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Effect of Strawberry Antioxidants against Oxidative and Inflammatory Stress

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A chi mi ha sempre sostenuto,
supportato e …sopportato. Grazie!

1. ABSTRACT

The first aim of this PhD project was to assess and compare the nutritional and phytochemical quality of strawberry fruit extracts of different cultivars and/or varieties, obtained through specific breeding programs: the main purpose of such approach was to evaluate the influence of genetic background on these parameters. The total antioxidant capacity, the radical scavenging activity, the content of total phenolics, flavonoids, anthocyanins, vitamin C and folates were measured in the different strawberry extracts. Among the varieties studied, the cultivar Alba was chosen for its nutritional value and was used in the second part of the Thesis.

The second aim of this project was to evaluate the effects of methanolic purified extracts from Alba cultivar on inflammatory status induced by *E.Coli* lipopolysaccharide (LPS) on two different cell lines, Human Dermal Fibroblasts (HDF) and RAW 264.7 macrophages. The cell viability, apoptosis rate and Reactive Oxygen Species (ROS) intracellular production were assessed. The protective role of strawberry extracts was estimated by the evaluation of the principal biomarkers related to inflammatory and oxidative stress and the activity of the principal antioxidant enzymes. Moreover, protein expression was evaluated to analyze and clarify the principal molecular pathways involved in strawberry and LPS mechanisms of action. Finally, the oxygen consumption rate related to mitochondria functionality was evaluated.

The results obtained demonstrated that strawberry extracts had an anti-inflammatory effect on LPS-treated cells, through a reduction of ROS and inflammatory and oxidative damages. Strawberry extracts also counteracted the inflammatory response increasing the antioxidant activities, through AMPK-related pathways. An improvement of mitochondria functionality was also demonstrated.

The results obtained with this work highlight and confirm the potential health benefit of strawberry against inflammatory and oxidative stress.

2. ITALIAN SUMMARY

Il primo obiettivo dello studio è stato quello di valutare e confrontare la qualità fitochimica e nutrizionale di diversi estratti di fragola, prodotti da varietà commerciali ottenue da specifici programmi di incrocio genetico. Nei vari estratti sono state misurate la capacità antiossidante totale, l'attività antiradicalica e il contenuto di polifenoli, flavonoidi, antociani, vitamina C e folati. Sulla base dei risutlati ottenuti la cultivar Alba è stata scelta per condurre le analisi nella seconda parte del progetto.

Il secondo obiettivo è stato quello di valutare gli effetti di estratti metanolici della cultivar Alba sull'infiammazione indotta dal LPS di *E.Coli* in cellule di fibroblasti di derma umano e macrofagi RAW 264.7. Dopo analisi preliminari di vitalità, apoptosi e produzione di ROS intracellulari, il ruolo protettivo degli estratti è stato stimato attraverso la valutazione dei principali biomarcatori collegati allo stress infiammatorio e ossidativo e in relazione all'attività dei principali enzimi antiossidanti. Analisi di espressione proteica sono state effettuate per identificare le principali vie molecolari coinvolte nell'azione degli estratti di fragola e del LPS E' stato inoltre misurato il consumo di ossigeno correlato alla funzionalità mitocondriale.

I risultati hanno evidenziato come gli estratti di fragola esercitino un effetto anti-infiammatorio sulle cellule trattate con LPS, riducendo la produzione di ROS e abbassando i livelli dei biomarcatori infiammatori e ossidativi. La risposta infiammatoria è stata altresì contrastata rafforzando l'attività antiossidante e regolando le vie molecolari collegate all'AMPK, registrando inoltre un miglioramento della funzionalità mitocondriale.

I risultati ottenuti sottolineano e confermano il potenziale beneficio per la salute di un consumo di fragola, in particolare nei confronti delle alterazioni indotte da stress infiammatori.

3. INTRODUCTION

3.1. General overview

A growing number of epidemiological studies suggest a strong association between a diet rich in fruits and vegetables and a lower incidence of different chronic pathologies, such as obesity (Kovacs et al., 2014; Charlton et al., 2014), infections (Siegel et al., 2010; Crowe et al., 2014), inflammation (Anderson et al., 2012; Urpi-Sarda et al., 2012; Lamprecht et al., 2013; Macready et al., 2014), cardiovascular (Threapleton et al., 2013; Tanaka et al., 2013; Wallace et al., 2013; Eilat-Adar et al., 2013; Wang et al., 2014) and neurodegenerative diseases (Elwood et al., 2013; Marder et al., 2013) and cancer (Grosso et al., 2013; Park et al., 2013a; Leenders et al., 2013; Kruk, 2014; Deschasaux et al., 2014). Taking into consideration the remarkable socio-economic and public health impact of these chronic pathologies, the comprehension of the molecular and biochemical mechanisms that underlie the beneficial effects of a plant-based diet has encouraged different basic, clinical and epidemiological research (Tulipani et al., 2009a). Focusing on fruits, it is quite complex to explain their potential health benefits, given the wide variety of fruits available for consumption and their complex composition. For these reasons, in recent decades, individual subgroups of fruits have been taken into account, to facilitate the observation and promote their specific health benefits. Nowadays, the synergistic and cumulative role in health promotion of the fibers, micronutrients and phytochemicals compounds present in fruit is an established evidence (Giampieri et al., 2012a). A deep sifting of the plant compounds potentially expressing biological activities has been performed, with particular attention to the phytochemical compounds, which are not designated as traditional nutrients. Despite this, the complete identification of the protective substances present in plants and the mechanism by which they can protect against disease still remain a great challenge, that requires a planned and strict collaboration among experts in plant food, nutrition and medical research. In particular, a full understanding of the etiologic pathways related to the different chronic disease could help in the characterization of the health-promoting compounds present in dietary plants.

3.2. Oxidative stress in health and disease

A common denominator in the pathogenesis of most chronic diseases is the involvement of oxidative stress, connected to the production by all aerobic organisms of reactive oxygen species (ROS) and reactive nitrogen species (RNS) including free radicals. These substances are implicated in human physiological and phatological conditions, ranging from rheumatoid arthritis and haemorrhagic shock through cardiomyopathy and cystic fibrosis to gastrointestinal ischaemia, cancer and also aging. Oxidative stress was originally defined by Sies (Sies, 1985) as an extreme

situation in terms of the organism oxidation, which occurs with a change in the balance between the generation of oxidants and control by antioxidants. In fact it could result from (i) a reduction in antioxidant defense; (ii) an increase in the production of ROS and RNS or even (iii) a combination of both events. The excessive production of reactive species, no longer adequately controlled by antioxidant defense systems, can lead to a wide range of effects: adaptation by up-regulating the natural defence system, which may completely or in part protect beside damage, tissue injury to different molecular targets and cell death by activating apoptosis and necrosis processes.

At the cellular level the main targets of the attack of ROS are lipids, proteins and nucleic DNA. All cellular membranes are especially vulnerable to oxidation due to their high concentrations of polyunsaturated fatty acids (PUFAs). The damage to lipids, usually called lipid peroxidation, occurs in 3 stages. During the first stage, called initiation, it occurs the attack of a reactive oxygen metabolite, capable of abstracting a hydrogen atom from a methylene group in the lipid. The presence of a double bond near to the methylene group weakens the bond between the hydrogen and carbon atoms, so that it can be easily removed from the molecule. Following hydrogen abstraction, when oxygen is in sufficient concentration in the surroundings, the remaining fatty acid radical reacts with it to form ROO•: this is the second step, called the propagation stage. These radicals themselves are capable of abstracting another hydrogen atom from a close fatty acid molecule, which leads again to the production of fatty acid radicals that undergo the same reactions and interaction with oxygen. The ROO• becomes a lipid hydroperoxide that can further decompose into several reactive species including lipid alkoxyl radicals (LO•), aldehydes (e.g., malonyldialdehyde, 4-hydroxy-2-nonenals), alkanes, lipid epoxides, and alcohols. The propagation stage allows the reaction to continue. The last stage, or chain termination, arises following interaction of one ROO• with another radical or antioxidants (Davies, 2000; Kohen and Nyska, 2002; Zhong and Yin, 2015).

Proteins, in particular their SH-groups, can serve as possible targets for attack by ROS, especially OH•, RO•, and nitrogen-reactive radicals. Proteins can undergo direct and indirect damage following interaction with ROS, as well as peroxidation, damage to specific aminoacid residues, changes in their tertiary structure, degradation, and fragmentation. These consequences are considered as a response mechanism to stress and can lead to interference with the creation of membrane potentials, loss of enzymatic activity, altered cellular functions such as energy production and changes in the type and level of cellular proteins. The principal products obtained by protein oxidation are usually aldehydes, keto compounds, and carbonyls (Kohen and Nyska, 2002).

Finally, ROS can interact also with DNA causing several types of damage, such as modification of DNA bases, loss of purines, single- and double-DNA breaks, DNA-protein cross-linkage and damage to the deoxyribose sugar and to the DNA repair system. The main responsible of DNA

damage is the hydroxyl radicals, that can determine the formation of a wide variety of oxidation product as 8-hydroxydeoxyguanosine, if the guanine at its C-8 position is attacked, or 8 (or 4-, 5-)-hydroxyadenine, if the hydroxyl radicals attack other bases like adenine, in different position. Other less reactive ROS (i.e., O2•- or H_2O_2), that can direct interact with DNA, are not dangerous at their physiological concentrations, but could serve as sources for other reactive intermediates that can easily attack and cause damage (Kohen and Nyska, 2002)

However, today we know that the damage to various cell components is not the only important effect of these reactive molecules. ROS production, at moderate levels, can be useful to regulate several signaling pathways under both physiological and pathological conditions, affecting different cellular processes, such as proliferation, metabolism, differentiation, and survival, antioxidant and anti-inflammatory response, iron homeostasis and DNA damage response (Trachootham et al., 2008, Ray et al., 2012) (Figure 1).

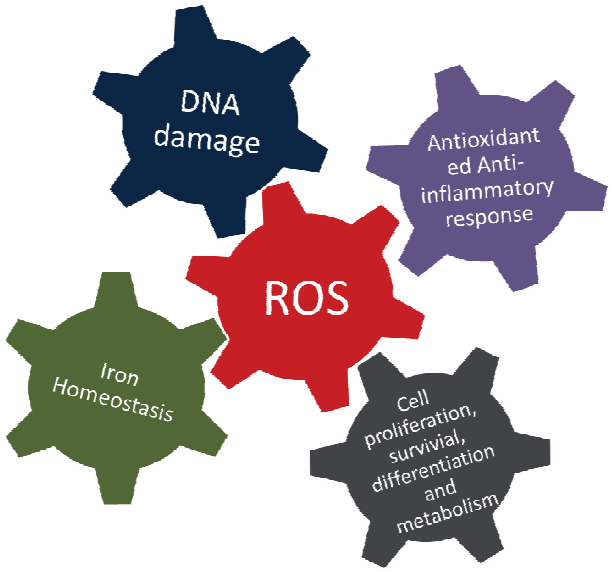


Figure 1. Cellular signalling pathway regulated by ROS (adapted from Ray et al., 2012).

