%, 45.5%, 45.7% and 50.7% reduction for BT, DT, OT, YT, WT and GT respectively.	Wang et al., 2018
	Charcoal grilled porck	Pilsner beer, non-alcoholic Pilsner beer, and Black beer	16PAHs	13%, 25% and 53% reduction in 8PAHs for Pilsner beer, non-alcoholic Pilsner beer and Black beer respectively.	Viegas et al., 2014

	Charcoal grilled chicken wings	Heineken, Tsing tao, Budweiser, Corona, Snow an Harbin extract	8PAHs	67% reduction with Heineken exhibiting the major effect	Wang et al., 2019c
	Charcoal grilled chicken wings	Marinades with 8 phenolic compounds existing in green tea: (epigallocatechin gallate (EGCG), gallocatechin (GC), catechin (C) epicatechin gallate (ECG), catechin gallate (CG), naringenin, and quinic acid (QA)	PAH8 BaP	Reduction of BaP in the range from 20.5% (GC) to 71% (QA); Reduction of PAH8 in the range from 15% (GC) to 54.5% (QA)	Wang et al., 2019b
	Charcoal-grilled pork belly	Tea marinade with green tea (GT) and yerba made (YM) (1%)	BaP	Reduction:24.5% (GT) and 31.5% (YM)	Park et al., 2017
Spice application	Charcoal-grilled-pork sausage	Garlic (0.05-0.15%, w/w) Garlic essential oil (GEO) (0.002-006%, w/w)	BaP	Reduction of BaP 37.2-62.3% (garlic) 29.1-57.1% (GEO)	Hu et al., 2021
	Barbecued beef	Sage and thyme extracts prepared at 0.5 to 2.0^0 Brix	7PAHs	41.7% and 46.05% reduction in sage and thyme treated barbecue sample at 2^0 Brix.	Buyukkurt et al., 2017

Seasoning	Barbecued beef patties	Red wine pomace seasoning (addition of 2 % w/w) and high-oxygen atmospheric storage	16PAHs	44.99% reduction in high oxygen atmosphere for 9 day compared to control and single treatments.	Garcia-Lomillo et al., 2017
	Charcoal grilled pork	Meat sprayed with white wine vinegar (WWV), red wine vinegar (RWV), apple cider vinegar (ACV), elderberry vinegar (EV), apple cider vinegar with raspberry juice (ACVR)	16PAHs	higher inhibition (82%) for EV followed by WWV (79%), RVW and ACV (66%) and ACVR (55%).	Cordeiro et al., 2020

1.4.10.3 Vitamin E as a PAHs reduction strategy

It has been discovered that plant oils include at least eight substances with vitamin E action. These substances are α, ß, γ, δ-tocotrienol and a, ß, γ, and ß-tocopherol. Each one differs chemically. The main distinction between tocopherols and trienols is that the carbon atoms at positions 3, 7, and 11 in the chain are unsaturated in trienols, whereas they are saturated in tocopherols. For tocopherols and tocotrienols, the nomenclature a, ß, γ and δ denotes the quantity and location of the methyl groups (Berges, 1999). Both molecules' hydrophilic and hydrophobic components can be observed in the figure below (Figure 12).

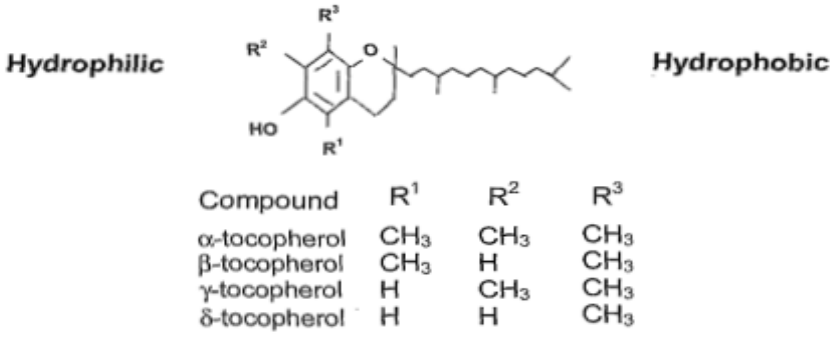


Figure 12. Tocopherols chemical structures. *(Source: Berges, 1999)*

As they all have the same biological effect, tocopherols and tocotrienols are collectively referred to as vitamin E. Tocopherols, particularly α-tocopherol, are much more active and potent and are responsible for practically all of the vitamin E action in live tissues. Only photosynthetic organisms, including higher plants, can produce tocopherols. All green tissues contain significant amounts, but seeds contain the most of them (Maiorano and Tavaniello, 2016). Animals must consume plants or animals that have consumed plants in order to obtain vitamin E.

Sources of Vitamin E can be classified in:

- Natural vitamin E, an alcohol that naturally occurs as a single stereoisomer and is known as d- α -tocopherol (RRR alpha tocopherol). This form derives from vegetable and vegetable oils (Machlin, 1991).
- Semi-synthetic: manufacturers frequently convert the vitamin's phenol form, which has a free hydroxyl group, to esters by using acetic or succinic acid to create d- α tocopheryl acetate or d- α tocopheryl succinate, respectively. In their esterified state, tocopheryl esters are more stable (less prone to oxidation) during storage.
- Synthetic: trimethyl hydroquinone, a reduced benzoquinone, and isophytol (acyclic terpenoid) are combined to create dl- α -tocopherol, a synthetic version of vitamin E. Since synthetic vitamin E is a racemic mixture that contains roughly equal levels of all eight racemic isomers of α -tocopherol, it has about half the biological activity of natural vitamin E.
- Fractionated forms. The two most popular are rich d-gamma-tocopherol and natural mixed tocopherols.

To effectively protect living systems from free radicals and the cellular damage they cause, vitamin E works in conjunction with other lipid- and water-soluble antioxidants. Chemical entities with one or more unpaired electrons that are capable of independent existence are known as free radicals. Free radicals are typically produced in living tissues in some of the following ways, according to Bramley et al. (2000): 1) in the mitochondria, where normal respiration produces superoxide and hydrogen peroxide (H_2O_2); 2) in the phagocytes, where the respiratory burst results in the production of hydrogen peroxide (H2O2), nitric oxide (NO), and hypochlorite (ClO^-); 3) in the peroxisomes, where a variety of substances, including fatty acids, are broken down to produce hydrogen peroxide (H_2O_2); 4) the catalysis of different oxidation processes by the cytochrome P-450 enzyme;5) low-wavelength radiation that produces hydroxy radicals (OH) from water; 6) ultraviolet radiation that causes the

breaking of the O-O covalent link in H_2O_2 to form two OH radicals. The main prooxidants that cause oxidative stress in living systems or start autoxidation reactions in raw and processed foods are the reactive oxygen species (ROS) and reactive nitrogen species (RNS). Since free radical formation can take place in both hydrophilic and hydrophobic environments, water- and lipid-soluble antioxidants are needed to reduce the risk of free radical damage. As the main lipid-soluble antioxidant in human and plant cells, α-tocopherol is present in cell membranes available to protect lipoproteins (Traber and Atkinson, 2007). Scavenging peroxy free radicals, it serves as a main, chain-breaking antioxidant, protecting cell membranes from oxidative damage (Maiorano and Tavaniello, 2016). Being a natural antioxidant, Vitamin E is known as well as for enhancing the nutritional content, color, flavor, texture, and shelf-life of meat (Kasapidou et al., 2012). It is generally recognized that oxidation is one of the main reasons for the decline of meat products' quality, leading to the development of off flavors, discoloration, the generation of harmful chemicals, a short shelf life, nutritional and drip losses, and other issues. Since animals are unable to produce vitamin E, the manner and form in which it is administered have a significant impact on the levels of serum, plasma, and muscle vitamin E concentrations, and consequently in meat products. Vitamin E can be provided to animals as part of a concentrate or liquid supplement or by injection (Maiorano and Tavaniello, 2016). In fact, several studies have studied the effect of Vitamin E supplementation in meat quality. According to Liu et al. (1995), by successfully preventing lipid oxidation and met-myoglobin accumulation in meat, vitamin E intake prevents meat from becoming discolored. This demonstrates the value of vitamin E in maintaining meat redness, which is one of the most significant meat-related commercial qualities as consumers tend to connect it with meat freshness (Carpenter et al., 2001). Maiorano et al. (2015) found that lambs that received injection of vitamin E delivered a meat with a more intense color and were more protected from lipid oxidation. In addition, the composition of fatty acids in muscle was found to be significantly affected by vitamin E with increasing of total PUFA and the PUFA to saturated fatty acids (SFA) ratio, suggesting a protective effect of vitamin E against PUFA peroxidation. Regarding the poultry sector, it has been shown that vitamin E supplementation increases the amount of the vitamin in muscles, improving the oxidative stability of poultry meat during storage. It lowers membrane-associated polyunsaturated fatty acid lipid peroxidation and improves color stability and quality of poultry meat (Pecjak et al., 2022). The intramuscular injection of vitamin E (dl-tocopheryl acetate) has been suggested in a number of studies as a means of reducing vitamin E activity losses in feedstuffs and ensuring a more uniform administration in animals (Maiorano and Tavaniello, 2016).

Muscle α -tocopherol contents are markedly increased when vitamin E is administered, either through diet or injection (Salvatori et al., 2004; Kasapidou et al., 2012).

Concerning diet and impact on endogenous tissue levels of vitamin E, in particular pasture feeding results in increased levels of α-tocopherol in meat muscles as green plant materials and seeds are the primary source of natural tocopherols. The greater amounts of natural antioxidants such as Vitamin E, confer on meat a superior resistance to oxidative deterioration (Maiorano and Tavaniello, 2016). This allow to extend the aging of meat to increase the tenderness of meat from pasture-fed animals which is characterized by lower tenderness compared to meat of grain-fed animals. In addition, Vitamin E acts post-mortem to delay color deterioration of the meat due to the conversion of oxymyoglobin into brown metmyoglobin.

Despite the impact on meat quality, has gained great interest in the study of the effect of vitamin E as an antioxidant in the generation of toxic compounds during meat processing. Some studies have reported inhibitory effects of vitamin E added during preparation or supplemented to animals on formation of carcinogenic compounds such as heterocyclic aromatic amines (HAAs), subgroup like PAHs, of the chemical group of polycyclic aromatic compounds (Sugimura et al., 2004; Ruan et al., 2014).

Concerning PAHs there is a lack of studies regarding the effect of vitamin E, either added prior cooking or its endogenous tissue level in animals on PAHs generation. In a meat model heated at 200°C for 30 min in dry conditions, Min et al. (2018) observed the effect of α-tocopherol and other antioxidants as epigallocatechin gallate (EGCG), (butylated hydroxyanisole (BHA), 3,5-di-tert-4-butylhydroxytoluene (BHT) and sesamol on PAH8 formation. PAH8 significantly decreased in all treatments compared to the control and antioxidants differed slightly from one another. The decrease range was from 11.62 ng/g to 14.37 ng/ compared to the 22.98 ng/g of control samples, with values for α-tocopherol dropped to 14.09 ng/g. As far as we know, it remains unstudied the effect of vitamin E administered to animals by diet or injections on PAHs formation, pointing out the need to better understand the relationship between feeding of animals, the endogenous composition in vitamin E of meat muscles and relative safety of meat product.

PART 2

RESEARCH WORK

2.1 Introduction

It is well known the effort of the worldwide scientific community to study the relationship of diet and nutrition to cancer. It is generally accepted that diet plays an important role in cancer development. In the last decades, the presence of mutagenic/carcinogenic substances in foods has become a major concern for consumers. In particular, has gain great interest the study of polycyclic aromatic hydrocarbons (PAHs), well-known carcinogens, presenting different pathways of contamination among different foods (Purcaro et al., 2013, Bansal and Kim., 2015).

In fact, food constitutes the major route of human exposure, particularly among non-smoking people. These toxic substances can be naturally found in foodstuff or generated during their processing process. Man-made activities contributing to the high occurrence of PAHs in the environment has become a matter of high concern among scientists and consumers. Actually, the environmental route, pertaining to PAHs present in soil, air and water, constitute the commonest source pathways, especially to raw food. On the other hand, the synthetic route, due to processing (industrial level) or to cooking process undergoing high-temperature (grilling, barbecuing, roasting, frying etc), presents an efficient pathway of PAHs intake. Among foods, those of animal origin such as meat and fish due to the presence of PAHs precursors and being major food consumed over the world, represent the most common route of human PAH intake (Ferguson, 2010; Alomirah et al., 2011, Oostindjer et al., 2014).

In the light of this, the study of PAHs has gain particular importance given the controversy around meat consumption and its effects on human health that tends to sway the public perceptions of risk more towards the meat itself than on the way it is processed or cooked. In this regard, the emerging issue of health risk from exposure to PAHs has led to various studies to better understand how the cooking methods influence their formation and how mitigation strategies consisting on the use of antioxidants could reduce their generation on cooked meat.

Among antioxidant, vitamin E has been pointed out as a principal natural antioxidant, able to protect from oxidative damage lipid molecules and cell membranes (Castellini et al., 1998). Vitamin E's chain breaking antioxidant ability is known to halt the self-sustaining formation of lipid peroxides that

causes food deterioration (Benzie, 1996). It is also acknowledged as a necessary nutrient for the wellbeing and growth of all animal species. These functions of vitamin E are brought about through its participation in immunoreactivity, nutritional myopathy, and prostaglandin production (Adebiyi et al., 2011).

Concerning the formation of carcinogenic compounds during meat processing, studies have reported an inhibitory effects of vitamin E (Liao et al., 2009; Sugimura et al., 2004). Wong et al. (2012) observed an inhibitory effect of Vitamin E in cooked beef added with vitamin E during cooking. However, no studies have yet looked into how endogenous tissues levels of vitamin E affect the production of PAHs in meat. In addition, the organism's level of vitamin E needs to be higher than usual for the excess to be deposited in the cellular membranes in order for it to be as effective as possible in avoiding lipid oxidation, promoting health, growth, and limiting the formation of PAHs. This can be accomplished either by injecting intramuscularly or by supplementing the diet.

This study aimed to assess the effect of vitamin E on PAHs formation through the development of two trials:

3. Beef and chicken meat model systems: vitamin E was added to the ground meat prior cooking. The aim of these two trials was to investigate how PAHs formation respond to different concentrations of vitamin E added before meat grilling;
4. Animal model: vitamin E was intramuscularly injected to broiler chickens in order to study how endogenous tissues levels of vitamin E could affect PAHs formation.

2.2 Material and methods

2.2.1 Experimental design

Assays on beef and chicken meat model systems were carried out from October 2021 to October 2022 within the collaboration between the research group of the Department of Agricultural, Environmental and Food Sciences of the University of Molise and the Laboratory of Bromatology and Hydrology of the Faculty of Pharmacy of University of Porto. Within this trial, two experiments were performed to assess the effect of the addition of different concentration of vitamin E in hamburgers of beef and chicken meat prior grilling. Vitamin E was added as *dl*–α–tocopheryl acetate (50 mg/ mL) (Vitalene® E, Fatro, Bologna).

Trial 1: beef meat model system

Beef meat samples used in this study were obtained from the *Longissimus dorsi* muscle from Marchigiana young bulls intensively reared and slaughtered at 12 months (live weight: approximately 620 ± 15 Kg; carcass weight: 402 ± 9 kg. Meat was grounded in a food mixer obtaining a homogenous representative mix meat sample. Approximately 50 g of this mix was used to carried out the analyses for the determination of the total lipid content, fatty acid profile and Vitamin E (α-tocopherol) content.

The homogenous meat sample was divided into five batches corresponding to the five experimental groups: control group (C); group added with 2 μg/g of *dl*–α–tocopheryl acetate (C1); group added with 3 μg/g of *dl*–α–tocopheryl acetate (C2); group added with 4,5 μg/g of *dl*–α–tocopheryl acetate (C3) and group added with 6 μg/g of *dl*–α–tocopheryl acetate (C4). Using a hamburger press kitchen food mold (diameter 8,6 cm and thickness 1 cm) for each group were shaped two hamburgers of 80 g each, as showed in figure 13.

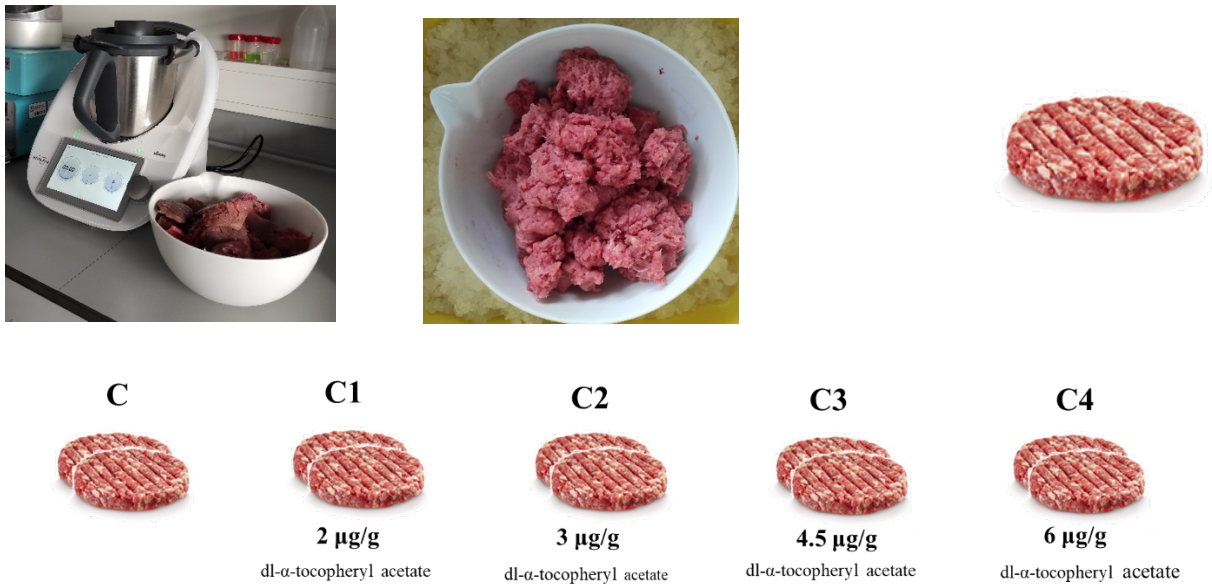


Figure 13. Beef meat sample preparation

Cooking conditions

The ten hamburgers prepared were cooked directly in disposable barbecues Algon (Soler Hispania S.L, contents: 70% nuts /coconut shell, 30 % bamboo charchoal) where fire was ingnited according to the manufactures instructions. The grilling temperatures was around 200 °C, with internal temperature of minimum 75 °C measured by a digital thermocouple (0560 9260, Testo 926, Lenzkirch Germany) with a surface probe (0603 1992, Testo 926, Lenzkirch, Germany). Samples were turned once during grilling (11 minutes each side) reaching well-done level of donenness (Figure 14).

Each cooked hamburger after cooling was mixed with a knife mill (Grindomix GM 200, Retsh, Hann, Germany) and frozen until PAHs analysis.



Figure 14. Grilling process of beef meat hamburgers.

Trial 2: chicken meat model system

Chicken meat samples were obtained from the breast muscles of chicken carcasses, purchased from a butchery in Porto, Portugal. Meat was grounded in a food mixer obtaining a homogenous representative mix meat sample. Approximately 50 g of this mix was used to carried out the analyses for the determination of the total lipid content, fatty acid profile and vitamin E (α-tocopherol) content.

The homogenous meat sample was divided into five batches corresponding to the five experimental groups: control group (C); group added with 2 µg/g of *dl*–α–tocopheryl acetate (C1); group added with 3 µg/g of *dl*–α–tocopheryl acetate (C2); group added with 4.5 µg/g of *dl*–α–tocopheryl acetate (C3) and group added with 6 µg/g of *dl*–α–tocopheryl acetate (C4). Using a hamburger press kitchen food mold (diameter 8,6 cm and thickness 1 cm) for each group were shaped two hamburgers of 80 g each. The sample preparation scheme was the same as for beef meat. Likewise, cooking conditions (Figure 15) were similar to those used for beef meat samples.

Each cooked hamburger after cooling was mixed with a knife mill (Grindomix GM 200, Retsh, Hann, Germany) and frozen until PAHs analysis.



Figure 15. Grilling process of chicken meat hamburgers

Trial 3: animal model system

Ten 43-d-old Ross 308 male chickens were divided into two equal weight groups. Each week, from the beginning of the study, 5 birds received intramuscular injections, in the left Pectoral muscle, of *dl*–α–tocopheryl acetate (50 mg/ mL) (Vitalene® E, Fatro, Bologna). The total doses for birds were as follows: 0 IU (Control group) and 100 IU (Group Vit E). Control group birds were given injections

of physiological saline. Birds were reared under semi-intensive conditions in a private poultry farm located in Matrice municipality (41°62′80.4″ N 14°72'81.2″ E, Italy), in a hilly area at an altitude of about 660 m. Animals were fed ad libitum a commercial diet according to their age. The basal diet was formulated to meet the dietary requirements of the selected broiler genotype according to the nutritional recommendations provided by the breeding company (Aviagen Group, Huntsville, AL). The animals were conventionally slaughtered at 66 days of age, after two days from the last injection. Breast muscles (including pectoralis major and pectoralis minor muscles) were collected from all carcasses for chemical analysis.

Preparation of samples and cooking methods followed the same procedure as for the first trial. Breast meat samples from animal control and animal supplemented with vitamin E were grounded in a food mixer obtaining a homogenous representative mix meat sample. A small part of this mix was used to determine total lipid content, fatty acid profile and Vitamin E (α-tocopherol) content. Using a hamburger press kitchen food mold (diameter 8,6 cm and thickness 1 cm) for each group were shaped two hamburgers of 80 g each.

Cooking process followed the conditions of the first trial.

2.2.2 Meat analyses

Total lipid and fatty acids

Total lipids were determined by the method of Folch et al. (1957) as modified by Bligh and Dyer, (1959). Ten grams of pectoral muscle were weighed out and cut into small pieces. The pieces were placed in a 500 mL Pyrex bottle and mixed with methanol/chloroform mixture (1:1, 200 mL). A 2-minute homogenization process was performed on the mixture using an ultra-turax (Ika® Ultraturax T25). The bottle was closed and kept in an oven at 60ºC for 20 minutes, removed and cooled under water to ambient temperature. Chloroform (100 mL) was added and the mixture was shaken and subsequently filtered through the Buchner funnel with a Whatman No 1 paper. The filtrate was transferred into a 500 mL bottle and mixed with aqueous potassium chloride (1 M, 100 mL). The mixture was shaken for 1 minute and kept overnight in a refrigerator at 4ºC. The day after, the mixture was w transferred into a 250 mL separating funnel arranged under the hood and the lower layer was

collected into a 250 mL bottle. Anhydrous sodium sulphate (2 spatula end fulls) was added and the mixture was shaken for about 3 minutes and kept at 4ºC for 2 hours. Labeled round bottomed flasks were dried in an oven set at 105ºC for 40 minutes, cooled under the dessicator, weighed (*Pi*) and used to collect the filtrate from the bottle. The filtrate was evaporated to dryness under a rotary evaporator set at 40ºC. The flasks were cooled under the dessicator and weighed again (*Pf*). The percentage of the extracted total lipids in the samples were then calculated using the formula:

$$TEL = \frac{P_f - P_i}{P_c} \times 100$$

Where *Pc* = mass of the sample used.

Analyses were carried out in triplicates.

Fatty acids (FA) were quantified as methyl esters (FAME) using a gas chromatograph GC Trace 2000 (ThermoQuest EC Instruments, Milan, Italy) equipped with a flame ionization detector (260 ◦C) and a fused silica capillary column (Zebron ZB-88, Phenomenex, Torrance, CA, USA) 100 m × 0.25 mm × 0.20 µm film thickness. Helium was used as a carrier gas. The column temperature was held at 100 ◦C for 5 min, then raised 4 ◦C/min up to 240 ◦C and maintained for 20 min. The individual FA peaks were identified by comparison of retention times with those of FAME authentic standards run under the same operating conditions. Results were expressed as percentage of the total FA identified. To assess the nutritional implications, the ratio of n-6 to n-3 FA (n-6/n-3) and the ratio of polyunsaturated fatty acids (PUFA) to saturated fatty acids (SFA) (P/S) were calculated. Moreover, to evaluate the risk of atherosclerosis and the potential aggregation of blood platelets, the atherogenic index (AI) and the thrombogenic index (TI) were calculated according to the formulas suggested by Ulbricht and Southgate (1991). Analyses were carried out in triplicates.

Vitamin E

The levels of vitamin E (α-tocopherol) were determined as described by Prates et al. (2006) with some modifications. In a screw teflon lined cap tube were placed 0.75 g of homogenized meat sample to which were added 0.2 g L-ascorbic acid and 5.5 mL saponification solution. Freshly prepared each week, the saponification solution, contained 11% (w/v) potassium hydroxide in a mixture of 55%(v/v)

absolute ethanol and 45% (v/v) distilled water. The sample was then vortexed, degassed with nitrogen gas, and shaken until the L-ascorbic acid was completely dissolved. The saponification process took place for 15 minutes at 80° in an ultrasonic (US) water bath device. Samples were chilled in water for 1 minute after being saponified. 4 mL of n-hexane was added after cooling. In order to hasten phase separation, the materials were vigorously vortexed for 2 min and centrifuged at 1500 g for 5 min. The upper layer containing n-hexane was transferred into vials of 5 mL capacity, evaporated to dryness and subsequently dissolved in 240 mL diethyl ether and 640 mL methanol. The sample then quantified by reverse-phase HPLC (Kontron Instruments, Milan, Italy) model 535 equipped with a C18 reverse-phase column (150 cm × 4.6 mm × 5 μm) (Phenomenex, Torrance, CA). The mobile phase was acetonitrile/methanol (55:45 v/v) at a flow rate of 1.0 mL/min, isocratically. The injection volume for all samples was 20 μL. Identification and quantification of the peaks were done by comparison with vitamin E standards (Perna et al., 2014). Results were expressed as mg/g freshwater for vitamin E. Analyses were carried out in triplicates.

2.2.3 Determination of PAHs

Reagents and Standards

The standard mixture (PAH Calibration Mix) containing 10 μg/mL of naphthalene, acenaphthene (Ac), acenaphthylene, fluorene (F), phenanthrene (Pa), anthracene (A), fluoranthene (Fl), pyrene (P), benzo(a)anthracene (BaA), chrysene (Ch), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), indeno(1,2,3-cd)pyrene (IP), benzo(ghi)perylene (BgP), dibenzo(ah)antracene (DhA) was supplied by Supelco (47940-U, Bellefonte, PA, USA). Working solution (1 μg/mL) was prepared with acetonitrile. Tryphenylene (purity ≥ 98%) from Sigma was used as internal standard (IS), and solution of 10 ng/mL prepared in acetonitrile was used. PAHs standard solutions were stored in amber flasks at − 20 °C. All the solvents used for PAHs analysis were of HPLC grade (Merck, Darmstadt, Germany) and water was purified using a Milli-Q System (Millipore, Bedford, MA, USA). The glassware, mostly amber, was carefully washed and rinsed with HPLC grade acetone and hexane before use. A chromatogram showing the 14 PAHs assayed is shown in Figure 16. Naphtalene and and acenaphtylene were not assayed as they are volatile and not fluorescent compound respectively.

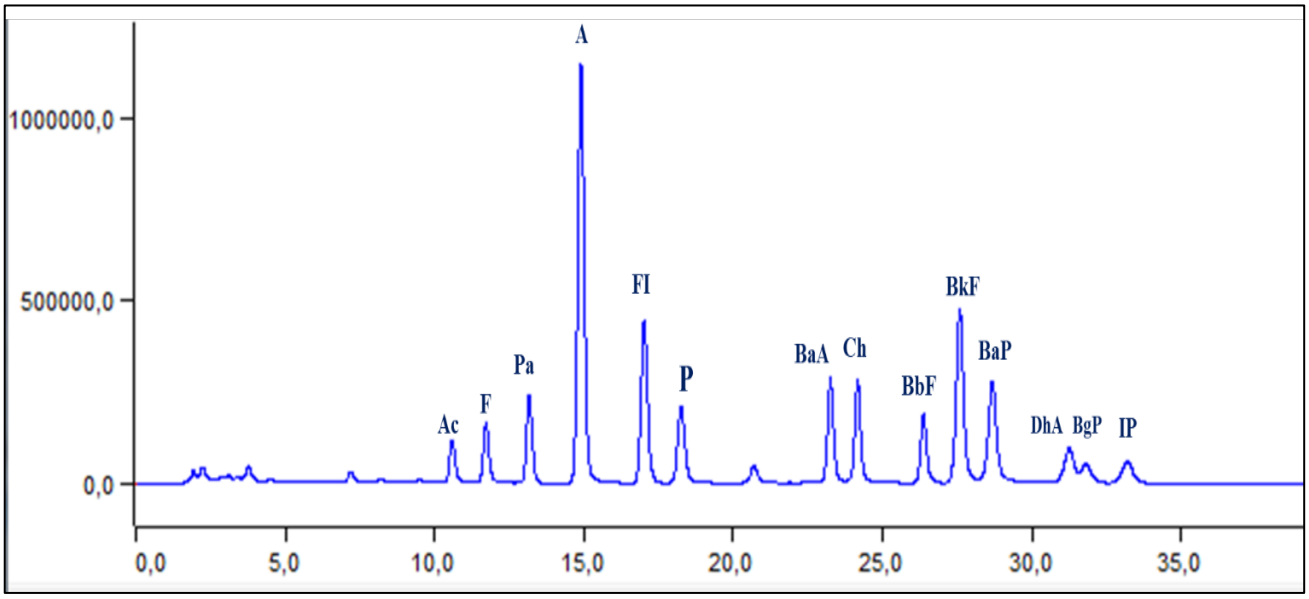


Figure 16. Chromatogram of the corresponding14 PAHs standarts: acenaphthene (Ac), acenaphthylene, fluorine (F), phenanthrene (Pa), anthracene (A), fluoranthene (Fl), pyrene (P), BaA (benz[a]anthracene) Ch (chrysene) , BbF (benzo[b]fluoranthene), BkF (benzo[k]fluoranthene) , BaP (benzo[a]pyrene), DhA (dibenzo[a,h]anthracene), BgP (benzo[g,h,i]perylene) and IP (indeno[1,2,3-cd]pyrene).

Blank matrix

Fresh beef hamburgers obtained from meat purchased in a butchery of Porto were used to prepare a blank matrix. They were immediately oven cooked slowly at a low temperature (120 °C) directly on the oven grid with a tray full of water below in order to collect the drips from meat and covered with aluminum foil to avoid roasting. The meat was not seasoned in any way. Following cooking, the meat was allowed to cool at room temperature before being minced in a knife mill (Grindomix GM 200, Retsh, Hann, Germany), and kept frozen until additional analyses. The blank matrix was used to optimize PAHs extraction, to prepare matrix-standard calibration solutions.