In other words, the ROS generation, maintained within certain limits, seems to be essential to regulate homeostasis. For these reasons, the importance of a balanced equilibrium between oxidant production and antioxidant defences is essential for preserving health and longevity: in this context dietary antioxidants from fruit may fill an important beneficial role, and clinical and epidemiological data from literature seem to corroborate this hypothesis (Hoelzl et al., 2005; Giacosa et al., 2012; Iqubal et al., 2014; Sadowska-Bartosz and Bartosz, 2014; McCarty et al., 2015).

3.3. Oxidative stress and inflammation

A sustained pro-inflammatory state is a major contributing factor to the development, progression, and complication of the most known chronic diseases such as cardiovascular disease, Alzheimer's, and type 2 diabetes. In normal conditions, inflammation is the common, protective, and temporary response of the innate immune system to pathogens and injury stimuli (Joseph et al., 2014).

On the contrary, the interaction of the cellular immune system with endogenous or exogenous antigens results in the generation of ROS and RNS, leading to signaling cascades that can result in hyperactivation of inflammatory responses inducing tissue damage and oxidative stress phenomena (Khansari et al., 2009; Schieber and Chandel, 2014).

Quantifiable inflammatory responses are characterized by the production of cytokines, which are small soluble proteins secreted by different cells that influence the behavior of other cells involved in immunity and inflammation; they also play an important role as hormonal mediators for host defense, growth and repair processes within injured tissues (Liu and Lin, 2012; Dia et al., 2014). Cytokines act as signals between immune cells to coordinate the inflammatory response, and they can take a pro- or anti- inflammatory role (Joseph et al., 2014): interleukin 1β (IL-1 β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) are commonly induced together and act as pro-inflammatory and alarm cytokines. IL-1 β is produced by activated macrophages, while IL-6 is produced by both immune and non-immune cells in response to infection and injury (Dia et al., 2014). Moreover, TNF-α, a pleiotropic cytokine produced by many cell types, including macrophages, monocytes, smooth muscle cells, lymphoid cells and fibroblasts, when released in response to sepsis, can cause shock, disseminated intravascular coagulation and multiorgan failure (Funakoshi-Tago et al., 2011; Dia et al., 2014).

In contrast, interlukin 10 (IL-10) is recognized as an anti-inflammatory cytokine produced by T helper type 2 lymphocytes, T regulatory cells, macrophages and some B cells which inhibit the synthesis of other cytokines and macrophage functions during the late inflammation phase (Liu and Lin, 2012).

The central orchestrator of the inflammatory response is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a redox-sensitive transcription factor (Joseph et al., 2014). In unstimulated cells, NF-κB remains inactive in the cytoplasm by the association with inhibitor proteins of the Inhibitor of κB (IκB) family (IκBα and IκBβ). In inflammatory conditions IkB kinase is activated by pro-inflammatory stimuli and phosphorylates IκBs, leading to their ubiquitination and proteasomal degradation (Funakoshi-Tago et al., 2011). These events release free NF-κB dimers in the cytosol, allowing them to translocate into the nucleus where they stimulate the expression of a wide number of genes including those responsible for the production of cytokines

and other inflammatory-molecules, as chemokines, cell adhesion molecules, soluble intercellular adhesion molecule-1 and acute phase proteins (Funakoshi-Tago et al., 2011; Joseph et al., 2014). (Figure 2)

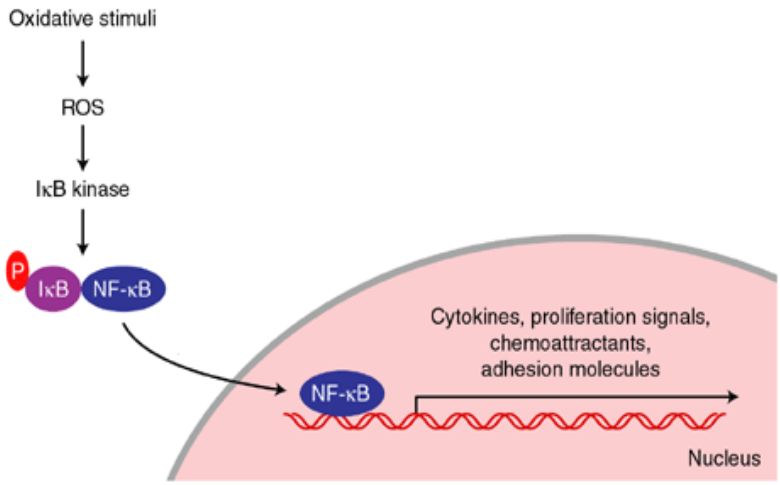


Figure 2. Simplified scheme of oxidant regulation of activation of the transcription factor NF-κB. (taken from Cassidy et al., 2003).

Inflammation can be triggered by different stimuli such as endotoxins (i.e., lipopolysaccharide from bacteria), viruses, and changes in levels of ROS, cellular redox status, fatty acids, cytokines, growth factors, and carcinogens (Giampieri et al., 2015a).

In addition to these classic inflammatory stimuli, inflammatory stress can also result from excess body fat and poor diet. Excess body fat and obesity are associated with a concomitant and persistent increase in low-grade inflammation (Giampieri et al., 2015a). In obesity, morphological changes in adipocytes result in altered secretory responses favoring an inflammatory state: elevated circulating inflammatory proteins (TNF-α and IL-6) are in fact observed in this condition and this may explain the critical link between obesity and the development of insulin resistance, type 2 diabetes, and cardiovascular disease (CVD) (Joseph et al., 2014).

Energy intake excess and poor dietary composition typical of the Western diet model may also promote acute (postprandial) and cumulative sustained inflammatory responses in both obese and normal weight individuals. The ROS overproduction, stimulated by an excess of energy intake, results in metabolic oxidative stress and cellular redox imbalance that activate redox-sensitive signaling molecules and ultimately leadto an increased expression of inflammatory genes and oxidative stress markers (Joseph et al., 2014) (Figure 3).

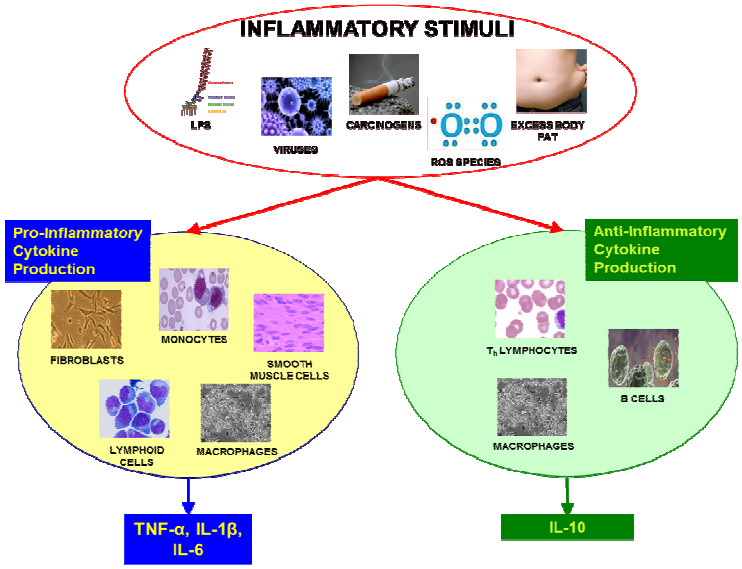


Figure 3. Cytokine production by different stimuli (adapted from Forbes-Hernández et al., 2015).

Therefore, during inflammation, cells involved in the inflammatory process are recruited to the damaged site, take up oxygen and release ROS. In addition, inflammatory cells secrete cytokine, which help to further recruit inflammatory cells, generating yet more ROS. Consequently, transcription factors such as NF-κB that encode pro-inflammatory genes, are activated, leading to an increased secretion of cytokines. This vicious cycle supports a protracted environment of oxidative and inflammatory stress, which contributes to several chronic diseases (Joseph et al., 2014) (Figure 4).

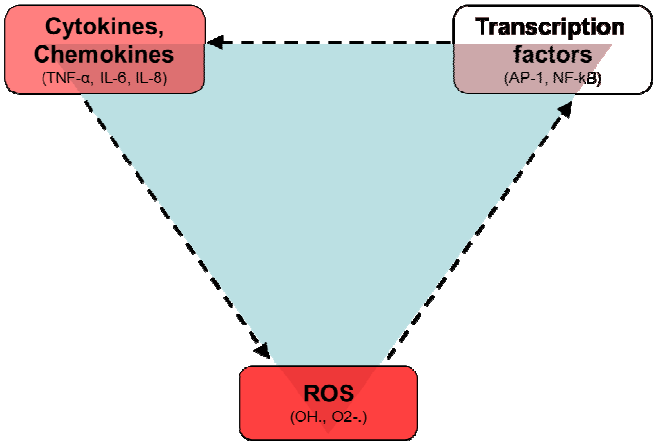


Figure 4. Schematic representation of the vicious cycle that supports a protracted environment of oxidative and inflammatory stress (taken from Forbes-Hernández et al., 2015).

Therefore, the control of excessive inflammation, through the modulation of pro- and anti-inflammatory cytokine expression in immune cells by potential food components, may represent a strategic tool to avoid immune disorder diseases and maintain health and wellness (Giampieri et al., 2015a).

3.4. ROS and RNS

Free radicals represent reactive chemical species capable of independent existence (Gutteridge, 1994), that possess an unpaired electron in the external orbit (Riley, 1994; Poljsak et al., 2013). An unpaired electron is one that occupies an atomic or molecular orbital by itself; its presence usually causes free radicals to be attracted slightly to a magnetic field and makes them highly reactive, although the chemical reactivity of radicals varies over a wide spectrum.

There are many free radicals in chemistry and biology. ROS is a collective term used to represent both free radical and non-free radical oxygenated molecules such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), singlet oxygen (1/2 O_2), and the hydroxyl radical ($HO^{\bullet}$) (Table 1).

Oxygen radicals	Non-radical oxygen derivatives
Oxygen (bi-radical) ($O_2^{\bullet\bullet}$)	Hydrogen peroxide (H_2O_2)
Superoxide ion ($O_2^{\bullet-}$)	Hypochlorous acid (HOCl)
Hydroxyl ($HO^{\bullet}$)	Ozone (O_3)
Peroxyl ($RO_2^{\bullet}$)	Aldehydes (HCOR)
Alkoxyl ($RO^{\bullet}$)	Singlet oxygen ($^1O_2$)
Nitric oxide (NO)	Peroxynitrite ($ONOO^-$)

Table 1. Radical and non-radical oxygen species (adapted from Kohen and Nyska, 2002).