PAHs extraction

The PAHs extraction was performed through an acetonitrile-based extraction of PAH as described by Silva et al., 2018. Briefly, 5 g of sample, with the addition of 20 µL of internal standard (10 µg/mL in

acetonitrile) and 5 mL of purified water was mixed in a 50-mL centrifuge tube. The mixture was vortexed for 1 min and 5 mL of acetonitrile were added. Afterwards, 2.5 g anhydrous magnesium sulphate and 2.5 g sodium chloride were added, followed by shaking and centrifugation at 4000 rpm for 5 min. Subsequently, 2 mL of the upper layer acetonitrile extract were evaporated to dryness under nitrogen stream at room temperature. The residue was dissolved in 100 µL of acetonitrile (Figure 17)

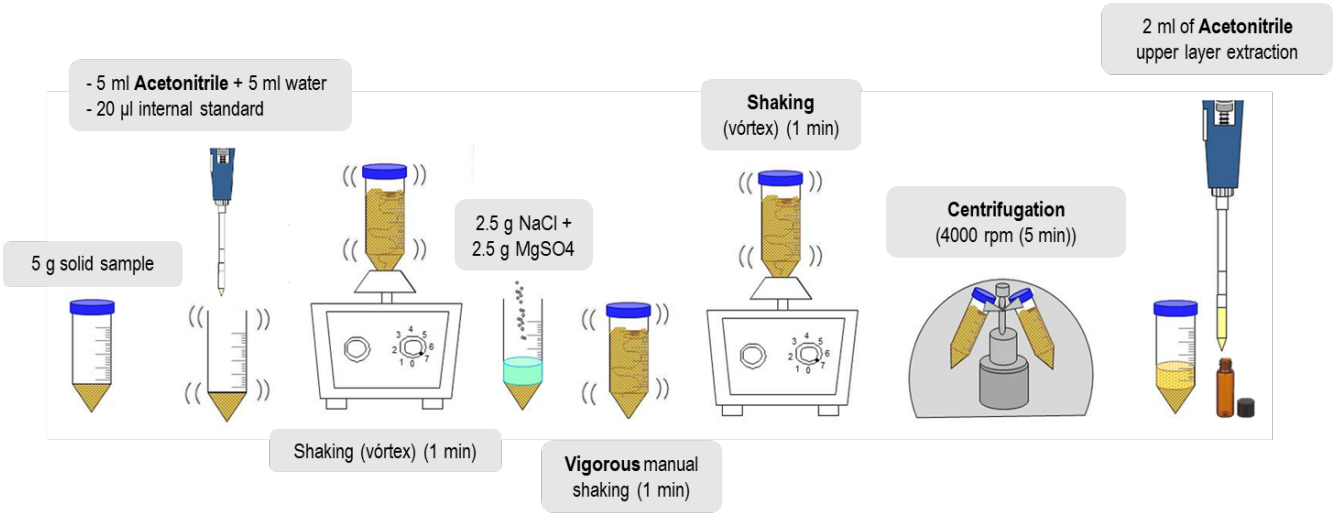


Figure 17. Scheme of PAHs acetonitrile-based extraction.

HPLC-FLD conditions

The HPLC unit (Jasco, Japan) was equipped with one PU-1580 HPLC pump, an AS-2057 auto sampler with a 20-µL loop and a FP-4020 fluorescence detector. The system was controlled by Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France). The column was a C18 Supelcosil LC-PAH (25 cm length; 4.6 mm internal diameter, 5-µm particle size) (Supelco, Bellefonte, PA, USA) thermostated at 32 °C. The gradient elution and fluorescence detection excitation/emission program were set up according to the method described by Viegas et al. (2012a) The mobile phase was as follows: solvent A: 75% methanol (in water); solvent B: 100% methanol, solvent C: 100% ethyl acetate with a flow rate 1 mL min−1. Mobile phase gradient program used to separate PAHs is shown in Table 11. Mobile phases degasification was performed using a magnetic stirrer coupled with a vacuum system, ESP Stirrer from VELP Scientifica (Usmate, Italy).

Table 11. Mobile phase gradient program used to separate PAHs.

Time (min)	%Eluent A	%Eluent B	%Eluent C
0	100	0	0
18	20	80	0
19	0	100	0
20	0	90	10
28.5	0	82	18
37.5	0	80	20
40	0	100	0
45	100	0	0

Excitation/emission wavelengths selected for PAHs are shown in Table 12. The detection for Fl and IP had a gain tenfold higher than for the other PAHs (Silva et al., 2018). The PAHs identification was based on retention times and co-elution with standards.

Table 12. Fluorescence program used to separate PAHs.

PAHs[1]	λ_{ex} (nm)	λ_{em} (nm)
Na, Ac, F	276	330
Pa	250	336
A	250	402
Fl	270	460
P, BaA, Chr	270	390
BbF	260	430
BkF, BaP, DhA, BgP	290	410
IP	290	470

[1]acenaphthene (Ac), acenaphthylene, fluorine (F), phenanthrene (Pa), anthracene (A), fluoranthene (Fl), pyrene (P), BaA (benz[a]anthracene) Ch (chrysene) , BbF (benzo[b]fluoranthene), BkF (benzo[k]fluoranthene) , BaP (benzo[a]pyrene), DhA (dibenzo[a,h]anthracene), BgP (benzo[g,h,i]perylene) and IP (indeno[1,2,3-cd]pyrene).

Quality Control and Criteria

The quality control of the extraction method was performed through the analysis of blank samples and grilled samples with and without spiking. The identification criteria for PAHs were based on retention times and on co-elution with standards. In order to study the linearity and obtain regression equations and correlation coefficients, matrix-match calibration curves were prepared, using 5 g of blank samples spiked with appropriate amounts of PAH calibration mix and internal standard (4 ng/g), extracted using the established conditions. Calibration curves with seven concentration levels were prepared in the interval between 0.05 and 50 g/g. Weighted linear regression model was selected as calibration model, to minimize the errors at the lower end of the calibration line that coexist with acceptable correlation coefficients (rw) and are underestimated in analyzing the dispersion of the regression parameters of least squares linear regression (Mulholland and Hibbert 1997).

Limits of detection (LOD) and limits of quantification (LOQ) were calculated based on the calibration curve parameters. LOD and LOQ are fundamental elements of method validation that define the limitations of an analytical method. In general, LOD is taken as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified under the stated conditions of the test (Alankar and Vipin, 2011). The IUPAC defines limit of detection as the smallest concentration or absolute amount of analyte such that the probability of type 1 and type 2 errors are equal. By convention and from statistics the limit of detection is the concentration corresponding to a signal three times the noise level of the background. LOQ on the other hand is generally taken as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of the test. It is mathematically taken as equal to ten times the standard deviation of the results for a series of replicates used to determine a justifiable LOD. It may also be taken as ten times the signal to noise ratio. Both LOD and LOQ are matrix, method and analyte specific (Harvey, 2000).

Recovery studies were carried out to determine the accuracy of the extraction procedure; for this purpose, different types of grilled and smoked samples were analyzed with and without spike at one level of 4 ng/g depending on PAHs concentration in unspiked samples.

2.2.4 Statistical Analysis

Data regarding the PAHs concentration in beef and chicken samples from the first 2 trials were analyzed by one-way ANOVA using GraphPad Prism 9.5.1. Tukey's test was used for comparing mean values. The data from the animal model trial were analyzed by one-way ANOVA using the SPSS package (SPSS/PC1Statistics 18.0; SPSS Inc., Chicago, IL, USA, 2010).

PART 3

RESULTS AND DISCUSSION

3.1 Trial 1: Beef meat model system

3.1.1 Vitamin E (α-tocopherol), total lipid, fatty acid profile and nutritional indices

Vitamin E (α-tocopherol) content, total lipid content, fatty acid composition and nutritional indices of the homogenous beef sample used to prepare beef burgers are presented in Table 13.

Vitamin E has been recognized as an essential nutrient for the growth and health of all animal species. Vitamin E is an essential nontoxic fat-soluble micronutrient stored in tissues, the highest levels occurring in fatty tissues (Fuchs et al., 2003). It acts directly on reactive oxygen species (ROS), inhibiting lipid peroxidation and oxidative damage (Evans, 2000). Oxidative stress is caused by the imbalance between prooxidants and antioxidants at either cellular or individual level (Voljc et al., 2011). For example, 3.0 to 3.5 μg/g of α-tocopherol are considered the threshold level to extend beef oxidative stability effectively (Arnold et al., 1993; Liu et al., 1995). Ruminants do not store appreciable levels of vitamin E (Pehrson and Hakkarainen, 1986; Luciano et al., 2011). The results of the present study confirmed this previous finding carried out on cattle. In particular, the concentration of α-tocopherol found in Longissimus dorsi muscle samples from cattle reared indoor was low (1.32 μg/g). This value is within the range of values reported by Marino et al. (2014) in different muscles of Podolian young bulls reared indoor. Generally, pasture-based feeding systems produce greater amounts of natural antioxidants, e.g. vitamin E (Wood et al., 2003). High concentrations of vitamin E in muscle have been shown to increase the oxidative stability of beef (Faustman et al., 1989). However, cattle are seldom raised only on pasture, and they often receive grass silages or concentrates when reared in housing. In fact, the mean values of vitamin E in beef reported in the literature vary between fed exclusively grazed pasture or concentrate offered indoors or pasture with supplementation. Generally, in muscle from grazed pasture beef is more than 2-fold greater (3.5-2.59 μg/g tissue) than that measured in muscle from cattle fed concentrate (1.8 - 1.15 μg/g tissue) (Yang et al., 2002; De la Fuente et al., 2009; Luciano et al., 2011; Marino et al., 2014). Fresh and processed meats are highly vulnerable to decay by oxidative degeneration. The principal quality parameter of

meat is its oxidative stability (Estévez et al., 2005). As above mentioned, vitamin E inhibits the oxidative process reducing the quantity of generated oxidative products. In particular, vitamin E is mostly concentrated in the unsaturated phospholipid bilayer of the cell membranes where it inhibits lipid oxidation by functioning as a free-radical scavenger (Azzi and Zinggl, 2005; Birben et al., 2012; Niki. E, 2014) that could deteriorate the quality of meat and meat products including taste, aroma, color, and texture before consumption (Decker et al., 2000; Kennedy et al., 2005; Maiorano et al., 2016).

Nutritional properties of meat and meat products are very important in purchasing behaviour because consumers are even more concerned their healthiness (e.g. fat and fatty acids composition, etc.). In fact, the amount and type of dietary fat in the diet are important factors influencing adipose tissue function and whole-body metabolism, with important health ramifications.

Lipids, essential molecules that function as the major structural components of living cells and play important roles in various cellular functions (Fahy et al., 2011), are among the bioactive components those that have received most attention, in quantitative and qualitative terms, with respect to the development of healthier meat and meat products. The fat content amount observed in the present study (1.36 g/100g) indicate a lean meat. The Food Advisory Committee (1990) reported that meat containing less than 5% total lipid could be regarded as lean. The low-fat content in meat is important from nutritional point of view. Moreover, nutritionists recommend not only limiting fat intake but also consuming large amounts of polyunsaturated fatty acids (PUFA), especially ω3 PUFA rather than ω-6 PUFA (Simopulos, 2002). On the other hands, numerous evidences have shown that ω-3 and ω-6 PUFAs exhibit significant effects in inhibiting various types of cancers (Huerta-Yépez et al., 2016).

It is recognised that over many years of evolution, humans have adapted to consuming large quantities of lean red meat (Mann, 2000) becoming super carnivores. With regard to the physiological effects on humans, it is widely recognized that fat plays important biological functions and a strong interest in human nutrition. However, red meat is often perceived as a fatty source with certain health risks associated with its consumption, and mainly qualitative intake of fat as the total fat content, SFA composition, and cholesterol found in beef and their relationship with diseases (Fernandez-Gines et al., 2005).

Table 13. Nutritional profile of beef meat

Item	
α-tocopherol (µg/g)	1.32 ± 0.02
Total lipids (g/100g)	1.36 ± 0.09
Fatty acids (FA; % on total FA)	
C10:0	0.04 ± 0.02
C12:0	0.06 ± 0.01
C14:0	2.61 ± 0.29
C16:0	28.17 ± 1.16
C16:1n7	2.59 ± 0.09
C17:0	1.14 ± 0.06
C17:1	0.88 ± 0.01
C18:0	20.91 ± 0.54
C18:1 trans9	0.58 ± 0.01
C18:1 cis9	30.95 ± 0.35
C18:2 n-6	7.19 ± 0.09
C18:3 n-3	0.91 ± 0.00
C18:3 n-6	0.04 ± 0.00
C20:1 n-9	0.04 ±0.00
C20:3 n-6	0.51 ± 0.07
C20:4 n-6	2.11 ± 0.23
C20:5 n-3	0.40 ± 0.07
C22:4 n-6	0.14 ± 0.05
C22:5 n-3	0.66 ± 0.21
C22:6 n-3	0.06 ± 0.04
Total fatty acids[1]	
SFA	52.94 ± 1.03
MUFA	35.04 ± 0.27
PUFA	12.02 ± 0.76
n-6 PUFA	9.98 ± 0.44
n-3 PUFA	2.04 ± 0.32
Nutritional indices[2]	
n-6/n-3	4.94 ± 0.56
P/S	0.23 ± 0.02
TI	1.80 ± 0.12
AI	0.82 ± 0.07

[1]SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
[2]P/S, PUFA/SFA ratio; AI, atherogenic index; TI, thrombogenic index.

The fatty acid profile found in the present study showed that the SFA (52.940%) and MUFA (35.04%) were the most abundant fatty acids followed by PUFA (12.02%). Considering the individual fatty acids, the major fatty acids were palmitic (28.17%) and stearic (20.91%) followed by myristic (2,61) and margaric (1.14%) in the SFA category; oleic (30.95%) and palmitoleic (2.59) acids in the MUFA category; and linoleic (7.19), arachidonic (2.11) and linolenic (0.91) acids in the PUFA category. Low values were found for eicosapentaenoic acid (EPA), docosanpetaenoic acid (DPA) and docosahexaenoic acid DHA (Table 13). A similar trend was found in Podolian young bulls reared indoor, while the animals reared at pasture showed a reduction in total SFA and an increase in total MUFA and PUFA (Marino et al., 2014). Similarly, was found in another study conducted with Charolais and Limousin castrated bulls fed differently with grass silage and concentrate supplements containing either barley/soybean meal (Salami et al., 2021). Regarding the human health, current nutritional guidelines recommend to consume no more than 25–35% of total daily calories from fat, with most coming from sources heavily endowed with MUFA and PUFA (US Department of Health, 2005). The beneficial effects of PUFA have been associated to a protection against cardiovascular diseases (Sokoła-Wysoczánska et al 2010), whereas for MUFA to a beneficial effect on insulin sensitivity and cholesterolemia (Nettleton et al., 2017). EPA and DHA are considered to be important in prevention of heart diseases and some cancers and obesity/type-2 diabetes (Simopulos, 2002). DHA is also important during pregnancy for cerebral and retinal tissues development and reduces the incidence of premature birth (Givens et al., 2006). Furthermore, different biological effects on human health are recognised even to n-3 fatty acids (Simopoulos, 2016). The n-3 fatty acids and n-6 fatty acids values found in the present study are similar the values found in previous studies carried out on beef (Rule et al., 2002; Marino et al., 2014)

The nutritional indices of the meat, such as the ratios n-6/n-3, polyunsaturated to saturated ratio (P/S), and TI and AI indices are presented in Table 13. The value of the n-6/n-3 ratio observed in this study (4.94) is similar than those found for Podolian bulls (Marino et al., 2014) and Charolais and Limousin castrated bulls (Salami et al., 2021), and it is close the maximum value of 4 (Wood et al., 2004). Polyunsaturated to saturated ratio (PUFA/SFA) in ruminant meat is unfavourably low because dietary unsaturated fatty acids are hydrogenated by rumen micro-organisms (Choi et al 2000), however in the present work the P/S ratio is close within the recommend range (0.3 - 0.6) being 0.23. The atherogenic and thrombogenic indices quantifies the fatty acids that can promote or prevent atherosclerosis and

coronary thrombosis. The values found in the present work (AI = 0.82; TI = 1.80) can be considered low for AI and slightly high for TI. Similar values for both indices are reported by Salami et al. (2021). It is generally agreed that fat content is an important parameter affecting PAHs (highly lipophilic molecules) formation in charcoal grilled muscle foods (Hamidi et al., 2022). A recent study (Onopiuk et al., 2022) have shown a relationship between the fatty acid profile and the PAH content (especially of the heavy PAHs). In particular, this study aimed to analyze the impact of partial replacement of beef tallow with sunflower, canola, linseed, olive oil and milk fat on fatty acid composition and PAHs formation in beef burgers. Authors found a higher PAH content in samples with canola oil, which had the highest levels of monounsaturated fatty acids (MUFA), and in the control group without fat substitution, which had the highest levels of saturated fatty acids (SFA) and trans conformations.

3.1.2 Determination of PAHs

Quality control of analytical method

A fast and simple method for analysis of 14 PAHs in grilled and smoked muscle foods using an acetonitrile based-extraction was carried out according to Silva et al. (2018). It is important to emphasize that the method used responds to the rigorous performance criteria established by European Commission for foodstuffs analyses (Commission Regulation (EU) No 836/2011), since no reference method is available. As reported by the aforementioned Regulation, for the four polycyclic aromatic hydrocarbons (PAH4: benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene), LODs and LOQs should be lower than 0.30 μg/kg and 0.90 μg/kg, respectively for each of the four substances. Recovery which is another parameter to meet in the Regulation, should be in a range of 50–120% to be acceptable. In the present study, the analytical performance of the extraction method for reliable chromatographic quantification of PAHs in grilled beef and chicken was evaluated. Linearity was observed using matrix-match calibration curves in the range between 0.05 and 50 ng/g for PAHs. The results of LOD, LOQ and linearity are reported in Table 14. Good linear responses were obtained for all compounds with coefficient correlations (rw) above 0.99. The LODs and LOQs for the 14 analyzed PAHs ranged from 0.004 to 0.06 ng/g and from 0.01 to 0.18 ng/g, respectively. The results are much lower than the performance criteria established by the Commission Regulation (EU) No 836/2011. This suggests that the developed method offered high sensitivity for the detection of the PAH compounds in the samples (Silva et al., 2018).

Table 14. Calibration parameters, limit of detection (LOD) and quantification (LOQ) obtained for quantification of PAHs.

PAHs	b_w	a_w	r_w	LOD (ng/g)	LOQ (ng/g)
Ac	0.0726	0.0401	0.9996	0.04	0.13
F	0.0997	0.1799	0.9944	0.03	0.1
Pa	0.1469	0.3367	0.9994	0.02	0.07
A	0.8549	0.2186	0.9993	0.004	0.01
Fl	0.3245	0.3005	0.9995	0.01	0.03
P	0.1679	0.113	0.9996	0.02	0.06
BaA	0.1896	0.0189	0.9987	0.02	0.05
Ch	0.1805	0.0432	0.9986	0.02	0.05
BbF	0.1275	0.0112	0.9996	0.02	0.08
BkF	0.3892	0.0022	0.9995	0.01	0.02
BaP	0.2486	0.0017	0.999	0.01	0.04
DhA	0.1035	-0.0156	0.9995	0.03	0.09
BgP	0.0531	0.0049	0.9994	0.06	0.18
IP	0.0706	-0.0018	0.9994	0.04	0.14

acenaphthene (Ac), fluorene (F), phenanthrene (Pa), anthracene (A), fluoranthene (Fl), pyrene (P), benzo(a)anthracene (BaA), chrysene (Ch), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), indeno(1,2,3-cd)pyrene (IP), benzo(ghi)perylene (BgP), dibenzo(ah)antracene (DhA);
bw weighted slope, aw weighted intercept, rw weighted correlation coefficient;
LOD = limit of detection, LOQ = limit of quantification

As there is a lack of certified reference materials for PAHs in processed muscle foods, in order to demonstrate the reliability of the data provided, the accuracy of the method was verified via spiking experiments in beef and chicken meat. Recovery studies were carried out analysing grilled samples with and without spike at 4 ng/g, depending on PAHs concentration in unspiked samples. The recovery values for beef and chicken meat are reported in figure X with a range between 76% and 126%. Recoveries were within the control limits established by ICH guidelines (reference ICH) between 70 and 120 % and the criteria established by the Commission Regulation (EU) No 836/2011for PAH4 analysis (50-120%).

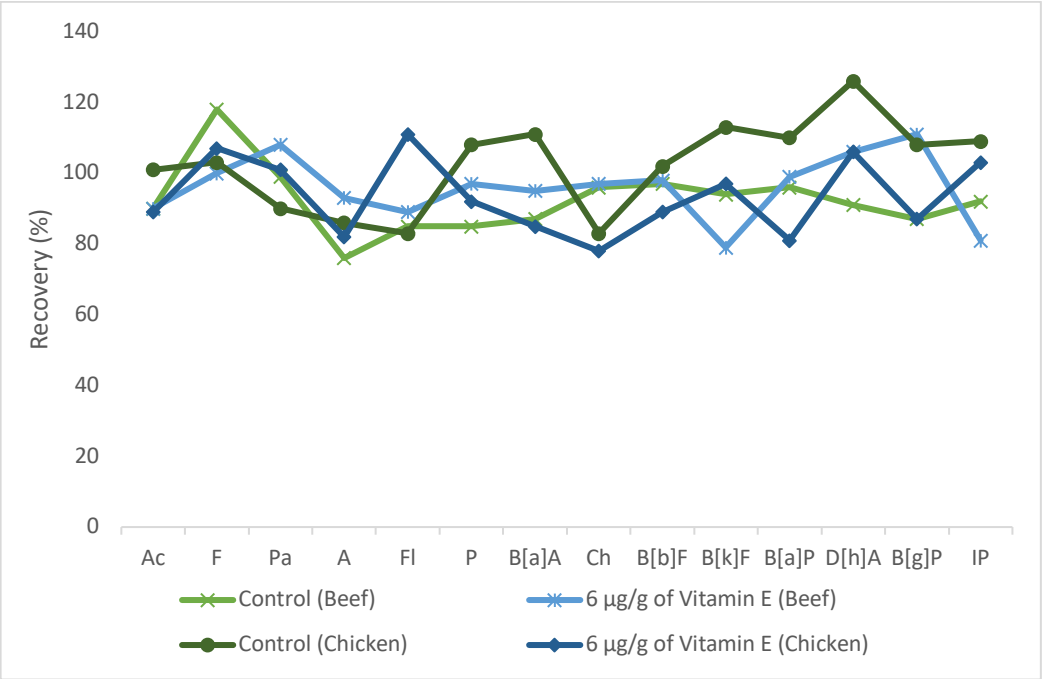


Figure 18. Recovery values (%) of PAHs in grilled beef and chicken burgers spiked at 4 ng/g.

Effect of vitamin E on formation of polycyclic aromatic hydrocarbons in grilled beef burgers

In the last decades, several studies have proven the effect of antioxidant in preventing the formation of heterocyclic aromatic amines (HAs) and PAHs formation. The main mechanism of PAHs formation is the recombination of free radicals produced during the combustion of food molecules under harsh conditions; antioxidants are able to interfere with these radical reactions or trap free radicals (Singh et al., 2023). In particular, different studies have shown that vitamin E added during preparation or cooking has inhibitory effects on the formation of HAAs in the Maillard reaction and reduces the formation of HAAs in cooked beef, pork, chicken and fish (Liao et al., 2009; Sugimura et al., 2004). As regard HAAs formation, Balogh et al. (2000) found that the addition of vitamin E at two concentrations 1% and 10% (based on fat content), added directly to the ground beef patties, reduced the 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) concentrations in the cooked patties by 69% and 72%, respectively. Smaller but more variable reductions were achieved for 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). Comparable inhibition of HAAs formation was achieved by the direct addition of vitamin E (1% based on fat content) to the surface of the patties

before frying, with average reductions ranging from 45% to 75%. In line with the above-mentioned study, the first in vitro trial of this research study was designed to determine the effect of different doses of vitamin E added to beef patties before grilling on the PAHs formation. The tested doses of 2, 3, 4.5 and 6 μg/g of *dl*–α–tocopheryl acetate were chosen considering the vitamin E muscle content of grazing or intensively raised cattle. As reported in a review paper by Daley et al. (2010), the concentration of natural α-tocopherol (vitamin E) found in grain-fed beef ranged between 0.75 to 2.92 μg/g tissue, whereas in pasture-fed beef ranged from 2.1 to 7.73 μg/g tissue depending on the type of forage. Grass finishing increases α-tocopherol levels three-fold over grain-fed beef and places grass-fed beef well within range of the muscle α-tocopherol levels needed to extend the shelf-life of retail beef (3 to 4 μg α-tocopherol/g tissue). The present study represents a starting point for future investigations in order to better understand how the muscular endogenous levels of vitamin E (grain-fed vs. pasture-fed beef) could be a preventive agent for mutagen formation in grilled meat. Considering that there is a relatively small body of literature concerning the effect of vitamin E on PAHs formation it was not easy to compare the data obtained in the present study with other findings. In general, it is difficult to compare PAH results between studies due to the large number of factors affecting the formation of PAHs such as: type of meat, fat content, presence of other ingredients, heat treatment conditions and many others. The concentrations of the PAHs compounds that were present in the samples at or above their limits of quantitation are shown in Table 15. Among the 14 PAHs assayed, only the dibenzo(ah)antracene (DhA) was not detected.

Table 15. Concentrations (ng/g) of polycyclic aromatic hydrocarbons formation in grilled beef patties by the direct addition of different doses of vitamin E (dl-α-tocopheryl acetate)

		Vitamin E				
PAHs[1]	**Control**	**C1 (2 µg/g)**	**C2 (3µg/g)**	**C3 (4.5µg/g)**	**C4 (6 µg/g)**	**P-value**
Ac	16.85 ± 1.21^{B}	22.25±0.57^{A}	14.16±1.59^{BCa}	10.25±1.45^{Cb}	13.77±1.89^{BCb}	0.0001
F	0.49±0.06^{B}	0.77±0.04^{A}	0.29±0.02^{C}	0.13±0.00^{D}	0.14±0.02^{D}	0.0001
Pa	41.70±1.20^{Aa}	41.65±1.98^{Aa}	32.48±0.49^{B}	24.80±0.86^{C}	37.98±1.72^{Ab}	0.0001
A	4.37±0.09^{Aa}	3.93±0.23^{ABb}	3.08±0.06^{C}	2.28±0.06^{D}	3.80±0.15^{B}	0.0001
Fl	5.35±0.33^{A}	5.49±0.20^{A}	4.46±0.21^{B}	3.25±0.42^{Db}	4.25±0.20^{BCa}	0.0001
P	4.79±0.41^{A}	4.16±0.13^{a}	3.59±0.28^{B}	3.35±0.32^{Bb}	4.05±0.11	0.0007
BaA	0.42±0.01^{a}	0.34±0.01^{bc}	0.32± 0.00^{c}	0.42±0.02^{ab}	0.38±0.06	0.0075
Ch	0.30±0.04^{Aa}	0.26±0.04^{A}	0.13±0.01^{B}	0.20±0.03^{ABb}	0.10±0.02^{Ba}	0.0001
BbF	0.20±0.05^{A}	0.10±0.02^{B}	0.08±0.01^{B}	0.07±0.01^{B}	0.11±0.01^{B}	0.0007
BkF	0.05±0.01^{A}	<0.02	<0.02	0.05±0.00^{A}	0.02±0.00^{B}	0.0001
BaP	0.06 ±0.03^{A}	0.05 ±0.01^{A}	<0.04	<0.04	0.04 ±0.02^{B}	0.0001
BgP	0.29±0.02	<0.18	<0.18	0.35±0.08	<0.18	0.0003
DhA	n.d	n.d	n.d	n.d	n.d	-
IP	0.90±0.11^{Aa}	0.81 ±0.09^{Aa}	0.44 ±0.13^{Cb}	0.95 ±0.19^{A}	0.35 ±0.10^{BC}	0.0005

[1] acenaphthene (Ac), fluorene (F), phenanthrene (Pa), anthracene (A), fluoranthene (Fl), pyrene (P), benzo(a)anthracene (BaA), chrysene (Ch), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), indeno(1,2,3-cd)pyrene (IP), benzo(ghi)perylene (BgP), dibenzo(ah)antracene (DhA);

^{A,B,C,D} Means within a row lacking a common superscript differ (P < 0.01).

^{a,b,c}Means within a row lacking a common superscript differ (P < 0.05).

Analysis of individual PAH content showed that light PAH (Pa > Ac > A > F) were the most abundant as compared to heavy ones (Table 15) and except for Pa and A, C and C1 groups showed different values for Ac, and F ($P < 0.01$); in general, lower values ($0.0001 < P < 0.05$) were found for the other vitamin E added groups (C2, C3 and C4). To better understand the differences among treated groups and control one the sum of different categories of PAHs (total, light, heavy, PAH4, PAH8) are reported in graphs.

The total content of the 14 PAHs assayed ranged from 46.14 ng/g to 80.00 ng/g (Figure 19). In particular, both C and C1 groups showed a higher ($P < 0.001$) total PAHs content (75.88 ng/g and 80.00 ng/g, respectively) as compared to the other vitamin E added groups, with the lowest ($P < 0.0001$) value for the C3 group (46.14 ng/g). Intermediate values were found for C2 (59.23 ng/g, $P < 0.0001$) and C4 (65.08 ng/g, $P < 0.001$). It is worth noting that the addition of 2 µg/g of vitamin E had no effect ($P>0.05$) on the reduction of the total PAHs as compared to control group, while the addition of 4.5 µg/g seems to be more effective in the PAHs reduction.

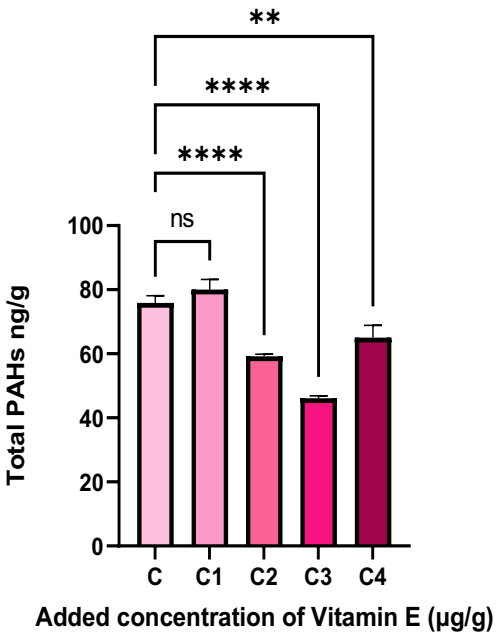


Figure 19. Total PAHs content in beef burgers. C = control; C1 = 2 µg/g; C2 = 3µg/g; C3 = 4.5 µg/g; C4 = 6µg/g. ns-not significant; *P ≤0.05; **P ≤0.01; ***P≤0.001; ****P≤0.0001

PAHs comprise fused aromatic rings, those containing two to four benzene rings are called ‘‘light PAHs’’, and those containing more than four aromatic rings are known as heavy PAHs, which are more stable and more toxic than the light ones. The incomplete combustion of the heat source (charcoal) may generate light PAHs and the melted fat that drips from patties to the heat source generates heavy PAHs which are carried up by the smoke to the meat surface (Viegas et al., 2012b).
In the present study, the mean concentrations (ng/g) of light PAHs (Ac, F, Pa, A) in control and treated samples were in the following range: Ac (10.25 - 22.25 ng/g), F (0.13 – 0.77 ng/g), Pa (24.8 – 41.7 ng/g), A (2.28 – 4.37) ng/g, while those for heavy PAHs (FI, P, BaA, Ch, BbF, BkF, BaP, BgP, IP) ranged between the following values: Fl (3.25 - 5.49 ng/g), P (3.35 - 4.79 ng/g), BaA (0.32 - 0.42 ng/g), Ch (0.10 - 0.30 ng/g), BbF (0.07 - 0.20 ng/g), BkF (<0.02 - 0.05 ng/g) BaP (<0.04 – 0.06 ng/g), BgP (<0.18 - 0.35 ng/g), IP (0.35 - 0.95 ng/g) (Table 15). The total content of light PAHs ranged from 37.46 ng/g to 68.61 ng/g. The addition of vitamin E to ground beef patties provided a significant inhibition of light PAHs formation at the concentration of 4.5 μg/g (37.46 ng/g, $P < 0.0001$) and 3μg/g (50.01 ng/g, $P < 0.001$), as well as with the highest concentration 6μg/g (55.69 ng/g, $P < 0.05$), compared to C group (63.41 ng/g). On the contrary, the lowest concentration (2μg/g) was not effective in the reduction of light PAHs formation (68.61 ng/g, $P > 0.05$) compared to C group. These results clearly indicate that the antioxidant or even the prooxidant effects are dose-dependent. Similarly, Liao et al. (2009) found that the inhibition of HA formation was dependent from the concentration of vitamin E added during processing and cooking of meat. The values for the single light PAHs found in the present study are quite similar to those reported by Silva et al. (2018) in charcoal-grilled beef for Pa (43.54 ng/g) and Ac (26.17 ng/g, comparable to C1 group); while different values were reported for F (8.07 ng/g) and A (< 0.17 ng/g). Also, the values found by Viegas et al. (2012b) in grilled beef samples using two different types of charcoal (wood and coconut) were in different ranges from our results. These differences are related to different variables such as the heat source, barbecue geometry, time-temperature combination, meat type in particular related to fat content (Duedhal-Olesen and Ionas, 2022), which makes difficult the comparison among different studies.