In the aerobic process, molecular oxygen is reduced to a series of intermediate reactive species through the mechanism indicated (Gutteridge, 1994):

$$O_2 + e^- + H^+ \longrightarrow HO_2^{\cdot}$$

$$HO_2^{\cdot} \longrightarrow H^+ + O_2^{\cdot -}$$

$$O_2^{\cdot -} + 2\,H^+ + e^- \longrightarrow H_2O_2$$

$$H_2O_2 + e^- \longrightarrow HO^- + HO^{\cdot}$$

$$HO^{\cdot} + H^+ + e^- \longrightarrow H_2O$$

The superoxide radical anion is considered the precursor of all radicals because, in most cases, is the first radical that is produced by cellular oxidase (Ardanaz and Pagano, 2006). It results from a single electron reduction of oxygen by various oxidases, such as dihydro nicotinamide adenine dinucleotide phosphate oxidase, xanthine oxidase, cyclooxygenase, and can act both as reducing agent, yielding in turn an electron to an oxygen molecule, and as an oxidizing agent, with the formation of hydrogen peroxide. This reaction is known as dismutation, which can occur spontaneously but extremely slowly (2×10^5 m/sec) or it can be catalyzed by the superoxide dismutase enzymes, able to accelerate the process up to 10^4 times.

$$2\,O_2^{\cdot -} + 2H^+ \longrightarrow O_2 + H_2O_2$$

The superoxide anion is able to oxidize many biologically important compounds, such as catecholamines, polyphenols, leucoflavine, and to reduce cytochrome c, tetranitromethane and nitroblue tetrazolium (Fridovich, 1986).

Superoxide radical anions may also be formed in the mitochondrial electron transport chain, during the oxidative phosphorylation process (Dröse and Brandt, 2012; Evans et al., 2003).

H_2O_2, because of its small size and the lack of charge, is much more stable and has a higher diffusion when compared to superoxide anion. H_2O_2 is able to produce highly reactive radicals as a result of its interaction with metal ions (Gutteridge, 1994). Direct action of H_2O_2 involves the attack on heme proteins structure with release of iron, enzyme inactivation and oxidation of DNA, lipids, -SH groups, and keto-acids (Kohen and Nyska, 2002). H_2O_2 can be depleted by catalase, through its conversion into water (Gandhi and Abramov, 2012; Droge, 2002).

It is considered toxic for its ability to convert into hydroxyl radical, considered the most reactive and damaging between the oxygen radicals, through exposure to ultraviolet light or through interaction with metal ions (Fenton reaction).

The superoxide anion can cooperate with the hydrogen peroxide in the formation of the hydroxyl radical. This reaction, known as the Haber-Weiss one, takes place both *in vitro* and *in vivo* (Liochev andFridovich, 1994).

HO· is a highly aggressive radical species, responsible for the oxidative damage of the most biological macromolecules. HO· has been reported as the most powerful oxidizing radical: it is characterized by high reactivity and short life-span that allows it to interact indiscriminately at the site of its generation with most organic and inorganic molecules, such as DNA, proteins, lipids, amino acids, sugars, and metals (Kohen and Nyska,2002; Halliwell and Gutteridge, 1999).

Finally, molecular oxygen is not a free radical, but it is considered an oxygenated species endowed with high reactivity (Gutteridge, 1994).

Alongside ROS, an important biological relevance also have RNS. In particular, nitric oxide (NO•), a colourless gas which can diffuse readily between and within cells. NO•, which is toxic in moderate concentrations, possesses different physiological roles: (i) it is an important vasodilator, inhibiting platelet adhesion and aggregation; (ii) it is produced in the brain as a neurotransmitter, (iii) it is involved in immune responses; (iv) it shows a protective effect during the process of ischemia/reperfusion (Wink et al., 1993; Ignarro, 2000). The NO• radical is synthesized by the oxidation of L-arginine by a family of synthases (NOS) of which there are three major isoforms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) (Stuehr, 1999). It is considered toxic because it is able to react with $O_2^{\bullet -}$, giving a highly reactive, damaging nitrogen species, namely peroxynitrite, a powerful oxidant versus many biological molecules: it determines depletion of sulfhydryl groups and oxidative damage on most biomolecules, acting similarly to hydroxyl radical, and it is also responsible for DNA damage, protein oxidation and nitration of aromatic amino acid in protein structure (e.g. 3- nitrosotyrosine) (Kohen and Nyska, 2002).

Peroxynitrite can be decomposed to hydroxyl radicals in presence, or not, of transition metals (Gutteridge, 1994; Hou et al., 1999; Stryer, 1995; Green et al., 1990).

$$ONOO^- + H^+ \longrightarrow HO^{\cdot} + {}^{\cdot}NO_2$$

Other important biochemical reactions involving nitric oxide are: (i) S-nitrosation of thiols and (ii) nitrosylation of transition metal ions. Moreover, the hemoglobin structure may be directly or indirectly altered by NO, respectively through the attachment to heme in the nitrosylation reaction, or by S-nitrosation of the thiol moieties, yielding S-nitrosothiols (van Faassen and Vanin, 2005).

ROS can be generated by endogenous or exogenous sources (Figure 5).

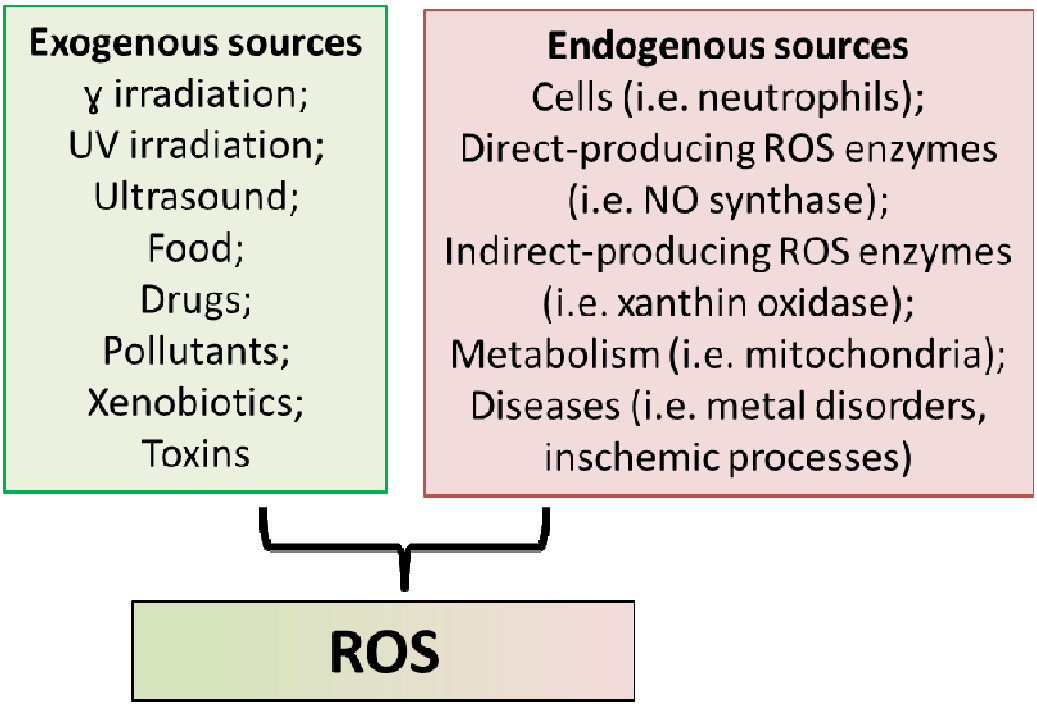


Figure 5. Exogenous and endogenous sources of ROS (adapted from Kohen and Nyska, 2002).

The endogenous production of ROS follows different metabolic pathways (Sauer et al., 2001); the most important site is represented by the electron transport chain (ETC) in the mitochondria (Beal, 2005). Indeed, in animals, about 85% of inspired oxygen is used by mitochondria, the major site of adenosine triphosphate (ATP) production in animals, in non-photosynthetic plant tissues and in leaves in the dark. Mitochondrial ETC consists of a series of redox reactions in which electrons are transferred from donors to acceptor molecules, resulting in a trans-membrane proton translocation which drives the formation of an electrochemical gradient (Forbes-Hernández et al., 2014).

ETC comprises the electron transport complexes I (NADH dehydrogenase), III (cytochrome bc_1 complex) and IV (cytochrome c oxidase) that constitute its actual energy–conserving centers and complex II (succinate dehydrogenase), glycerol phosphate dehydrogenase, ubiquinone and cytochrome c (cyt c) which, although incapable of pumping protons across the inner mitochondrial membrane per sé, are essential ETC constituents having critical roles in ETC function and efficiency (Forbes-Hernández et al., 2014). In mitochondrial ETC, electrons released (i) by the oxidation of NADH and (ii) by the oxidation of $FADH_2$ are transferred to the different complexes of the chain, having oxygen as the final acceptor, which is reduced to water (Pieczenik and Neustadt, 2007). The energy released by electrons while they pass through the ETC is used to pump protons from the mitochondrial matrix into the intermembrane space, creating an electrochemical proton

gradient across the inner mitochondrial membrane called mitochondrial membrane potential, that is essential to mitochondrial bioenergetics (Brand and Nicholls, 2011).

This electrochemical proton gradient permits ATP synthase, the final complex of the chain, to use the flow of H^+ through the enzyme back into the matrix to generate ATP from adenosine diphosphate (ADP) and inorganic phosphate. The complete process is the oxidative phosphorylation, since ADP is phosphorylated to ATP using the energy of hydrogen oxidation in many steps. But in this process a small percentage of electron do not complete the whole series of reactions and directly react with oxygen, resulting in the formation of several ROS as secondary ETC products (Frantz and Wipf, 2010). During normal oxidative phosphorylation 0.4–4.0% of all oxygen consumed in mitochondria is transformed into the $O_2^{\cdot -}$ which is transformed to H_2O_2 by the detoxification enzymes copper/zinc superoxide dismutase or manganese superoxide dismutase (MnSOD) and then to water by glutathione peroxidase (GPx) or peroxidation enzymes (PRx). When these enzymes cannot transform ROS fast enough, oxidative damage occurs and accumulates in mitochondria (Forbes-Hernández et al., 2014) (Figure 6).