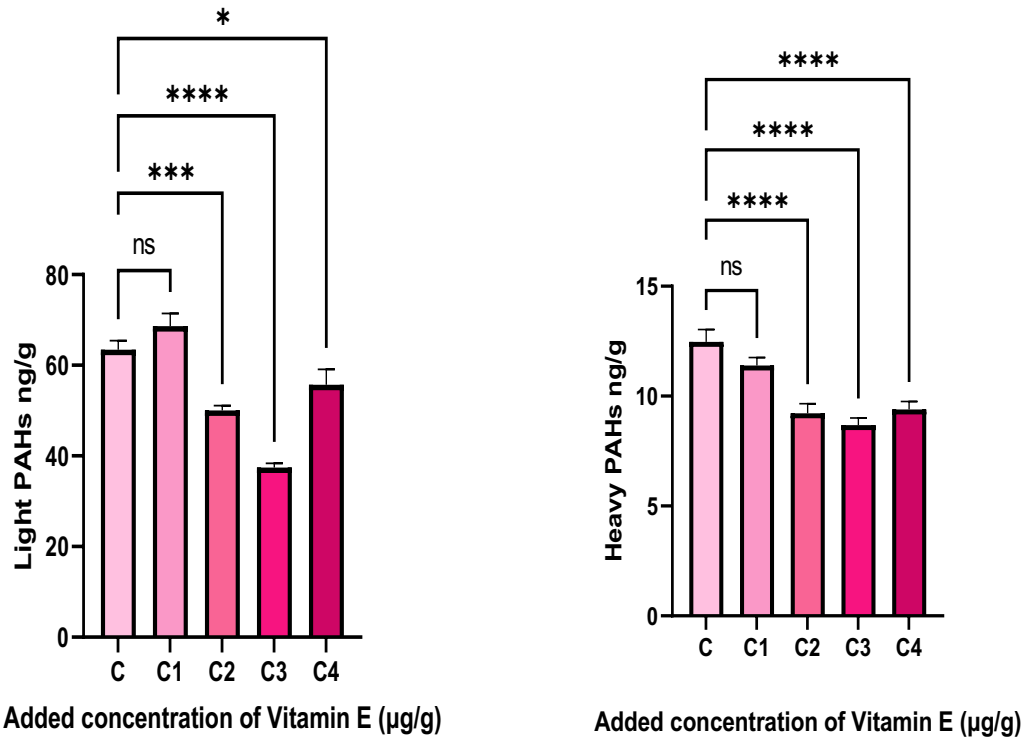


Figure 20. Light and Heavy PAHs content in beef burgers. Low molecular weight (light) PAHs includes the sum of Ac, F, Pa, A. High molecular weight (heavy) PAHs includes the sum of Fl, P and genotoxic PAHs (PAH8). C = control; C1 = 2 µg/g; C2 = 3µg/g; C3 = 4.5 µg/g; C4 = 6µg/g. ns-not significant; *P ≤0.05; **P ≤0.01; ***P≤0.001; ****P≤0.0001

The total content of heavy PAHs ranged between 8.68 to 12.47 ng/g. Concerning the effect of vitamin E, it was observed a similar trend to light PAHs (Figure 20). Not significant reduction (11.40 ng/g, P > 0.05) was observed at the lowest concentration of vitamin E added (2 µg/g), meanwhile their content decreased significantly (P < 0.0001) in the other ones (C2: 9.22 ng/g; C3: 8.68 ng/g; C4: 9.39 ng/g) as compared to C group (12.47 ng/g). The obtained values are similar to those reported by Silva et al. (2018) in charcoal-grilled beef and by Garcia-Lomillo et al. (2017) in barbecued beef patties.

As regard the risk to human health from PAHs in food, the European Food Safety Authority (EFSA) established the sum of the four specific substances (PAH4): benz[a] anthracene (BaA), chrysene (Chr), benzo[b]fluoranthene (BbA) and benzo[a]pyrene (BaP) as the most appropriate indicator for the occurrence and carcinogenic potency of PAH in food (EFSA, 2008). The EFSA also concluded that the sum of eight substances (PAH8 = PAH4 + benzo[k]fluoranthene (BkA) + dibenz[a,h]anthracene (DhA) + benzo[g,h,i]perylene (BgP) + indeno[1,2,3-c,d]pyrene (IP)) does not

provide additional relevant information compared to the system of four substances. Therefore, the Commission Regulation No. 835/2011 instituted a maximum limit for BaP and PAH4 as 2 μg/kg and 12 μg/kg wet weight for smoked meat and meat products, respectively; while for grilled or barbecued meat sold directly to consumers, these limits are set at 5 μg/kg and 30 μg/kg, respectively. Regarding the PAH4 found in the present study, the concentration ranged between 0.56 ng/g to 0.99 ng/g which are far from the maximum level established by the Reg. 835/2011. Furthermore, the addition of vitamin E to beef patties reduced significantly the total PAH4 amount at all the concentration used (Figure 21), with the lowest value for the C2 group (0.56 ng/g, $P < 0.0001$) as compared to C group (0.99 ng/g). According to Büyükkurt et al. (2017), the use of extracts of sage and thyme in commercial marinating preparations for meat significantly decreased the levels of PAHs in barbecued beef, lowering PAH4 the most, highlighting the role of antioxidant compounds as inhibitors in free radical reaction pathways. The values of PAH4 obtained in the present study are lower than those reported by Silva et al. (2018) in charcoal-grilled beef (2.61 ng/g). In a recent review, Duedahl-Olesen and Ionas (2022) reported the concentration (μg/Kg) of PAH4 in different barbecued meat types with indications of the use of heat source. In particular, for beef burger with different fat content and different heat source it was reported a wide range of variation for the PAH4 level from 0.86 μg/Kg to a maximum level of 144 μg/Kg (Maga, 1986; Viegas et al., 2012; Rose et al., 2015). Considering the qualitative profile, the following profile was found for control group: BaA (ranging from 0.32 to 0.42 ng/g) > Ch (ranging from 0.13 to 0.30 ng/g) > BbF (ranging from 0.07 to 0.20 ng/g) > BaP (ranging from 0.05 to 0.06 ng/g). Similar values were found by Garcia-Lomillo et al. (2017) in barbecued beef patties for BaA (0.42 ng/g vs 0.42 ng/g), while higher levels were found for Ch (0.63 ng/g vs 0.30 ng/g), BbF (0.30 ng/g vs 0.20 ng/g) and BaP (0.22 ng/g vs 0.06 ng/g).

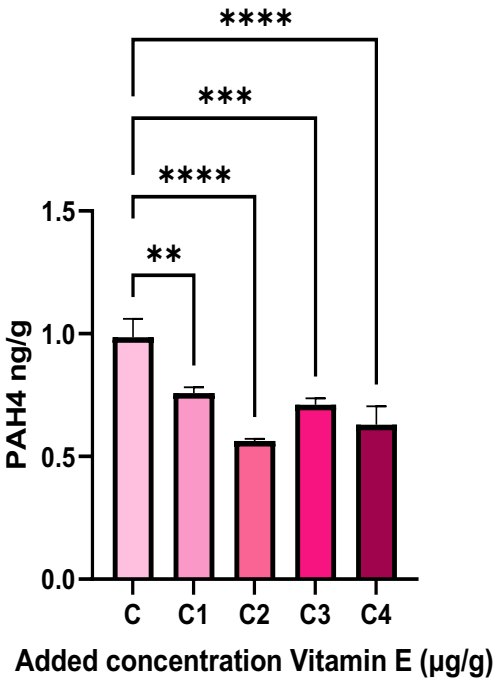


Figure 21. PAH4 content in beef burgers. PAH4 includes the sum of BaA, Ch, BbF, BaP. C = control; C1 = 2 µg/g; C2 = 3µg/g; C3 = 4.5 µg/g; C4 = 6µg/g. ns-not significant; *P ≤0.05; **P ≤0.01; ***P≤0.001; ****P≤0.0001

As regard the PAH8 content, the obtained values ranged between 1.10 ng/g to 2.33 ng/g. Viegas et al. (2012b) found a higher concentration of PAH8 in beef samples (3.22 ng/g) and a notable higher content in salmon samples (43.22 ng/g) which is mainly related to the fat content of samples. In particular, authors explained that these differences could be related to the fat dripping which led to flame formation, increasing release of smoke that carries HAs and PAHs on the surface of the food. It is generally agreed that the fat content is an important parameter affecting PAHs formation in charcoal grilled muscle foods. Since PAHs are highly lipophilic molecules, they were expected to accumulate in the fat contained in food material (Hamidi et al., 2022). Lean beef is known to form lower content of PAHs compared with pork, chicken and salmon (Chung et al. 2011; Viegas et al., 2012a; Viegas et al., 2014).

As regard the effect of vitamin E on PAH8 formation (Figure 22), no effect of vitamin E ($P > 0.05$) was observed in burgers added with 2 µg/g (C1: 1.75 ng/g) and 4.5 µg/g (C3: 2.08 ng/g), while significant reduction was noticed in burgers added with 3 µg/g (1.17 ng/g, $P < 0.01$) and 6 µg/g (1.10 ng/g, $P < 0.001$) as compared to C group (2.33 ng/g). These outcomes are consistent with those of

Min et al. (2017) who found in heated beef meat model system added with α-tocopherol a significant reduction in PAH8 content (from 22.98 µg/kg in control to 14.05 µg/kg α-tocopherol).

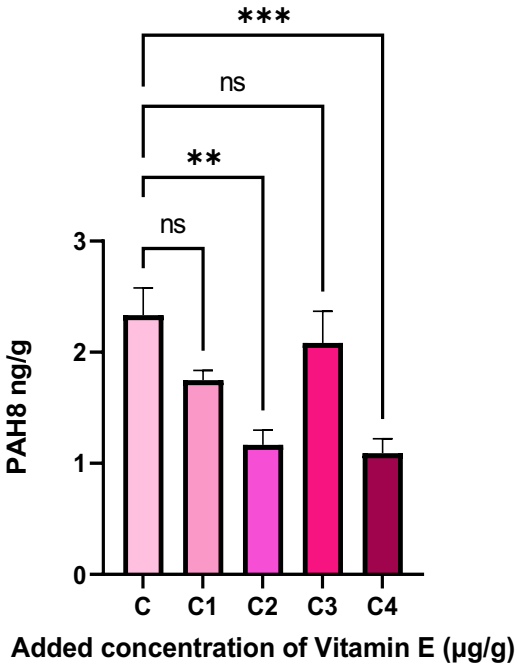


Figure 22. PAH8 content in beef burgers. PAH8 includes the sum of BaA, Ch, BbF, BkF, BaP, BgP, IP, DhA. C = control; C1 = 2 µg/g; C2 = 3µg/g; C3 = 4.5 µg/g; C4 = 6µg/g. ns-not significant; *P ≤0.05; **P ≤0.01; ***P≤0.001; ****P≤0.0001

Concerning the BaP concentration in control samples was 0.06 ng/g, far away from the maximum level (5.0 ng/g) established by the European Union. It was possible to detect it only in beef burgers added with 2 µg/g and 6 µg/g of vitamin E, while in other groups was below the LOQ (< 0.04 ng/g) (Table 15). These results are similar to those reported by Kazeoruni et al. (2001) who reported values of BaP in the range of 0.09 - 1.52 ng/g in grilled/barbecued hamburgers. Nonetheless burgers did not present significant exposure to BaP, Vitamin E showed a promising effect in reducing BaP concentration. A similar effect was observed by Min et al., 2017 in heated beef meat model system. The addition of α-tocopherol significantly reduced BaP concentrations from 6.58 µg/kg in control samples to 4.95 µg/kg in added samples (Figure 23).

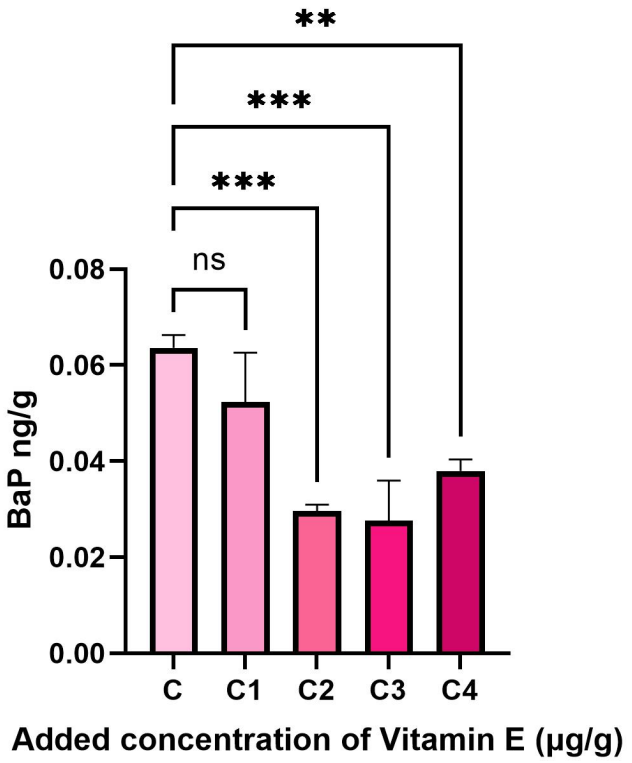


Figure **23.** BaP content in beef burgers. C-control; C1~2 µg/g; C2~3µg/g; C3~4.5 µg/g; C4~6µg/g ns-not significant; *P ≤0.05; **P ≤0.01; ***P≤0.001; ****P≤0.0001

3.2 Trial 2: Chicken meat model system

3.2.1 Vitamin E, total lipid, fatty acid profile and nutritional indices

Poultry meat can be enriched with vitamin E by providing it with feed (Bordoni and Danesi, 2017). Generally, the vitamin E content in feed vary to the age fed, for Ross broilers: started: 80 IU; grower: 65 IU; finisher: 55 IU (AVIAGEN). The concentrations of α-tocopherol found in breast muscle from Ross 308 broiler chickens are variable (287.3 µg/100g, Leskovec et al., 2019; 490.2 µg/100g, Pečjak et al., 2022). In the present study, the α-tocopherol content found in chicken meat (5.54 µg/g) is quite similar to that reported by Pečjak et al. (2022).