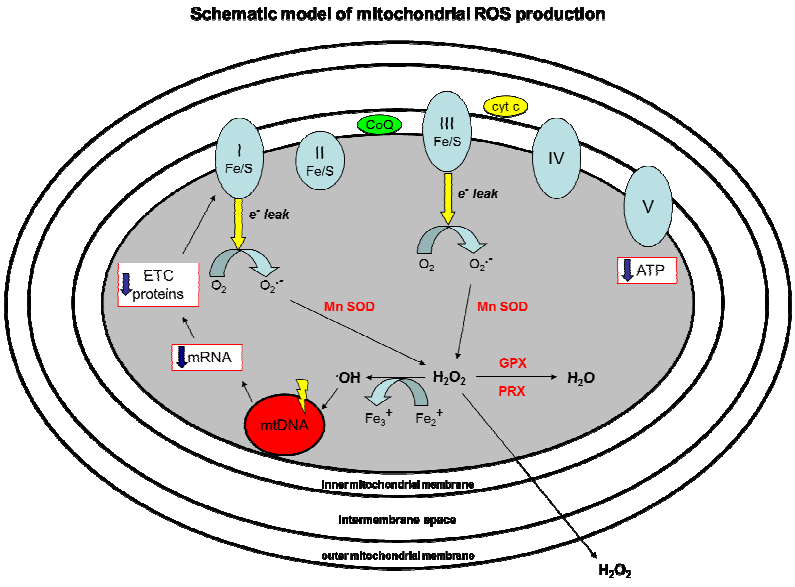


Figure 6. Schematic model of mitochondrial oxidative damage. During mitochondrial respiration a small amount of the molecular oxygen consumed by cells is converted into $O_2^{\cdot -}$ by reactions occuring in complex I and III. MnSOD enzyme converts $O_2^{\cdot}$ to H_2O_2 which can be converted into H_2O by GPx or PRx. H_2O_2 can also react with Fe_2^+ to produce $HO^{\cdot}$. This radical could attack mtDNA, decreasing mRNA and altering the expression of proteins essential for ETC (taken from Forbes-Hernández et al., 2014).

Additionally, NO is produced inside the mitochondria by mitochondrial nitric oxide synthase and also diffuses into the mitochondria from the cytosol (Forbes-Hernández et al., 2014).

Most of the 10-15% of O_2 taken up by aerobic eukaryotes that is not consumed by mitochondria is used by various oxidase and oxygenase enzymes, of which one of the most important is cytochrome P450, contained in the endoplasmatic reticulum of many animal and plant tissues.

The P450 superfamily has been encoded by over 150 genes; these enzymes are involved in the oxidation of a wide range of substrates at the expense of O_2. Substrates for cytochromes P450 include insecticides and pesticides, hydrocarbons, and drugs. For these reasons, liver endoplasmatic reticulum is especially rich in P450s. However the product of reaction with P450 is less toxic than the substrates.

Another important multienzymatic complex involved in the generation of ROS, especially during the transduction of intracellular signals, is NADPH oxidase, present in particular in phagocytic cells. This complex contains FAD and a cytochrome b, in form of a dimer, capable of reducing O_2 to $O_2^{\bullet-}$. Usually, up to 30% of neutrophil cytochrome is present in the plasma membrane and the rest in the cytoplasm, especially in specific granules, which can supply more b-type cytochrome to the plasma membrane. When the system is activated, the cytoplasmic constituents are phosphorylated and translocate on the membrane where they bind to the cytochrome b, allowing the electron transfer to molecular oxygen, thus producing superoxide. The activation of NADPH oxidase also requires the participation of two proteins of low molecular weight, Rac2, forming part of a cytoplasmic dimer with Rho-GDI, and Rap1A, a member of the Ras family located at the level of the cytoplasmic membrane in intimate contact with the cytochrome (Babior, 1999; Li et al., 2001). This complex is well characterized in phagocytic cells, where it is essential the bactericidal function performed by ROS ($O_2^{\bullet-}$, H_2O_2 and HOCl) produced by respiratory reactions, which represent a defense mechanism against invading microorganisms. Finally, in the case of ischaemia, the main source of ROS is the xanthine oxidase, which chairs the metabolism of purines. In healthy animal tissues, this enzyme system is present in endothelial cells of small vessels and in the epithelial cells of the breast tissue. Generally it exists in the form of xanthine dehydrogenase that transfers electrons not to O_2 but to NAD^+, as it oxidizes xanthine or hypoxanthine into uric acid. However, in certain conditions, as ischaemia, some of the xanthine dehydrogenase can be converted into xanthine oxidase by oxidation of essential –SH groups or by limited proteolysis. Xanthine oxidase produces $O_2^{\bullet-}$ and H_2O_2 when xanthine or hypoxanthine are oxidized.

In addition, ROS can be produced by a host of exogenous processes (Valko et al., 2006). Environmental sources including: x rays and γ rays, ultraviolet light (which produce free radicals when transfer their energy to cellular components or molecules of water (Giampieri et al., 2012b),

ionizing radiation, and pollutants such as paraquat and ozone. Even pesticides, tobacco, solvents, anesthetics, and in general all the aromatic hydrocarbons may be responsible for oxidative stress (Pagano, 2002). All of these sources of free radicals, both enzymatic and non-enzymatic, have the potential to cause oxidative damage on a wide range of biological macromolecules (Mateos and Bravo, 2007) (Figure 7).

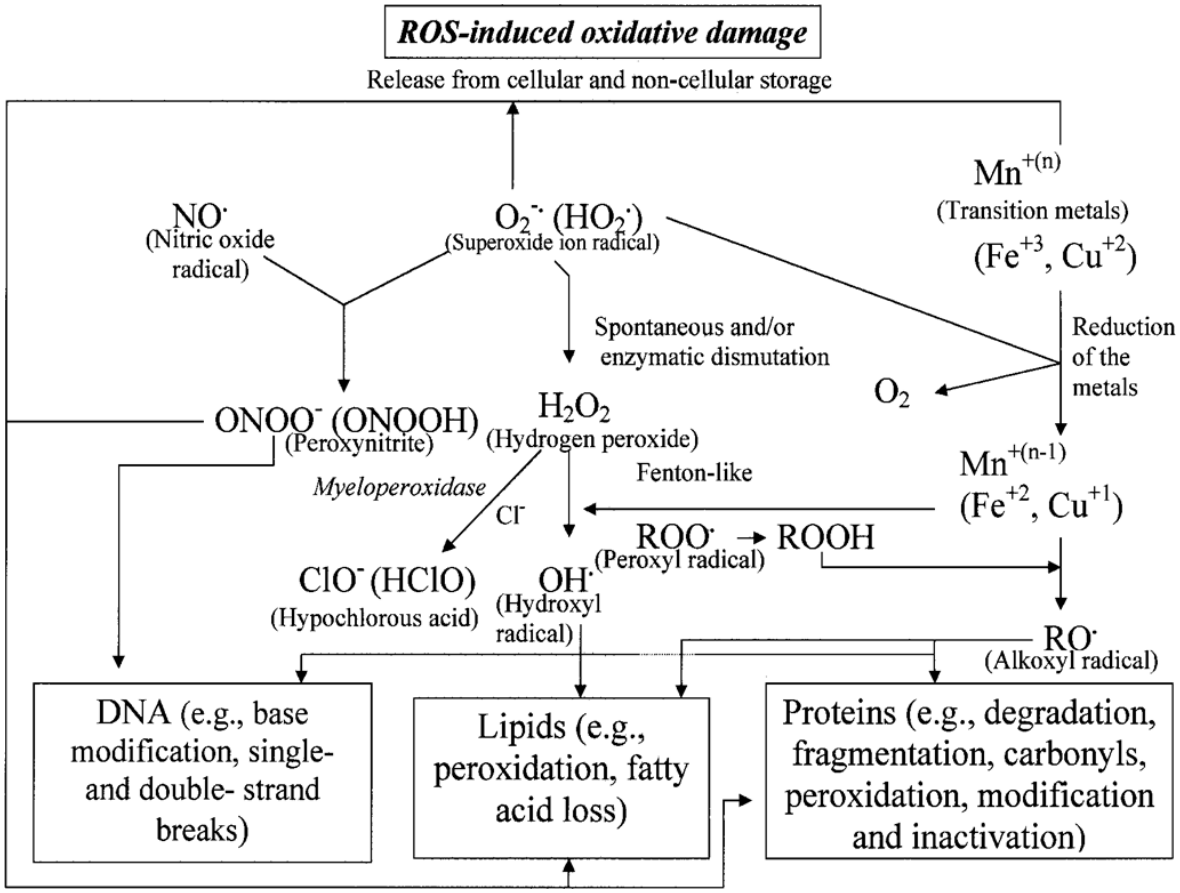


Figure 7. ROS damage in biological macromolecules (taken from Kohen and Nyska, 2002).

3.5. Antioxidant defences

In all aerobic organisms the production of ROS and RNS is inevitable and, within certain concentrations, essential (Feher, 1985). Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Cadenas, 1997).

Halliwell and Gutteridge proposed, in 1989, a comprehensive definition of an antioxidant as "any substance that, when present at concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell and Gutteridge, 1989).

Afterwards, the antioxidants were defined by a more general perspective as "all those compounds that protect biological systems against the harmful effects of excessive generation of oxidants," and this definition is currently the most widely used (Krinsky, 1992).

Antioxidant functions lead to a decrease of oxidative stress, DNA mutations, malignant transformations, as well as other parameters of cell damage. Some epidemiological studies highlight the antioxidant ability to contain the effects of ROS activity, reducing the incidence of cancer and other degenerative diseases (Godic et al., 2014).

There is no an universal and best antioxidant: the defenses against oxidative stress are not able to completely avoid damage. In fact, in a biological system, the function of an antioxidant depends on various parameters, such as the type of ROS generated, the manner and the place in which is formed, the extent of damage. The first types of antioxidant defense systems developed against oxidative damage are those that prevent ROS occurrence (preventative mechanisms) and those that block, capture radicals that are formed (repair mechansims) (Cheeseman and Slater, 1993).

These systems can be enzymatic (such as superoxide dismutase, catalase, glutathione peroxidase) and nonenzymatic, such as oxidative enzyme (e.g. cyclooxygenase or lipooxygenase) inhibitors, antioxidant enzyme cofactors (Se, coenzyme Q_{10}), ROS/RNS scavengers (vitamin C, vitamin E), and transition metal chelators (e.g. EDTA) (Huang et al., 2005).

Under physiological conditions, the balance between prooxidant and antioxidant compounds moderately favors prooxidants, thus inducing a slight oxidative stress, requiring the intervention of endogenous antioxidant systems of the organism (Dröge, 2002).

Redox homeostasis of the cell is assured by its complex endogenous antioxidant defense system, which includes endogenous antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and non-enzymatic compounds like glutathione, proteins (ferritin, transferrin, ceruloplasmin, and even albumin) and low molecular weight scavengers, like uric acid, coenzyme Q, and lipoic acid (Poljsak et al., 2013).