In terms of human health, total fat and fatty acids (FA) composition of meat products are very important parameters of meat quality. The fat content amount observed in the chicken meat (2.08 g/100g) is higher compared to the values reported in literature on breast muscle of Ross 308 broiler chickens (1.56g/100g, Cheng et al., 2022; 1.47g/100g, Cygan-Szczegielniak and Bogucka, 2021; 2.43g/100g, Tavaniello et al. 2022a). The high amount of fat found in the samples used in the present study could be due to low level of physical activity in the chickens, that favored the lipogenesis instead of myogenesis, and/or feed diet (e.g. high levels of fat). However, we did not have any information about the genotype used and rearing condition since chicken meat was bought in a supermarket.

Broiler fat is characterized by a significant amount of monounsaturated fatty acids (MUFA), and, in comparison with red meat, substantial amounts of polyunsaturated fats, especially the n-6 linoleic acid and arachidonic acid. Considering the general FA profile of breast meat (Table 16), total MUFA were the most abundant (42.12%), followed in descending order by SFA (- 7%) and PUFA (-20.7%). These findings are inconsistent with those reported by Tavaniello et al. (2022b), who found a higher value of SFA and followed in descending order by PUFA and MUFA in breast muscle from slow-growing chickens. In another work, Tavaniello et al. (2022a) found a quite similar values for SFA and PUFA and lower value for MUFA (-9.4%) in breast muscle from Ross 308 broiler chickens.

Considering the composition of single FA, among SFA, palmitic (C16:0), and stearic (C18:0) acids were the most abundant. Palmitic acid is thought to increase cholesterol levels together with lauric and myristic acid, while stearic acid has little or no effect. In fact, stearic acid is generally considered to be a neutral fatty acid because it has been shown to have no net impact on the plasmatic level of either LDL or HDL cholesterol in humans (Tavaniello et al., 2022a).

MUFA were mainly represented by oleic acid (C18:1 cis9; 37.58%). PUFA were mainly in the form of linoleic acid (C18:2 n-6; 17.14%) the precursor of the n-6 family, followed by the arachidonic acid (C20:4 n-6; 2.22%). A similar trend of single fatty acids was found by Tavaniello et al. (2020a, b). The values of the n-6/n-3 ratio observed in this study are very higher than those found for fast-growing chickens and slow-growing chickens (Tavaniello et al., 2020a, b). Because of the risk of coronary heart disease and cancer, the recommended values of the n-6/n-3 ratio should be lower than 4.0 (Wood et al., 2004). Generally, poultry is characterized by the highest n-6/n-3 ratio compared to other types of meat, essentially due to the higher amount of n-6 FA (20.34%) than muscles of the other species (Rule et al., 2002; Wood et al., 2004). In fact, linoleic acid is the predominant essential FA in poultry and as a result the n-6 PUFA are the primary products found in tissue lipids. For a nutritional point of view, the P/S value observed in the present study (0.59) is favorably, a higher P/S ratio is recommended and should be increased to above 0.4 (Wood et al., 2004), value very difficult to achieve in poultry and other meat products (Milícević et al., 2014). The AI and TI values, crucial for the health of consumers, found in the current study can be considered similar with those reported by Tavaniello et al- (2022b), in medium-growing broiler chickens fed soybean or pea bean and raised under semi-intensive conditions, and Banaszak et al. (2020) in ducks fed yellow lupin, and however better than those found in Ross 308 chickens, males and females, reared under organic system and slaughtered at 82 days of age (Cygan-Szczegielniak and Bogucka, 2021). In a previous work, Tavaniello et al. (2020) reported a better AI (0.42) and TI (0.75) indices in Ross 308 chickens reared in floor pens.

Table 16. Nutritional profile of chicken meat.

Items	
α-tocopherol (μg/g)	5.54 ± 0.58
Total lipids (g/100g)	2.08 ± 0.29
Fatty acids (FA; % on total FA)	
C12:0	0.03 ± 0.01
C14:0	0.59 ± 0.05
C16:0	27.94 ± 0.54
C16:1n7	4.37 ± 0.67
C18:0	7.90 ± 0.33
C18:1 cis9	37.58 ± 1.39
C18:2 n-6	17.14 ± 0.74
C18:3 n-3	0.75 ± 0.09
C18:3 n-6	0.09 ± 0.01
C20:1 n-9	0.18 ± 0.03
C20:3 n-6	0.45 ± 0.04
C20:4 n-6	2.22 ± 0.32
C20:5 n-3	0.10 ± 0.03
C22:4 n-6	0.44 ± 0.10
C22:5 n-3	0.14 ± 0.04
C22:6 n-3	0.08 ± 0.02
Total fatty acids[1]	
SFA	36.46 ± 0.90
MUFA	42.12 ± 1.99
PUFA	21.42 ± 1.24
n-6 PUFA	20.34 ± 1.12
n-3 PUFA	1.07 ± 0.18
Nutritional indices[2]	
n-6/n-3	19.21 ± 2.57
P/S	0.59 ± 0.02
TI	1.06 ± 0.03
AI	0.48 ± 0.02

[1]SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
[2]P/S = PUFA/SFA ratio; AI = atherogenic index; TI = thrombogenic index.

3.2.2 Polycyclic aromatic hydrocarbons in chicken burgers

Several studies have demonstrated that the final PAH concentration depend on many factors including the type of meat, which is mainly related to the lipid content and fatty acid composition. In fact, it has been found that the higher the content of unsaturated fatty acids, the higher the probability for benzene-like compounds and derivatives of PAHs to be formed (Chen and Chen, 2001). However, considering that many are the variables which can affect the PAHs formation and concentration, no clear trends for specific meat types and PAH concentrations have been identified (reviewed by Duedhal-Olesen and Ionas, 2022). In light of this, another in vitro trial was carried out to determine the effect of different doses of vitamin E added to chicken patties before grilling on the PAHs formation. The tested doses were the same used for grilled beef patties: 2, 3, 4.5 and 6 μg/g of *dl*–α–tocopheryl acetate.

As already reported in the paragraph 3.1.1.2.1 "Quality control of analytical method", the analytical performance of the extraction method for reliable chromatographic quantification of PAHs in grilled beef and chicken was evaluated. Linearity was observed using matrix-match calibration curves in the range between 0.05 and 50 ng/g for PAHs. The results of LOD, LOQ and linearity are reported in Table 14. Good linear responses were obtained for all compounds with coefficient correlations (rw) above 0.99. As regard the concentration of the PAHs compounds that were present in the samples at or above their limits of quantitation are shown in Table 17. Among 14 PAHs assayed, only DhA was not detected.

Table 17. PAHs content (ng/g) in chicken burgers added with different concentrations of vitamin E (µg/g).

PAHs[1]	Control	Vitamin E				P-value
		C1 (2 µg/g)	C2 (3 µg/g)	C3 (4.5 µg/g)	C4 (6 µg/g)	
Ac	54.23±2.56^{A}	36.81±0.67^{B}	50.62±3.7^{A}	22.12±1.17^{C}	26.36±0.82^{C}	0.0001
F	19.66±1.57^{A}	12.65±0.51^{B}	17.66±1.72^{A}	6.97±0.77^{C}	6.87±0.65^{C}	0.0001
Pa	70.76± 3.33^{A}	64.20±4.32^{A}	52.02±2.65^{B}	45.44±1.32^{BC}	41.81±3.34^{C}	0.0001
A	10.34±0.38^{A}	8.78±0.20^{B}	7.26±0.4^{C}	4.82±0.23^{D}	5.3±0.43^{D}	0.0001
Fl	19.92±0.8^{A}	12.38±0.84^{B}	12.43±1.57^{B}	6.81±0.61^{C}	6.96±0.6^{C}	0.0001
P	14.68 ±0.2^{A}	8.64±0.51^{C}	11.01±1.04^{B}	5.36±0.07^{D}	5.54±0.54^{D}	0.0001
BaA	1.68±0.09^{A}	0.83±0.02^{B}	0.85±0.09^{B}	0.55±0.02^{C}	0.55±0.07^{C}	0.0001
Ch	1.31±0.08^{A}	0.73±0.03^{BC}	0.84±0.11^{B}	0.35±0.02^{Cb}	0.59±0.09^{Ca}	0.0001
BbF	1.10±0.01^{A}	0.6±0.02^{B}	0.65±0.06^{B}	0.43±0.02^{C}	0.40±0.03^{C}	0.0001
BkF	0.18±0.00^{A}	0.11±0.01^{B}	0.08±0.01^{CDa}	0.06±0.00^{Db}	0.06±0.00^{D}	0.0001
BaP	0.45±0.01^{A}	0.26±0.01^{B}	0.19±0.02^{CDa}	0.15±0.00^{Db}	0.14±0.01^{D}	0.0001
BgP	0.54±0.02^{A}	0.28±0.04^{B}	0.26±0.05^{B}	< 0.18	< 0.18	0.0001
DhA	n.d	n.d	n.d	n.d	n.d	
IP	0.40±0.02^{A}	0.26±0.06^{B}	< 0.13	< 0.13	< 0.13	0.0001

[1] acenaphthene (Ac), fluorene (F), phenanthrene (Pa), anthracene (A), fluoranthene (Fl), pyrene (P), benzo(a)anthracene (BaA), chrysene (Ch), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), indeno(1,2,3-cd)pyrene (IP), benzo(ghi)perylene (BgP), dibenzo(ah)antracene (DhA);
^{A,B,C} Means within a row lacking a common superscript differ ($P < 0.01$).
^{a,b,c}Means within a row lacking a common superscript differ ($P < 0.05$).

Analysis of individual PAH content showed that light PAH (Pa > Ac > F > A) were the most abundant as compared to heavy ones (Table 17). Comparing the light PAHs content found in the present study between grilled beef and chicken meat, the latter was characterized by a higher PAHs content, e.g. for control group: Pa: 41.70 vs 70.76 ng/g; Ac: 16.85 vs 54.23 ng/g; F: 0.49 vs 19.66 ng/g; A: 4.37 vs 10.34 ng/g, respectively for beef and chicken meat. The observed differences could be related to the different lipid content, which was higher in breast chicken meat than in beef *longissimus dorsi* (2.08 % vs 1.36 %), and lipid composition. In general, for the individual PAHs content in chicken meat, C3 and C4 groups were characterized by the lowest ($P < 0.0001$) content as compared to C group, while intermediate values were found for C1 and C2 groups ($P < 0.0001$). To better understand the differences among treated groups and control one the sum of different categories of PAHs (total, light, heavy, PAH4, PAH8) are reported in graphs.

The total content of the 14 PAHs assayed ranged from 93.25 ng/g to 195.2 ng/g (Figure 24). These values are consistent with those reported by Khalili et al. (2023) in grilled chicken (183 µg/Kg) and hamburger chicken (115.7 µg/Kg). Similarly, in a study carried out by Shen et al. (2022) to compare the effects of different natural plant extracts on the formation of PAHs in roast duck, they found an average content of PAHs (16 PAHs) of 241.78 µg/Kg. On the contrary, lower PAHs content were found by Silva et al. (2018) in charcoal-grilled chicken.

Regarding the effect of vitamin on PAHs formation in grilled chicken, all vitamin E added groups were characterized by a significant lower ($P < 0.0001$) total PAHs content as compared to C group. In particular, C3 and C4 groups showed the lowest values (93.25 ng/g and 94.74 ng/g, respectively) followed by C1 and C2 groups (146.5 ng/g and 154.0 ng/g, respectively) compared to C group. The obtained values are higher than those obtained for grilled beef, ranging from 46.14 ng/g to 80.00 ng/g. Interestingly, the effect of different doses of vitamin E added to meat before grilling on PAHs formation is different in relation to meat type. In beef meat the lowest vitamin E concentration (2 µg/g) had no effect ($P>0.05$) on the reduction of the total PAHs as compared to control group, while the addition of 4.5 µg/g seems to be more effective in the PAHs reduction.

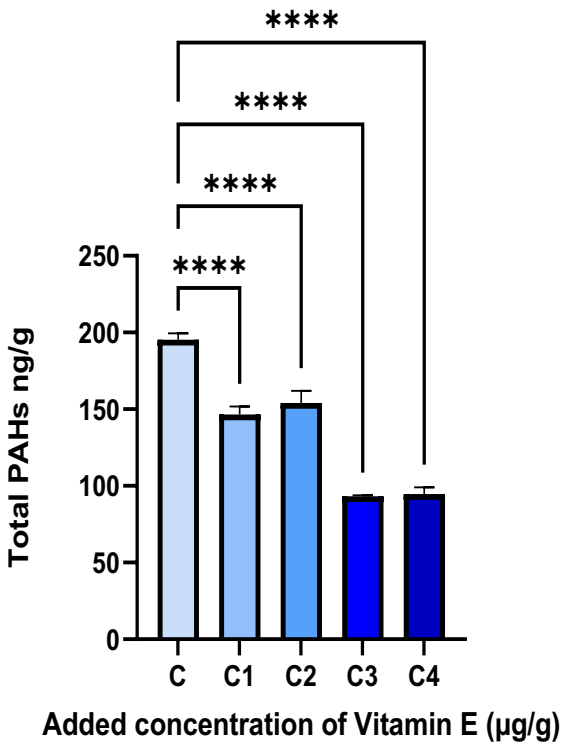


Figure 24. Total PAHs content in chicken burgers. C-control; C1~2 µg/g; C2~3µg/g; C3~4.5 µg/g; C4~6µg/g ns-not significant; *P ≤0.05; **P ≤0.01; ***P≤0.001; ****P≤0.0001

The total content of light PAHs ranged from 79.35 ng/g to 155.0 ng/g, while in beef we found lower values (37.46 ng/g - 68.61 ng/g). The mean concentrations (ng/g) of light PAHs (Ac, F, Pa, A) in control and treated samples were in the range Ac (22.12 – 54.23), F (6.97 – 19.66), Pa (45.44 – 70.76), A (4.82 – 10.34). The application of vitamin E to ground chicken patties provided a significant inhibition ($P < 0.0001$) of light PAHs formation at all the concentration used as compared to untreated group, following the same trend of total PAHs (Figure 25). Similarly, C3 and C4 groups showed the lowest values (79.35 ng/g and 80.39 ng/g, respectively) followed by C1 and C2 groups (122.4 ng/g and 127.6 ng/g, respectively) compared to C group (155.0 ng/g). On the contrary, in beef patties we found that the concentrations of 4.5 µg/g (37.46 ng/g, $P < 0.0001$) and 3µg/g (50.01 ng/g, $P < 0.001$) were the most effective, with intermediate values for the highest concentration 6µg/g (55.69 ng/g, $P < 0.05$) as compared to C group (63.41 ng/g). The lowest concentration (2µg/g) was not effective in the reduction of light PAHs formation (68.61 ng/g, $P > 0.05$).

The total content of heavy PAHs (FI, P, BaA, Ch, BbF, BkF, BaP, BgP, IP) ranged from 13.89 ng/g to 40.26 ng/g, while for beef we observed lower values ranged between 8.68 ng/g to 12.47 ng/g. The observed values for charcoal-grilled chicken are comparable with those reported by Viegas et al. (2012b) even if the concentration of the single heavy PAHs is quite different. In the above-mentioned study, chicken samples were collected in one of the traditional restaurants for chicken charcoal grilled to evaluate the effect of continuous barbecuing with the same charcoal. Chicken samples collected at the first period at the beginning of charcoal combustion displayed an average content of heavy PAHs of 51.92 ng/g; while chicken meat collected one and a half hour later of charcoal combustion displayed a significant higher content of heavy PAHs (149.26 ng/g). The authors, explained that these differences could be related to the fact that heavy PAHs are more stable than light PAHs (instable and volatile) and may accumulate in charcoal, being released later and then deposited on the surface of the later grilled chickens.

Concerning the effect of vitamin E (Figure 25), it was observed the same trend as for heavy PAHs. Highly significant reduction ($P \leq 0.0001$) of light and heavy PAHs content were observed in all burgers added with vitamin E (C1: 24.08 ng/g; C2: 26.41 ng/g; C3: 13.89 ng/g; C4: 14.35 ng/g) compared to the control group (40.26 ng/g).