On the contrary, the main exogenous antioxidants sources are vitamin C and E, carotenoids, and phenolics. Clinical studies proved that, together with omega-3 fatty acids, these antioxidants present in fruits, vegetables, whole grains, legumes, could work as preventative agents regarding disease occurrence, counterpartying the activity of the above-mentioned endogenous antioxidative defense (Willett, 2006).

Another source of exogenous antioxidants is constituted by dietary supplements, as vitamins, minerals, fibres, fatty acids or amino acids, which are either lacking or not found in sufficient amounts in the common diet (Poljsak et al., 2013).

The use of exogenous antioxidants may retard the uptake of endogenous antioxidants, for the total "cell antioxidant potential" to remain unaltered (Pisoschi and Pop, 2015). The use of antioxidant supplementation is indeed effective if the initial oxidative stress level is higher than normal or above the individual's set point of regulation (Pisoschi and Pop, 2015).

Thus, the antioxidant supplements may reduce the increased levels of oxidative stress that cannot be inactivated by the endogenous sources (Poljsak et al., 2013).

Another antioxidant classification regards their localization in aqueous (water-soluble) or in membrane cell compartments (fat-soluble) (Pisoschi and Pop, 2015). The "scavengers" are water soluble compounds of low molecular weight, as vitamin C, uric acid, glutathione, localized in various cell districts, that interact directly with ROS, neutralizing them. The fat-soluble molecules, such as vitamin E and β-carotene, instead, are located at the level of membranes and are used for the neutralization of lipid peroxidation. Coenzyme Q_{10} or ubiquinone is an essential component of the inner mitochondrial membrane (Fato et al., 1985; Battino et al., 1990; Lenaz et al., 1992; Rauchova et al., 1992; Samori et al., 1992) and can act neutralizing radical species independently or synergistically with vitamin E. In this sense, it is possible to detect the existence of "networks" of antioxidants, where some antioxidants interact with others to reconstitute their corresponding reduced forms in a coordinated and controlled process, that just now it has been understood in detail (Sen et al., 2000) (Figure 8).

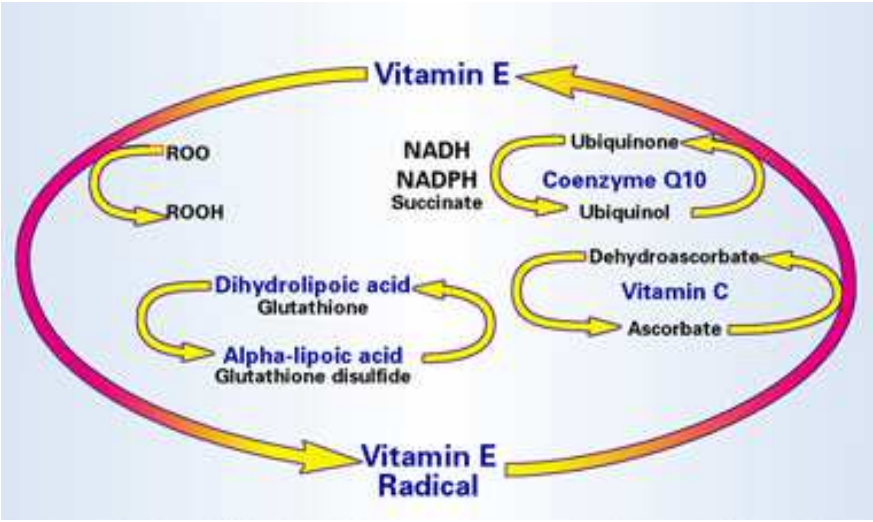


Figure 8. Exemple of antioxidant network. The radical species that are formed in the lipophilic cellular compartment are reduced by Vitamin E, with the formation of the Vitamin E radical. This can be reduced back to Vitamin E by another fat-soluble antioxidant such as ubiquinol, or by a cytoplasmic antioxidant such as ascorbate.

Antioxidants can be also classified according to their mode of action: we can identify primary, secondary and tertiary antioxidants. The primary antioxidants are those that convert ROS into less harmful molecules (before they could react with the vital structures), or prevent their production. In this group there are: superoxide dismutase, glutathione peroxidase, glutathione reductase, peroxidase, catalase, the glutaredoxin, thioredoxin, and proteins such as ferritin and ceruloplasmin (Figure 9).

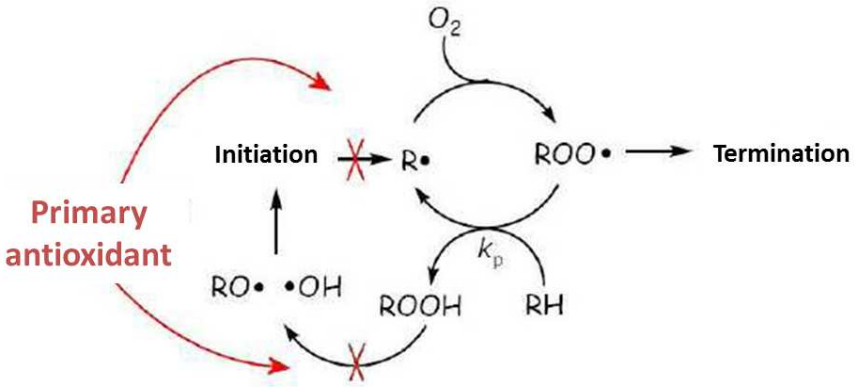


Figure 9. Mechanisms of action of primary antioxidants.

The secondary antioxidants, instead, oxidize and become weak and non-toxic free radicals when they react with ROS/RNS. Examples of these substances are: vitamins C and E, β-carotene, uric acid, bilirubin, albumin, ubiquinone and selenium and, among phenolic compounds, phenolic acids, phenols and flavonoids (Carr and Frei, 1999a; Catani et al., 2001; El-Agamey et al., 2004; Landis and Tower, 2005; Miller et al., 2005; Schrauzer, 2006) (Figure 10).

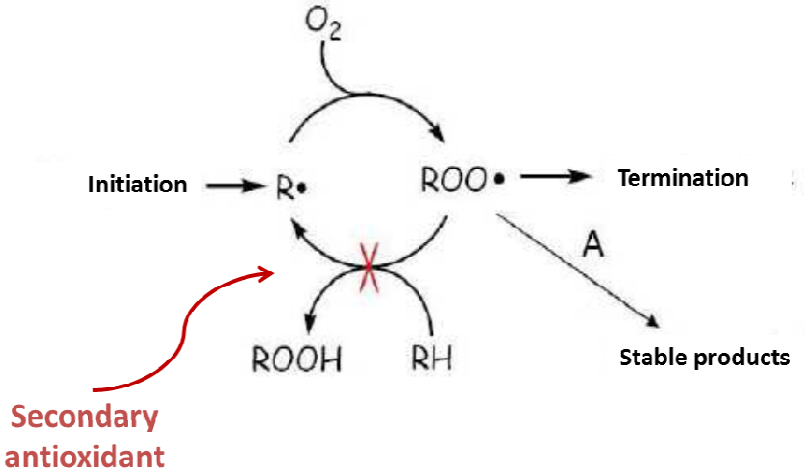


Figure 10. Mechanisms of action of secondary antioxidants.

The third system of antioxidants is mainly composed by phospholipase A2 and C, endo- and exonucleases, DNA glycosylase, peroxidase and methionine sulfoxide reductase, whose functions include (i) to repair direct damage to proteins, lipids and carbohydrates, DNA, RNA and (ii) to eliminate irreversibly damaged products (Sahnoun et al., 1997).

Finally, the antioxidants can be also classified taking into account their functions and we can distinguish between:

a) indirect antioxidants: they inhibit the formation of free radicals, by chelation of metals (i.g. Fe^{2+});

b) direct antioxidants: they "capture" ROS and RNS once generated;

c) a large group of compounds that modulate positively not only the cellular capacity to counteract the high levels of ROS and RNS, but also the ability to promote the repair of oxidized biomolecules (Aruoma, 1996). In this sense, the concept of antioxidant capacity is linked not only to the effects of decrease free radicals formation, but also to the prevention of oxidative damage in cellular functions (Aruoma, 1998).

At the end, it has been proposed that antioxidant defenses, in general, act in parallel (several antioxidants may play similar roles) and in serial mode (antioxidant enzymes operate in tandem during their catalytic action): this is the proof of the complexity of this system of molecules (Beckman and Ames, 1998). Secondary antioxidants, for example, may exhibit synergetic effects in combination with primary antioxidants, following several possible mechanisms (Pisoschi and Pop, 2015):

- stabilizing primary antioxidants through the creation of an acidic environment;
- regenerating primary antioxidants by hydrogen donation;
- chelating pro-oxidative transition metal cations;
- quenching molecular oxygen.

Moreover, it has been revealed that antioxidant enzymes can catalyse the synthesis or the regeneration of non-enzymatic antioxidants (Martysiak-Żurowska and Wenta, 2012).

The synergistic interaction between antioxidants is also favoured by the "protection" that occurs between each enzyme: for example, SOD "protects" GSH-Px and catalase from damage caused by $O_2^{\cdot-}$, while GPx and catalase "protect" in turn SOD from inactivation caused by hydroperoxides and H_2O_2 (Chaudieáre & Ferrari-Iliou, 1999).

The principal enzymatic and non-enzymatic antioxidants present in animals and humans are listed below:

Enzymatic:

- Superoxide dismutase (SOD) (E.C. 1.15.1.6). This antioxidant enzyme catalyzes the dismutation of $O_2^{\bullet-}$ to O_2 and to the less-reactive species H_2O_2 (Valko et al., 2006). In mammalian tissues, three types of superoxide dismutases can be found: copper-zinc containing superoxide dismutase (SOD1) present in the cytosol, manganese containing superoxide dismutase (SOD2) found in the mitochondrial matrix and extracellular superoxide dismutase (SOD3) (Ghezzi et al., 2005; Sung et al., 2013);
- Catalase (E.C. 1.11.1.6). Catalase is present in the peroxisome of aerobic cells and represents the enzyme involved in the reductive depletion of H_2O_2 to water. Catalase has one of the highest turnover rates for all enzymes: one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute (Rahman, 2007);
- Glutathione peroxidase (GPx) (E.C. 1.11.1.9). Glutathione peroxidase is a selenium-containing enzyme. It catalyses both the reduction of H_2O_2, and organic hydroperoxides to water or corresponding alcohols. All GPx enzymes are known to add two electrons to reduce peroxides by forming selenoles (Se-OH). Its antioxidant properties enable them to eliminate peroxides as potential substrates for the Fenton reaction (Chaudieáre & Ferrari-Iliou, 1999).