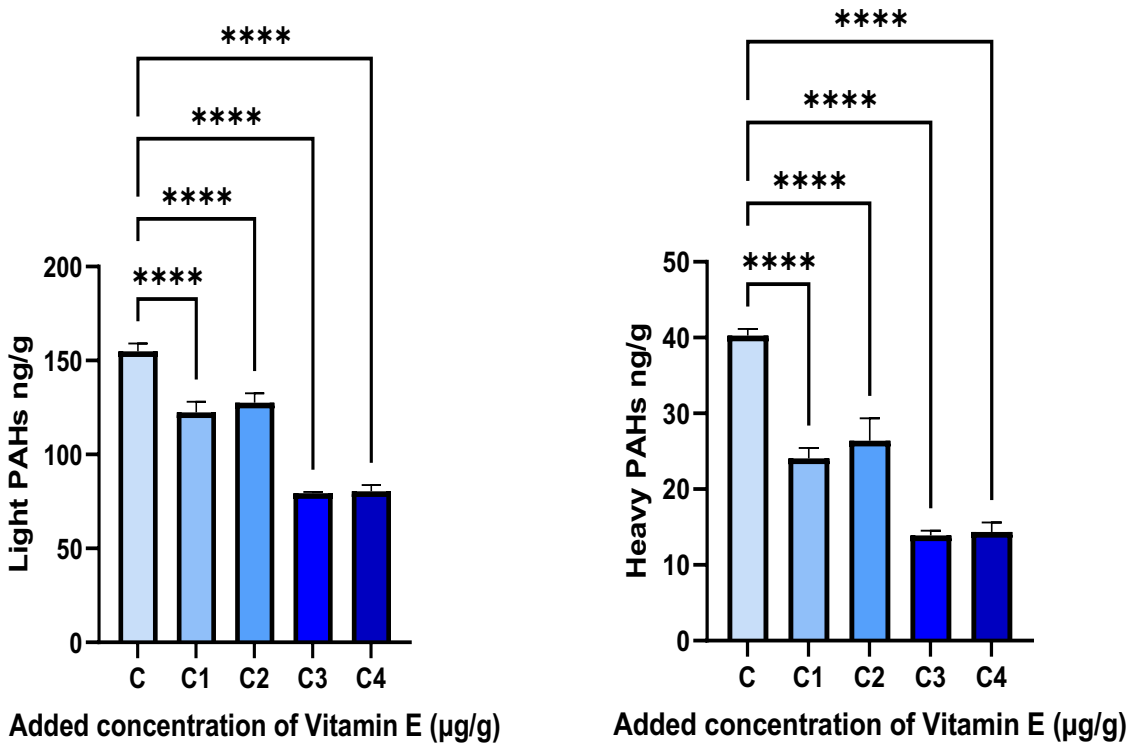


Figure 25. Light and Heavy PAHs content in chicken burgers. Low molecular weight (light) PAHs includes the sum of Ac, F, Pa, A. High molecular weight (heavy) PAHs includes the sum of Fl, P and genotoxic PAHs (PAH8). C = control; C1 = 2 µg/g; C2 = 3µg/g; C3 = 4.5 µg/g; C4 = 6µg/g. ns-not significant; *P ≤0.05; **P ≤0.01; ***P≤0.001; ****P≤0.0001

The concentrations of PAH4 ranged between 1.47 ng/g to 4.54 ng/g. The control group was characterized by the highest PAH4 content (4.54 ng/g), however, the addition of vitamin E to chicken patties reduced significantly ($P < 0.0001$) the total PAH4 amount at all the concentration used (Figure 26), with the lower value for the C3 and C4 group (1.47 ng/g and 1.69 ng/g, respectively), followed by C1 and C2 (2.40 ng/g and C2 2.54 ng/g, respectively). Similarly, in grilled beef patties the addition of vitamin E reduced significantly the total PAH4 amount at all the concentration used with the lowest value for the C2 group (0.56 ng/g, $P < 0.0001$) as compared to C group (0.99 ng/g).

Considering the qualitative profile, the following profile was found for control group: BaA > Ch > BbF > BaP, similar to grilled beef patties even if the concentration were higher in chicken meat: BaA (0.42 ng/g vs 1.68 ng/g), Ch (0.30 ng/g vs 1.31 ng/g), BbF (0.20 ng/g vs 1.10 ng/g) and BaP (0.06 ng/g vs 0.45 ng/g).

Silva et al. (2018) found an average content of PAH4 of 3.88 ng/g in charcoal-grilled chicken. El Husseini et al. (2021) in a study on traditional Lebanese barbecued products found that also the surface to mass ratio influenced the PAH4 formation; in fact, they found that the PAH4 concentrations were more than doubled with increased surface areas with PAH4 concentrations of 2 mg/kg for steak/ breast to 7.5 mg/kg for small beef pieces and 6.6 mg/kg for chicken pieces.

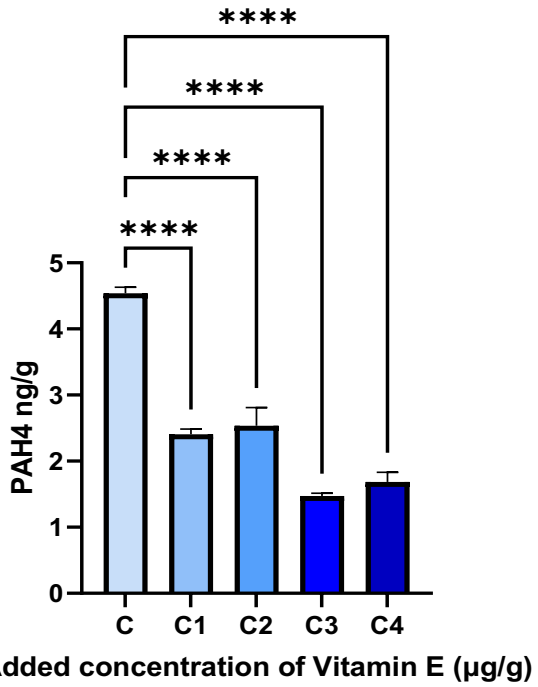


Figure 26. PAH4 content in chicken burgers. PAH4 includes the sum of BaA, Ch, BbF, BaP. C = control; C1 = 2 µg/g; C2 = 3µg/g; C3 = 4.5 µg/g; C4 = 6µg/g. ns-not significant; *P ≤0.05; **P ≤0.01; ***P≤0.001; ****P≤0.0001

Concerning the PAH8 content, the obtained values ranged between 1.73 ng/g to 5.66 ng/g. On the contrary, lower values were found in grilled beef patties (1.10 ng/g to 2.33 ng/g). Similarly, Wang et al. (2019c) in charcoal-grilled chicken wings found an average content of PAH8 in control group of 2.5 ng/g with a reduction range from 15% to 54.5% in marinades with 8 phenolic compounds existing in green tea. Different values of PAH8 were reported by Alomirah et al. (2011) in charcoal grilled chicken (20.3 ng/g), and by Badry (2010) in chicken grilled on direct flame butane gas (25.4 ng/g).

Viegas et al. (2012) found in chicken samples, grilled in the first period at the beginning of combustion when all flames had subsided, an average content of PAH8 of 24.97 ng/g; while chicken samples from second period exhibited approximately 2.5-fold the PAH8 value from first period samples.

As regard the effect of vitamin E on PAH8 formation (Figure 27) a significant reduction ($P \leq 0.0001$) of PAH8 content was observed in all burgers added with vitamin E (C1: 3.06 ng/g, C2: 2.97 ng/g, C3: 1.73 ng/g and C4:1.85) compared to the control group (5.66 ng/g). Lower values were found for beef burgers (2.33 ng/g and 1.75 ng/g for C and C1 respectively); a significant reduction was noticed only in burgers added with 3 µg/g (1.17 ng/g, $P < 0.01$) and 6 µg/g (1.10 ng/g, $P < 0.001$) as compared to C group.

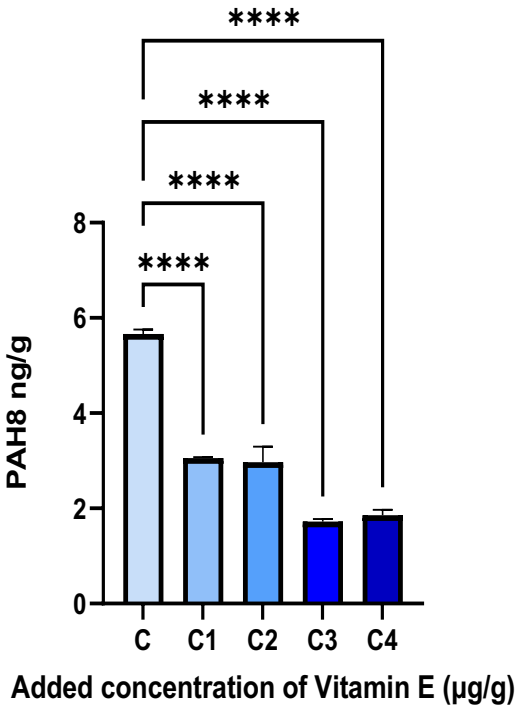


Figure 27. PAH8 content in chicken burgers. PAH8 includes the sum of BaA, Ch, BbF, BkF, BaP, BgP, IP, DhA. C = control; C1 = 2 µg/g; C2 = 3µg/g; C3 = 4.5 µg/g; C4 = 6µg/g. ns-not significant; *P ≤0.05; **P ≤0.01; ***P≤0.001; ****P≤0.0001.

Concerning BaP, concentration in control samples was 0.45 ng/g, far from the maximum level (5.0 ng/g) established by the European Union. According to Kazerouni et al. (2001), electric heating

resulted in BaP concentrations well below 0.5 µg/kg. These heat source results in line with the discardable grill used for the trial, filled with charcoal made by briquets with 30% of coconut. In accordance with Viegas et al. (2012b) this type of charcoal by itself serves as inhibitor of PAHs formation. Nonetheless, in all burgers added with vitamin E was observed a highly significant reduction ($P \leq 0.0001$) of BaP content: C1 0.26 ng/g; C2 0.19 ng/g, C3 0.15 ng/g; C4 0.14. A similar effect of vitamin E as antioxidant was observed by Wang et al., 2019a that observed the effect of the antioxidant activity of phenolic compounds in tea used to marinate meat, inhibiting in charcoal-grilled chicken wing BaP formation up to 70 %.

3.3 Trial 3: Animal model

3.3.1 Vitamin E (α-tocopherol), total lipid, fatty acid profile and nutritional indices

Animal models have long been extensively employed in biomedical research focusing on physiological, morphological, behavioral, and other aspects. The chicken (*Gallus gallus domesticus*) as a key animal model that is presumed to be typical of birds and generally applicable to mammals (Flores-Santin and Burggren, 2021).

In light of this, 10 Ross 308 chickens were used as animal model to study the effect of 100 IU of dl-α-tocopheryl acetate (Control: n = 5; vitamin E injected: n = 5) intramuscular injected on total lipid, fatty acid composition and nutritional indices of breast muscle (Table 18), and on formation of polycyclic aromatic hydrocarbons in grilled chicken burgers (results and discussion in the next paraph).

Vitamin E cannot be synthesized by animals and has to be supplied by the diet or injected; its presence in body tissues is related to the dietary availability or to the amount injected. According to NRC (1994) 10 IU of vitamin E/kg of diet are needed to meet the nutritional requirements of poultry. Even if the nutritional requirements of broiler chickens are likely to have changed in response to intense genetic selection that has been observed in poultry, it is generally agreed that the vitamin E amount present in the main ingredients utilized in poultry nutrition may be sufficient for the poultry needs (Pompeu et al., 2018). The establishment of isolated vitamin E requirements is difficult, mainly because of its interactions with diverse factors that may affect the necessity of diet supplementation and the physiological status of the broiler chickens (Kuttappan et al., 2012). This information is useful to

optimize the inclusion levels to maximize gains, because vitamin E is one of the most expensive vitamins in the commercial supplement for poultry. In light of these consideration, the intramuscular injection of vitamin E could be a valuable strategy to ensure a more standardized administration in the animals (Maiorano et al., 2015), especially for animal model studies.

In the present study, as expected, meat from vitamin group had higher content of tocopherol (5-fold) compared with the control one (P = 0.000). A similar trend was observed in crossbred lambs (Ile de France x Pagliarola) injected intramuscularly in the left gluteus with a total 1000 IU of dl-α-tocopheryl acetate (Salvatori et al., 2004). The authors found in semimembranosus muscle 2.76 and 9.90 μg/g of vitamin E in control and treated groups, respectively. In addition, they found lower content of vitamin E in longissimus dorsi and gluteus biceps of the same lambs. The concentration of α-tocopherol within membranes is tissue- and organ-dependent (Faustman et al., 1998). This variation is probably due to factors, such as functional and metabolic differences of fiber, and different fiber fat contents (Chan and Decker, 1994; Sales and Koukolová, 2011; Picard, 2012). These finding are consistent with those reported by Maiorano et al. (2016) who found higher tocopherol content (6-fold) in longissimus dorsi and vastus lateralis muscle of Laticauda male lambs injected intramuscularly with a total 1500 IU of dl-α-tocopheryl acetate.

Vitamin E did not affect total lipid content. In contrast to this result, in a study conducted on lambs Salvatori et al. (2004) found a higher amount of total lipid in meat from crossbred lambs treated in vivo with vitamin E compared with the control one. Differently, a recent study (Zayed and Estuty, 2022) reported that supplementation with various doses vitamin E in the diet reduced intramuscular fat in male kids, with the increase of vitamin E levels in the diet fat amount decreased. Authors suggesting the concentration of vitamin E in muscles depends on the level and duration of vitamin E supplementation.

Table 18. Nutritional profile of breast muscle from control chickens (C) and vitamin E injected chickens (T).

	C	T	p-value
α-tocopherol (μg/g)	2.93±0.52	14.59±0.61	0.000
Total lipids (g/100g)	1.49±0.14	1.43±0.13	0.550
Fatty acids (FA; % on total FA)			
C12:0	0.04±0.01	0.03±0.00	0.126
C14:0	0.94±0.21	0.75±0.04	0.083
C16:0	27.04±0.73	24.84±1.36	0.013
C16:1n7	2.90±0.61	2.41±0.27	0.135
C18:0	7.99±0.71	8.37±0.66	0.403
C18:1 cis9	32.71±2.43	33.10±1.75	0.782
C18:2 n-6	22.38±2.22	23.11±1.45	0.556
C18:3 n-3	1.03±0.23	1.01±0.17	0.883
C18:3 n-6	0.09±0.02	0.11±0.03	0.262
C20:1 n-9	0.30±0.08	0.31±0.07	0.804
C20:3 n-6	0.37±0.11	0.38±0.06	0.919
C20:4 n-6	3.09±0.48	4.05±1.24	0.146
C20:5 n-3	0.10±0.02	0.11±0.02	0.238
C22:4 n-6	0.49±0.09	0.71±0.19	0.052
C22:5 n-3	0.35±0.07	0.47±0.17	0.173
C22:6 n-3	0.17±0.03	0.24±0.09	0.149
Total fatty acids[1]			
SFA	36.02±0.50	34.00±1.92	0.053
MUFA	35.91±2.88	35.81±1.85	0.949
PUFA	28.07±2.70	30.19±1.32	0.154
n-6 PUFA	26.42±2.54	28.35±1.11	0.158
n-3 PUFA	1.66±0.18	21.84±0.23	0.193
Nutritional indices[2]			
n-6/n-3	15.98±0.81	15.53±1.36	0.544
P/S	0.78±0.07	0.89±0.07	0.041
TI	0.99±0.02	0.90±0.08	0.038
AI	0.48±0.02	0.42± 0.03	0.011

[1]SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
[2]P/S = PUFA/SFA ratio; AI = atherogenic index; TI = thrombogenic index.