Non-enzymatic:

- Water soluble
    - Glutathione: One of the main thiol antioxidants is the tripeptide glutathione (GSH), which is an intracellular antioxidant and is considered to be the major thiol-disulphide redox buffer GSH/GSSG of the cell (Masella et al., 2005). The basic protective functions of glutathione against oxidative stress are that it can act as a co-factor for several detoxifying enzymes, involved in amino acid transport through plasma membrane, scavenge hydroxyl radical and singlet oxygen directly, and regenerate vitamins C and E back to their active forms (Masella et al., 2005). The accumulated oxidized glutathione (GSSG) inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Dröge, 2002b).
    - Vitamin C (ascorbic acid): is a very important and powerful water-soluble antioxidant and thus works in aqueous environments of the body. It works synergistically with vitamin E (regenerating α-tocopherol from α-tocopherol

radicals in membranes and lipoproteins) and the carotenoids as well as working along with the antioxidant enzymes (Kojo, 2004; Carr and Frei, 1999b). Furthermore, it supports intracellular glutathione levels thus playing an important role in protein thiol group protection against oxidation (Rahman, 2007).

- Fat-soluble
    - Vitamin E: This is a fat-soluble vitamin that exists in eight different forms. In humans, α-tocopherol is the most active form, and is the main powerful membrane bound antioxidant hired by the cell (Hensley et al., 2004). The primary function of vitamin E is to protect against lipid peroxidation (Pryor, 2000). During the antioxidant reaction, α-tocopherol is converted to an α-tocopherol radical by the donation of an hydrogen to a lipid or lipid peroxyl radical. The α-tocopherol radical can subsequently be reduced to the primary α-tocopherol form by ascorbic acid (Kojo, 2004).
    - The other non-enzymatic antioxidants that are involved in oxidative stress defense are carotenoids and flavonoids. Carotenoids are pigments that are present in plants and microorganisms. They contain conjugated double bonds and their antioxidant activity result mainly as a consequence of the ability to delocalize unpaired electrons (Mortensen et al., 2001). Flavonoids are a large class of low molecular ubiquitous groups of plant metabolites and are an inseparable part of the human diet (Rice-Evans, 2001). They are benzo-γ-pyrone derivatives containing phenolic and pyran rings (Rahman, 2007). Their antioxidant activity is performed by the phenolic compounds that operate as inhibitors of free radical chain (Valko et al., 2006). Recently, the interest in flavonoids increased, due to their antioxidant capacity, chelating properties and their possible role in the prevention of chronic and age-related diseases.

3.6. Lipopolysaccharide

Lipopolysaccharide (LPS), usually referred to an endotoxin, is an outer membrane structure and an important virulence factor of the cell wall of Gram-negative bacteria (e.g. Escherichia coli, Chlamydia pneumoniae, and periodontopathogens that are common pathogens colonizing the human gastrointestinal tract, including the oral cavity and the gut); it is not found in Gram positive bacteria (Mayer et al., 1985). It may originate from several sources, including infections, diet, and commensal microbiota. The LPS molecule is essential for the viability of most Gram-negative

bacteria, exerting a crucial role in outer-membrane integrity as a permeability barrier, protecting bacteria from toxic molecules, bile salts and lipophilic antibiotics (Mayer et al., 1985).

In the circulation system, LPS is able to interact with several cell types, as epithelial cells, macrophages, smooth muscle cells, fibroblasts, B- and T-cells and endothelial cells (Whitfield and Trent, 2014).

3.6.1. Structure

LPS is a complex glycolipid in which the lipid and polysaccharide moieties are joined by a covalent bond. The three structural regions of LPS are: (i) lipid A, a hydrophobic lipid which is responsible for the toxic properties of the molecule, (ii) a hydrophilic core oligosaccharide and (iii) a hydrophilic O-specific side chain (O antigen) (Mayer et al., 1985) (Figure 11).

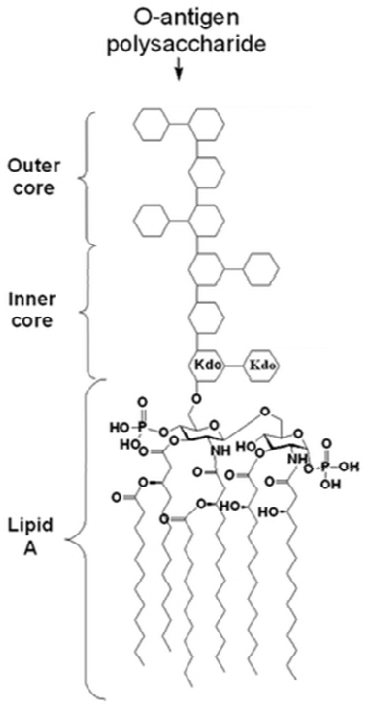


Figure 11. Chemical structure of LPS (taken from Qiao et al., 2014).

The biological activity of LPS is strictly dependent on the lipid A moiety, which is its most conserved part and connects the molecule to the outer membrane of bacterial. It is a phosphorylated glucosamine disaccharide acylated with hydroxyl saturated fatty acids that further 3-O-acylate the 3-hydroxyl groups of the fatty acids of lipid A (Raetz 1990). In 1986 Munford and Hall demonstrated that the removal of the O-acylated saturated fatty acids or their substitution with

unsaturated fatty acids leads to the disappearance of endotoxin activity (Munford and Hall, 1986). The core oligosaccharide is directly attached to lipid A. The O-side chain is a repetitive glycan polymer, which binds to the core oligosaccharide forming the most external part of the LPS (Manco et al., 2010). The repetitive units of the polymer may be linear or branched, producing homo- or heteropolymers (Raetz and Whitfield, 2002). Each repeating unit represents diverse antigen properties, and their sequence and their way of linkage determine the serological O specificity of respective strains (Mayer et al., 1985).

3.6.2. LPS-mediated signaling and the innate immune response

LPS induces the release of a large number of inflammatory cytokines, which play an important role in metabolic processes through the linkage to the pathogen-sensing system. The main elements of LPS-mediated signaling comprise lipoproteins, LPS-binding protein (LBP), cluster of differentiation 14 (CD14) and toll-like receptor 4 TLR4 (Bosshart and Heinzelmann, 2007). These elements act together to initiate a signaling pathway, that ultimately leads to the activation of NFκB transcription factor. In the circulation, LPS is transported to hepatocytes by LBP, phospholipid transfer protein, and lipoproteins (Munford et al., 1981; Hailman et al., 1996). Approximately 80–90% of the LPS is bound to the lipoproteins through lipophilic lipid A, including all main lipoprotein classes: (i) HDL, under physiological conditions, (ii) VLDL, when the serum HDL is low, or (iii) TG-rich lipoprotein-LPS complex, that is rapidly eliminated to reduce LPS-induced toxicity or is internalized by macrophages (Brown and Goldstein 1983; Levels et al., 2001; Harris et al., 2002). Therefore, the metabolic fate of LPS may be regulated by the lipoprotein profiles (Berbee et al., 2005).

LBP may transport LPS to lipoproteins or alternatively to soluble or membrane-bound CD14 (Stoll et al., 2004). In this case, the LPS-CD14 complex engages TLR4 via lipid A moiety (Chow et al., 1999). TLRs are necessary for the downstream signaling pathway, since that CD14 misses a transmembrane domain. The TLR4 activation leads to the employment of additional adaptor molecules, which further trigger a cascade that allows NFκB to diffuse into the nucleus and activate the transcription of proinflammatory cytokines, as TNFα, IL-1β, IL-6, and chemokines (Stoll et al., 2004; Parker et al., 2007; Lu et al., 2008) (Figure 12).

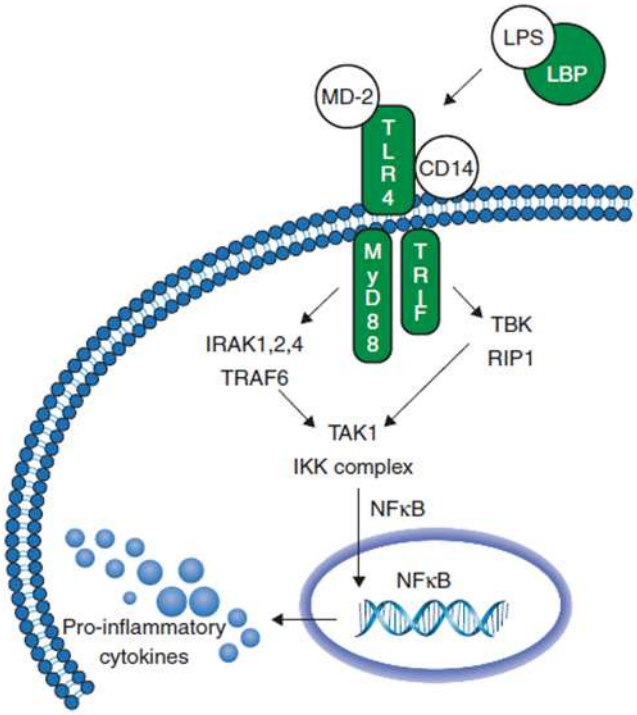


Figure 12. Schematic representation of LPS-induced pro-inflammatory cytokines production (taken from Neves et al., 2013).

Although activation of cytokines is a part of the host defense response to infections, their excessive production and secretion may result in septic shock, systemic inflammatory response syndrome, severe tissue damage and multiple organ dysfunction, including liver, lung, heart, gastrointestinal tract, and kidneys (Haghgoo et al., 1995; Shimazu et al., 1999; Cadenas and Cadenas, 2002; Yokozawa et al., 2003; Chen et al., 2007; Zhang et al., 2009).

3.6.3. LPS and nutrition

In addition to the oral cavity, the other main source of LPS is the gut. Even if LPS has a strong affinity for chylomicrons and is able to cross easily the gastrointestinal mucosa, under physiological conditions, the intestinal epithelium defends itself from LPS translocation (Ghoshal et al., 2009). However, possible mechanisms implicated in LPS translocation from the gut include uptake by intestinal enterocytes and microfold cells, and alterations in the gene expression of host epithelial cells by Gram-negative bacteria (Hathaway and Kraehenbuhl, 2000; Hooper and Gordon, 2001) (Figure 13).

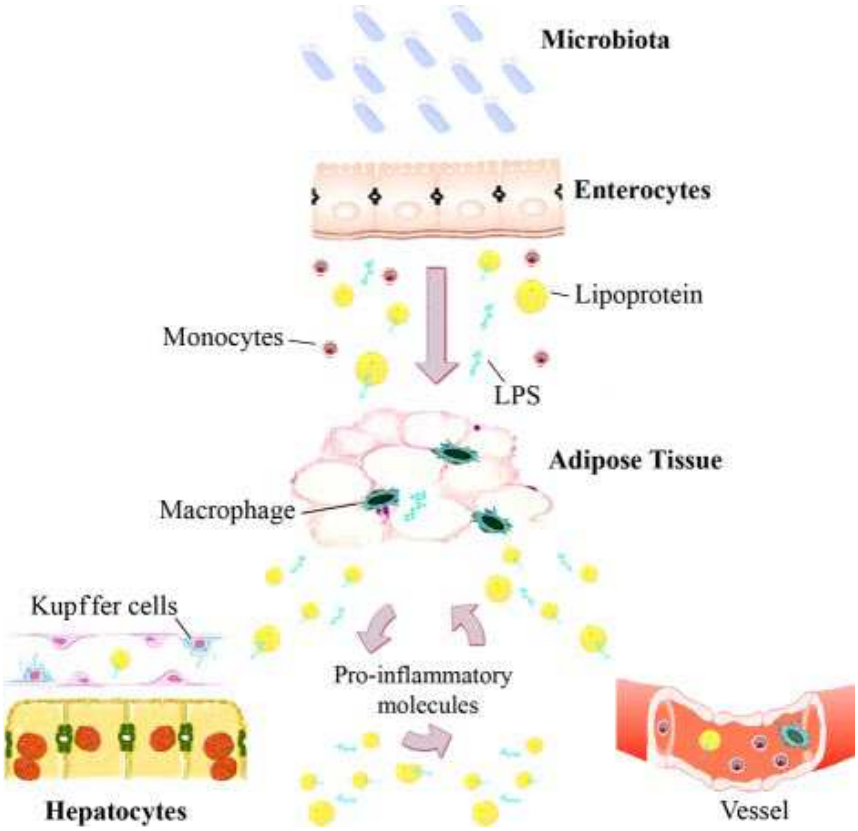


Figure 13. Gram-negative-derived LPS pass across the intestinal barriers into the lymphatic system and then into the bloodstream. Main target tissues are the adipose tissue, the liver, and the endothelium (taken from Manco et al., 2010).

A high-fat diet, obesity, diabetes, and non-alcohol fatty liver disease have been associated with increased permeability of the gastrointestinal mucosa, leading to metabolic endotoxemia (Neves et al., 2013). In mice, a chronic exposure to LPS appears to be related to the development of insulin resistance, weight gain and an insurgent inflammatory status.

The absorption of LPS through the intestinal barrier seems to be enhanched by an high-fat diet, determining a very strong association between this type of diet, the microbiota, and inflammation (Cani et al., 2007).

For this reason, LPS can be considered an important factor directly involved in the onset of obesity induced by a rich-fat diet and type 2 diabetes (Manco, 2009).

In addition, recent studies on mice and pigs fed with a saturated fatty acids-rich diet showed an increase in serum concentrations of LPS and a more active transport of this endotoxin to peripheral tissues through high levels of LBP and soluble CD14 low levels (Laugerette et al., 2012; Mani et al., 2013).

The capability of LPS to interact synergistically with this type of fatty acids is related to the strong influence that saturated fatty acids have on the immune system and on TLR4 activation (Fritsche, 2006; Suganami et al., 2007).

Human studies have confirmed the close relationship between high-fat or energy-rich diet and the occurrence of inflammation (Erridge et al., 2007; Amar et al., 2008; Ghanim et al., 2009; Pendyala et al., 2012). Studies on healthy subjects highlighted that an high-fat meal or excessive energy intake, leading to a significative increase in plasma concentration of LPS (Erridge et al., 2007; Amar et al., 2008; Ghanim et al., 2009), expecially in subjects with high metabolic risk, as impaired glucose tolerance and type 2 diabetes (Harte et al., 2012). Dietary fats in fact deeply increase LPS absorption through modification of the gut microbiota, raising the amount of chylomicrons, and increasing the permeability of the gastrointestinal mucosa (Manco et al., 2010).

3.7. Strawberry

Nowadays what we call "food" is not just a tool to satisfy the appetite of the consumer, but it is becoming an essential factor closely related to human health. In this context, the change of lifestyle in modern society has highlighted new crucial aspects from the nutritional point of view. The excessive or inadequate food intake could be one of the causes of the onset of various diseases and illnesses, while the careful choice of food can help to prevent or limit the onset of these diseases (Testoni and Lovati, 2004).

From this point of view, fruits and vegetables represent the foods with the highest amount of bioactive compounds, demonstrating the best beneficial effects on human health. Among commestible fruits, berries of the family Rosaceae (Rubus and Fragaria) are especially rich in antioxidant compounds, as reported by various systematic analysis of a large number of dietary plants (Halvorsen et al., 2002; Proteggente et al., 2002; Sun et al, 2002; Bagchi et al., 2003).

In addition, edible berries may represent a potential important contribution to the intake of fresh fruit for the populations in countries where, as declared by World Health Organization (WHO), there is a limited availability of fruits and vegetables, as in northern latitudes. For example, in a Norwegian diet, berries represent 21.7% of the total plant antioxidants in the diet (Halvorsen et al, 2002), with strawberries, black currant and cranberry being the most significant source among them. One of the most commonly consumed berries both in fresh and processed forms such as jams, yogurts, desserts or juice, is strawberry (*Fragaria x ananassa Duch*), due to the nutritional quality and the several bioactive compounds that it contains. Strawberries are also economically and commercially important and therefore represent one of the most studied fruit from the agronomic,

genomic and nutritional points of view. Strawberry is a relevant source of folate (Olsson et al., 2004; Tulipani et al., 2008a), is rich in vitamin C and contains various phytochemicals, which may greatly influence the nutritional and organoleptic qualities of this fruit (Scalzo et al., 2005a; Proteggente et al., 2002; Deighton et al., 2000; Tulipani et al. 2009a; Giampieri et al., 2012a).

Their health effects are mainly attributed to the high levels of antioxidant compounds, mainly phenolic compounds, such as anthocyanins, flavonols, flavanols, condensed tannins (proanthocyanidins, ellagitannins and gallotannins), hydroxybenzoic and hydroxycinnamic acid derivatives, and hydrolyzable tannins (Giampieri et al., 2012a; 2013). These compounds are reported to possess important antioxidant, anti-inflammatory, anticancer, and antineurodegenerative biological properties (Giampieri et al., 2013).

3.7.1. Nutritional aspects

The nutritional quality of the strawberry is related to the quantity and the quality of the phytochemical compounds that it contains (Table 2). This composition determines the nutritional profile and the possible preventive effect of strawberry against many chronic diseases (Tulipani et al., 2011):

- the content of vegetable fiber (1.6 g/100 g fresh weight (FW)) has a satiating effect, determining the reduction of the calorie intake in the diet. Fiber also allows for adjustment of the glycemic index, slowing the rate of digestion;
- strawberries are a natural source of essential fatty acids: in particular, the seeds are rich in unsaturated fatty acids;
- the high content of vitamin C makes the strawberry one of the main dietary sources of this compound and makes it particularly important from the nutritional point of view;
- in addition to traditional nutrients, strawberries are one of the richest dietary sources of phytochemicals known as non-nutritional compounds, represented in particular by the class of polyphenols, main responsible of the fruit color and the characteristic aroma (Mazzoni et al. 2013, 2016).

CHEMICAL COMPOSITION OF STRAWBERRY	
Strawberry nutrient (Fragaria vesca)	**Ammount**
Water	90.5 g
Protein	0.9 g
Lipid	0.4 g
Sugars	5.3 g
Fiber	1.6 g
soluble	0.45 g
insoluble	1.13 g
Energy	27 kcal
Sodium	2 mg
Potassium	160 mg
Iron	0.8 mg
Calcium	35 mg
Phosphorus	28 mg
Thiamin	0.02 mg
Riboflavin	0.04 mg
Niacin	0.5 mg
Folate	20 mg
Vitamin C	54 mg
Vitamin A	Trace

Table 2. Chemical composition of strawberry (Source INRAN). The data are expressed per 100 g of fresh weight.

3.7.1.1. Micronutrients in strawberry

3.7.1.1.1. Folates

The terms folate and folic acid are often both used to describe this water-soluble B-complex vitamin, also called vitamin M or B9. However, the term folates refers to all derivatives of tetrahydrofolic acid, which are present in food in about 150 different chemical forms, and to the metabolically active forms in the human body (Giampieri et al., 2012a). The elementary structure of these essential dietary compounds consists of a pteridine ring linked to a paraaminobenzoic acid, conjugated with usually five to eight L-glutamic acid residues. These compounds differ one to another in three points inside the molecule: i) the reduction-state of the pteridinic ring, ii) the type of mono carbonaceous unit bound to it, iii) and the number of glutamic acid residues. Commonly,

folates are present in conjugated form to one or more residues of glutamic acid (Bates and Heseker, 1994) (Figure 14).

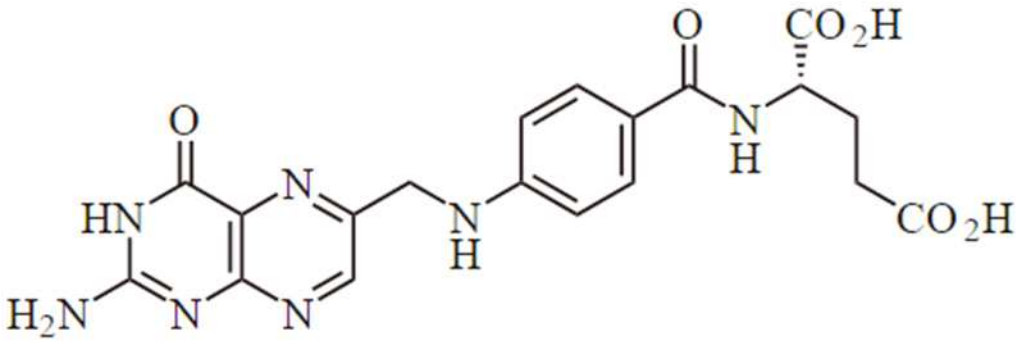


Figure 14. Chemical structure of folic acid.

The essential biological function of folate is to work as a coenzyme in transferring one-carbon units to a variety of target molecules. In particular folates can act as acceptors and/or donors of one-carbon units in the synthesis of nucleic acids (eg. Purine synthesis) or in the re-methylation of homocysteine to methionine. Folate is also very important to prevent the insurgence of diseases related to their deficiency, such as megaloblastic anemia and some types of cancer. Their assumption is strongly recommended in particular for women of childbearing age to prevent the terrible consequences that can be verified in the fetus in the early stages of pregnancy in the case of a deficiency of this compound (Giampieri et al., 2012a).