Regarding the fatty acid profile (Table 18), comparison of the experimental groups revealed no differences in MUFA and PUFA content ($P < 0.05$). On the contrary, lower SFA content was found in treated chickens compared to control group (- 2%; $P < 0:05$), mainly due to a lower proportion of

palmitic acid (C16:0; - 2.2%; $P < 0.05$). In a meta-analysis, Pompeu et al. (2018) studied the effect of vitamin E supplementation on the growth performance, carcass traits, meat quality, and immune response of male broiler chickens. Considering 51 research studies using a dosage of *all-rac* α-tocopheryl acetate from 0 to 960 mg/kg found that dietary vitamin E supplementation increased the vitamin E content in the muscle and did not change the PUFA concentration. The composition of PUFA in the meat is primarily dependent on dietary oil type, but independent of dietary vitamin E concentration. Overall, the SFA were the most abundant fatty acids (ranging from 34.00 to 36.02 %) followed by MUFA (about 36%) and PUFA (ranging from 28.07 to 30.19 %). The major SFAs observed were C16:0 and stearic acid (C18:0), for MUFA oleic acid (C18:1 cis9), and for PUFA linoleic acid (C:18:2 n-6) and arachidonic acid (C 20:4 n-6). These findings are in agreement with previous studies (Tavaniello et al., 2020, 2022; Kwiecień et al., 2021; Cabrol et al., 2022). From a nutritional point of view, oleic acid has a relevant importance in the human diet because it acts on lipaemia reducing both LDL cholesterol and the triglycerides (Mattson and Grundy, 1985) and providing other health benefit, such as a reduced risk of stroke and a significant decrease in both systolic and diastolic blood pressure in susceptible populations (Kris-Etherton, 1999). Considering the composition of single FA, among PUFA docosatetraenoic acid (C22:4 n-6) was found slightly higher ($P = 0.053$) in vitamin E group (0.71%) compared to control (0.49%). The content of total n-3 PUFA and n-6 PUFA were not influence ($P > 0.05$) by vitamin E treatment. Positive effect of ω-3 PUFA on coronary heart diseases has been shown in a lot of experimental studies in animals, humans, and tissue cultures (Bušová et al., 2020). In fact, to decrease the risk of cardiovascular diseases, n-3 PUFA intake is recommended (Yagi et al., 2017) because n-3 PUFAs could improve heart rate and blood pressure through improving left ventricular diastolic filling or augmenting vagal tone (O'Keefe et al., 2006). However, chicken meat contains a low content of omega 3 and a high content of omega 6. On the contrary, n-3 PUFA (including eicosapentaenoic, docosahexaenoic and docosapentaenoic acids) mainly exist in fatty fish. PUFA ω-3 and ω-6 are essential and have protective functions for the human organism (WHO, 2008).

The nutritional indices of the meat (Table 18), such as the ratios of PUFA/SFA, AI and TI indices were improved by vitamin E. In particular, PUFA/SFA was higher ($P < 0.05$) in vitamin groups, on the contrary AI and TI indices were lower ($P < 0.05$) compared with control group. Meat is an important source of fat and fatty acid for humans. Although opinions differ on the importance of the fatty acid composition for meat quality, consumers increasingly prefer meat products with higher proportion of unsaturated acid fat and prevalently PUFA because of their beneficial effects on health.

AI index expresses the ability of pro-atherogenic activity or preventive anti-atherogenic effect (inhibiting the aggregation of plaque, diminishing the levels of cholesterol, phospholipids, and so preventing coronary diseases) (Ulbricht and Southgate, 1991). TI index expresses the tendency to form clots in the blood vessels. Both, AI (0.48 and 0.42 in control and vitamin E groups, respectively) and TI (0.99 and 0.90 in control and vitamin E groups, respectively) values found in this study are comparable with the values found in other works on chickens (Kwiecień et al., 2021; Tavaniello et al., 2020, 2022a, b). Regarding other species, fish meat has lower indices values (AI = 0.58 - 0.68, TI = 0.44 - 0.53) compared to meat from warm-blooded animals (chicken: AI = 0.80, TI = 0.70; pork: AI = 0.59, TI = 0.71; beef: AI = 0.91, TI = 1.00). However, differences are dependent on the composition of fatty acids, which reflects feed, climate and the other living conditions (Stanek et al., 2019; reviewed in Bušová et al., 2020). Vitamin E had no effect ($P > 0.05$) on n-6/n-3 ratio. The values of the n-6/n-3 ratio observed in this study are higher than those found for fast-growing chickens (Tavaniello et al., 2020) and medium and slow-growing chickens (Tavaniello et al., 2022a,b), and are distant from the ideal value of 1 and the maximum value of 4 (Wood et al., 2004). A recent study (Liput et al., 2021) reported that n–6/n–3 ratio in our present diet should reflect the composition of human ancestors' diet, in which the ratio of n–6/n–3 PUFAs was about 1:1. In addition, the authors suggested PUFAs' biosynthesis from linoleic acid (LA, 18:2n–6) and α-linolenic acid (ALA, 18:3n–3) is not uniform among populations and is the result of the adaptation to nutrient exposure during Homo sapiens development.

3.3.2 Polycyclic aromatic hydrocarbons in chicken burgers

To date no studies have investigated the role of endogenous tissue levels of vitamin E on the formation of PAHs in poultry meat. The present trial was carried out to study the effect of intramuscular vitamin E injection on the formation of PAH in grilled chicken meat. The intramuscular injection of vitamin E ensures a more standardized administration in the animals (Maiorano et al., 2015). The tested dose of 100 IU was chosen considering our previous trail (Acaye 2018, PhD thesis) carried out on 28d old Cobb 500 chicks which were once intramuscularly injected with 0.25 mL (12.5 IU) (T1) and 0.5 mL (25 IU) (T2) of *dl* α-tocopheryl acetate (50 mg/mL). In the present trial the effect of multiple doses of vitamin E on the final intramuscular content was studied. As regard, the effect of vitamin E

treatment on PAH formation Table 19 showed the concentration of the PAHs compounds that were present in the samples at or above their limits of quantitation. Among 14 PAHs assayed, DhA and IP were not detected.

Table 19. PAHs content (ng/g) in chicken burgers obtained from meat of control chickens (C) and vitamin E injected chickens (T).

PAHs	C	T	p-value
Ac	36.33±3.68	31.74±7.79	0.268
F	8.81±3.49	6.98±2.37	0.361
Pa	59.78±4.34	50.21±7.37	0.037
A	7.2±0.93	6.32±0.95	0.177
FI	5.01±1.80	4.78±1.81	0.846
P	5.00±2.33	4.03±0.81	0.405
BaA	0.72±0.19	0.47±0.07	0.025
Ch	1.14±0.57	0.97±0.57	0.648
BbF	0.61± 0.13	0.54±0.22	0.562
BkF	0.09±0.04	0.07±0.04	0.301
BaP	0.21±0.10	0.17±0.06	0.497
BgP	0.33± 0.07	0.23±0.13	0.164
DhA	n.d	n.d	
IP	n.d	n.d	
Total	125.29±13.14	106.57±20.42	0.123
PAH4	2.68±0.89	2.15±0.82	0.355
PAH8	3.16± 0.92	2.50±0.96	0.304
Light PAHs	112.12±11.28	95.25±17.81	0.111
Heavy PAHs	13.17±2.90	11.31±3.28	0.372

[1] acenaphthene (Ac), fluorene (F), phenanthrene (Pa), anthracene (A), fluoranthene (Fl), pyrene (P), benzo(a)anthracene (BaA), chrysene (Ch), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), indeno(1,2,3-cd)pyrene (IP), benzo(ghi)perylene (BgP), dibenzo(ah)antracene (DhA).

Analysis of individual PAH content showed that light PAH (Pa > Ac > F > A) were the most abundant as compared to heavy ones (Table 19). The values obtained in this study were lower compared to those obtained from the grilled chicken meat with different doses of vitamin E (Table XX). For example, these differences are evident when comparing the values of the control groups of the two studies: Pa: 59.78 vs 70.76 ng/g; Ac: 36.33 vs 54.23 ng/g; F: 8.81 vs 19.66 ng/g; A: 7.20 vs 10.34 ng/g, respectively. The observed differences could be related to the different lipid content, which was higher in breast chicken meat of the previous trial (2.08 % vs 1.49 %), and lipid composition. Among light PAH, only the Pa content was lower ($P = 0.037$) in vitamin E treated chickens compared to control ones (50.21 vs 59.78 ng/g, respectively). The total content of light PAH was numerical reduced in the vitamin E group ($P = 0.111$). Differently, the addition of different doses of vitamin E directly into the chicken patties before grilling determined a significant ($P < 0.0001$) reduction of light PAHs formation at all the concentration used as compared to untreated group (Table 19), the reduction was more marked with the 4.5 ng/g of vitamin E (79.35 ng/g) compared to the control (155.0 ng/g).

The total content of heavy PAHs (FI, P, BaA, Ch, BbF, BkF, BaP, BgP, IP) was similar between groups 13.17 vs 11.31 ng/g, for control and vitamin E treated groups, respectively. On the contrary, in the trial carried out on chicken patties added with different doses of vitamin E before grilling control group showed a higher content of heavy PAH (40.26 ng/g) while a significant reduction ($P \leq 0.0001$) was observed in all burgers added with vitamin E (2 μg/g: 40.2%; 3 μg/g: 34.4%; 4.5 μg/g: 65.5%; 6 μg/g: 64.4%) compared to the control group (Table 19).

Among heavy PAHs only the BaA was reduced ($P = 0.025$) in vitamin E treated group compared to control one (0.47 vs 0.72 ng/g, respectively).

The concentrations of PAH4 were similar ($P = 0.355$) between groups (2.68 ng/g and 2.15 ng/g for control and vitamin E, respectively), values well below the maximum level of 30 ng/g established by the Reg. 835/2011. The qualitative profile trend found (Ch > BaA > BbF > BaP) was similar to the control chicken meat of the trial with different doses of vitamin E (Table 19), even if the values found in the present trial were lower: BaA (0.72 ng/g vs 1.68 ng/g), Ch (1.14 ng/g vs 1.31 ng/g), BbF (0.61 ng/g vs 1.10 ng/g) and BaP (0.21 ng/g vs 0.45 ng/g).

The concentration of BaP, similar between groups, was lower (0.17 – 0.21 ng/g) than the maximum levels of 5 ng/g established from the Reg. 835/2011.

Referring to the results obtained from our previous study on intramuscular injected chickens (Acaye, 2018; PhD thesis), the mean concentrations (μg/kg) of PAHs formed in grilled meat were in the range:

0.30 – 1.55 for BaP, 0.37 – 2.54 BbF 0.80 – 1.19 for BgP, 0.89 – 1.97 for BkF, 0.94 for DhA and 1.18 – 1.57 for IP. In particular, the mean contents of BaP found in breast muscle were 0.3 μg/kg for group injected with 12.5 IU of of *dl* α-tocopheryl acetate and 1.55 μg/kg for the control group, with no detection for group injected with 25 IU of *dl* α-tocopheryl acetate. The mean concentration of BaP was within the tolerable limit set up by EU regulation (5 μg /kg). Even if not significant, an apparent trend of vitamin E treatment towards inhibition of the formation of these carcinogenic compounds appeared to have emerged. In fact, more of the PAHs were detected in the control as opposed to the vitamin E treated groups.

PART 4

CONCLUSIONS

The emerging issue of health risk from exposure to PAHs has drawn the attention of the scientific community to finding effective strategies to inhibit or reduce the formation of these toxicants, such as the use of antioxidants. The results of this research could provide a theoretical basis to better understand the potential inhibitory role of vitamin E on the PAH formation and to gain insight into how the muscular endogenous levels of vitamin E could be a preventive agent for mutagen formation in grilled meat. In the light of this, three different trials were carried out, and the results can be summarized as follows:

Trial 1:

Raw beef samples used presented an endogenous vitamin E content of 1.32 μg/g; total lipid content was 1.36g/100g and the fatty acid profile was characterized by a higher proportion of SFA (52.940%) followed by MUFA (35.04%) and PUFA (12.02%). In relation to the total content of PAHs, no effect was observed in hamburgers added with 2 μg/g of vitamin E, while a significant reduction was found in those added with 3 and 4.5 μg/g compared to control samples. A similar trend was observed for heavy and light PAHs: vitamin E at the lowest concentration did not reduce their formation, while their content decreased with the higher vitamin E levels. Despite low values of PAH4 were observed in control samples, far from the maximum established by the European Union, the addition of vitamin E further and significantly reduced PAH4 in all experimental groups, especially in those added with 3 and 4.5 μg/g. Although, the cooking did not generate in the hamburgers high PAHs amount, the effectiveness of vitamin E in inhibiting PAHs formation in grilled beef hamburger depends on the concentration used. Concerning the BaP concentration in control samples was 0.06 ng/g, far away from the maximum level established by the European Union. It was possible to detect it only in beef burgers added with 2 μg/g and 6 μg/g of vitamin E, while in other groups was below the LOQ (< 0.04 ng/g).

Considering that several are the factors which can influence the formation of PAHs, such as the total lipid and fatty acid composition of meat, the same study was carried out on chicken meat, and the results are showed in the following trial.

Trial 2:

Raw chicken meat samples used presented an endogenous vitamin E content of 5.54 μg/g; total lipid content was 2.08 g/100g and the fatty acid profile was characterized by a higher proportion of MUFA (42.12%), followed by SFA (36.46%) and PUFA (21.42%).

Regarding the effect of vitamin on PAHs formation in grilled chicken, all vitamin E added groups were characterized by a significant lower total PAHs content as compared to C group. In particular, groups added with 4.5 and 6 μg/g showed the lowest values followed by groups added with 2 and 3 μg/g as compared to control group. The concentrations of PAH4, ranged between 1.47 ng/g to 4.54 ng/g, were higher as compared to beef hamburger, but well below the maximum values set by the current EU regulation. The control group was characterized by the highest PAH4 content (4.54 ng/g), while the addition of vitamin E to chicken patties reduced significantly the total PAH4 amount at all the concentration used, with the lower value for the groups added with 4.5 and 6 μg/g, followed by 2 and 3 μg/g vitamin E added groups. Concerning the BaP, concentration in control samples was 0.45 ng/g, far from the maximum level (5.0 ng/g) established by the European Union. In all burgers added with vitamin E was observed a highly significant reduction of BaP content.

In light of the obtained results in trials 1 and 2, a future aim is to replicate the experiment considering different muscles characterized by a different average lipid content and fatty acid composition (e.g. *Longissimus* vs *Semimembranosus* for beef; *Pectoralis major* vs *Biceps femoris* for chicken).

Trial 3: Animal model

Chicken breast samples from animals that received intramuscular injections of vitamin E into the left pectoral muscle, and chicken breast samples from animals that received intramuscular injections of physiological saline solution were used for this study.

As expected, meat from vitamin E treated group had higher content of tocopherol (5-fold) compared with the control one (2.93 μg/g). Total lipid content was similar between groups. Regarding the fatty acid profile, comparison of the experimental groups revealed no differences in MUFA and PUFA content; while a lower SFA content was found in treated chickens compared to control group

In grilled chicken, the concentrations of PAH4 were similar between groups (2.68 ng/g and 2.15 ng/g for control and vitamin E, respectively), values well below the maximum level of 30 ng/g established by the EU regulation. As well as, the concentration of BaP was similar between groups and lower (0.17 – 0.21 ng/g) than the maximum levels of 5 ng/g established by the EU regulation. Considering these preliminary results, carried out on a small number of samples, we can hypothesize that high intramuscular levels of vitamin E such as those found in treated chickens (14.59 μg/g) are not effective

for reducing the formation of PAHs in grilled meat, this is most likely due to the mechanism of action of this antioxidant which, having reached a maximum of activity, higher concentrations are no longer effective or even pro-oxidants. In light of this, further research is needed with a higher number of samples and with a wider range of doses of vitamin E and a different timeline to better understand the pharmacokinetics of vitamin E in relation to its role on formation PAHs in grilled meat.

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