On the other hand, folic acid is the more stable, fully oxidised monoglutamate form of folate (Pteroyl-L-Glutamic acid) most often used in dietary supplements and fortified foods. Folic acid occurs rarely in foods or in the human body, and requires the reduction to tetrahydrofolic acid for assimilation into the folate body pool.

In association with vitamin C, folate plays a crucial role in emphasizing the nutritional quality of strawberry. Among fruit, strawberry is one of the richest natural sources of this indispensable micronutrient; its content is considered in the range of 20-25 µg/100 g (FW). To date, still few studies are focused on the evaluation of the folate content of fresh strawberries (Muller, 1993; Vahteristo et al., 1997; Konings et al., 2001; Tulipani et al., 2008a), of its stability during storage and of its retention after fruit processing. According to the existing data (Tulipani et al., 2008a) the intake of dietary folate through strawberry consumption (in 250 g of strawberries are present ~60 µg folate on average) can supply 30% of the European folate RDA (Giampieri et al., 2012a). The

increasing awareness of the relevance of folate content in strawberry as an interesting nutritional quality parameter makes strongly necessary the optimization of correct, accurate and reliable analytical methods for its determination, in order to confirm and support the available data.

3.7.1.1.2. Vitamin C

Vitamin C (or ascorbic acid) is a water-soluble vitamin with several biological functions in humans (Figure 15).

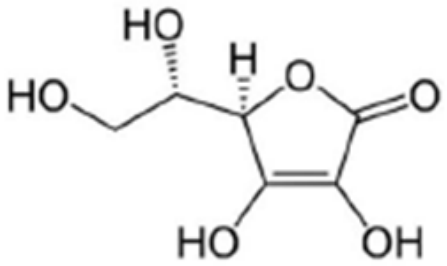


Figure 15. Chemical structure of vitamin C.

First of all, vitamin C is a highly reactive and effective antioxidant, able to act both directly and indirectly by ROS scavenging through enzymatic and non-enzymatic reactions. Even in small quantities it plays an important role in protecting essential biomolecules in the body (i.e. proteins, carbohydrates, lipids, DNA and RNA) from damages by free radicals and ROS generated (i) during normal metabolism, (ii) in pathological conditions and (iii) through exposure to toxins and polluants (i.e. smoking). Furthermore, due to its one electron reduction potentials, vitamin C plays a crucial role in regenerating the active form of other antioxidant molecules (i.e. vitamin E), and in scavenging the relative antioxidant-derived radicals: for this reason it is defined “the terminal water-soluble small molecule antioxidant” (Carr and Frei, 1999a).

Vitamin C is also an essential cofactor for the synthesis of hormones, collagen, neurotransmitter norepinephrine or small intracellular molecules like carnitine, and seems to be involved in the functionality of the immune system. In Europe the recommended dietary allowance (RDA) for vitamin C was recently revised upward from 60 mg daily. This RDA value continues to be calculated as the daily amount necessary for the prevention of vitamin C deficiency disease, such as scurvy, rather than the prevention of chronic disease and the promotion of optimum health. Nevertheless, the results of different prospective, observational and case-control studies indicate that higher intakes of vitamin C from diet are frequently correlated with a lower incidence of cardio

and cerebrovascular diseases (Carr & Frei, 1999b; Yokoyama et al., 2000), most types of cancers (Steinmetz and Potter, 1996), and other health problems such as lead toxicity. Moreover, vitamin C supplementation seems also to be a beneficial adjunt to conventional therapies for individuals with atherosclerosis (Dawson et al., 1999), hypertension and diabetes mellitus (Duffy et al., 1999).

However, discrepancies among different observational studies and randomized controlled trials still persist, and further studies are required to evaluate the potential effects of vitamin supplementation. At the moment, there is no reason to limit high vitamin C intakes by a fruit and vegetables-rich diet since no toxicity and pro-oxidative events have been ever observed. Notwithstanding, it is still difficult to distinct the health-promoting effects of vitamin C from the potential effects of the other nutrient and non-nutrients compounds present in foods.

One of the aspects of major nutritional relevance in strawberry fruit is the extremely high content of vitamin C (even higher than citrus fruit). It is interesting to note that a cup of strawberries (corresponding to two servings) is sufficient to cover the daily RDA for vitamin C, since its content varies between 0.1 and 1 mg g^{-1} FW (Cordenunsi et al., 2002; Kafkas et al., 2007), with an average of 0.6 mg g^{-1} FW (Kalt et al., 1999; Proteggente et al., 2002; Keutgen & Pawelzik, 2007) for several strawberry genotypes (Table 3).

Dietary source of Vitamin C	Ammount
Kiwi	85 mg
Strawberries	54 mg
Oranges	50 gm
Bananas	16 mg
Apricots	13 mg
Cherries	11 mg
Apples	5 mg
Peaches	4 mg

Table 3. Contents of vitamin C (mg/100g of FW) in strawberry and other fruits (Source INRAN)

Vitamin C is one of the most important strawberry antioxidant components and is responsible, per sé, for more than 20% of the total antioxidant capacity (TAC) of strawberry extracts (Tulipani et al., 2008b). Thus, the vitamin C content in strawberry is a crucial factor affecting the nutritional quality of the fruit, and its variation is an important parameter when comparing both commercial varietes and new selections; in this context the new research lines in the agronomic field are pushing more and more towards the selection of varieties rich in vitamin C (Mazzoni et al., 2013, 2016).

3.7.1.1.3. Other vitamins

Despite the main interest is focused on the determination of vitamin C and folate in the fruits, it is important to note that other vitamins, even if in a lower extent, are present in strawberry, such as thiamin (B1), riboflavin (B2), niacin (B3), vitamin B6, vitamin K, vitamin A and vitamin E (Giampieri et al., 2015a) (Figure 16).

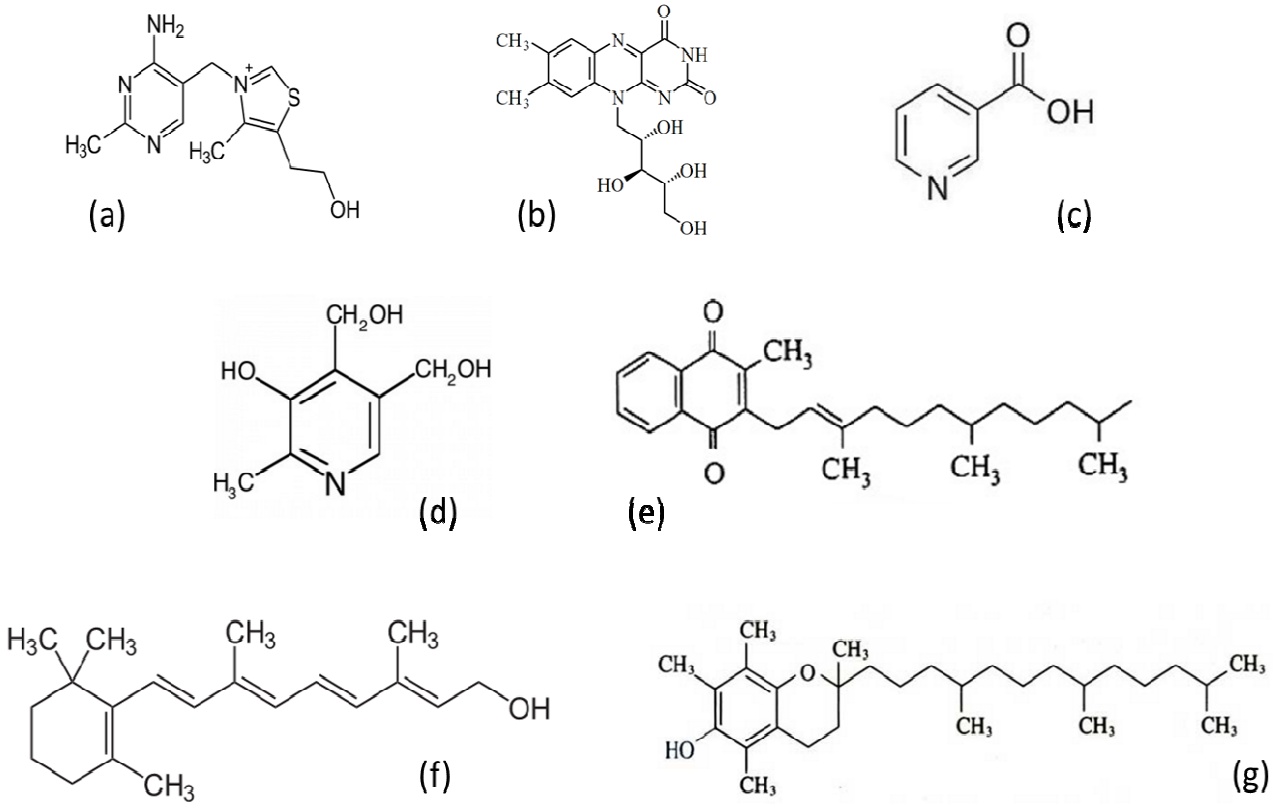


Figure 16. Chemical structure of vitamin B1 (a), vitamin B2 (b), vitamin B3 (c), vitamin B6 (d), vitamin K (e), vitamin A (f) and vitamin E (g).

3.7.1.1.4. Essential minerals

The strawberry represents also an excellent source of microelements, especially manganese, so that a serving of strawberries (eight medium berries, corresponding to about 150 g) can provide more than 20% of the daily requirement for this mineral. The same amount of this berry is able to provide about 5% of the necessary intake for potassium, and appears to be also a good source of iodine, magnesium, copper, iron and phosphorus (Giampieri et al., 2015a).

3.7.1.2. Phytochemicals in Strawberry

In addition to traditional nutrients, strawberries are among the richest dietary sources of phytochemicals. These compounds can be defined as secondary metabolites produced by plants but the term is commonly used to describe chemicals from plants that may affect health, even if are not designated as traditional nutrients. In the last decades, with the introduction of highly sensitive and accurate analytical methods, knowledge on the composition of strawberry fruits has rapidly expanded, allowing to obtain the phytochemical profiles and characterization of these fruits.

3.7.1.2.1. Classification of phenolics

Strawberry phytochemicals are mainly represented by the wide class of phenolic compounds, a large and heterogeneous group of biologically active non-nutrients, with many non-essential functions in plants but characterized by huge biological potentialities in humans (Häkkinen, 2000). In particular, strawberry phenolics are well known for their antioxidant and anti-inflammatory actions, directly and indirectly antimicrobial activity, anti-allergy and anti-hypertensive properties, as well as the capacity of inhibiting the activities of some physiological enzymes and receptors (Wang et al., 1996; Alvarez-Suarez et al., 2011).

The major class of phenolic compounds in strawberry is represented by **flavonoids** (mainly anthocyanins, secondly flavonols and flavanols), followed by hydrolyzable tannins (**ellagitannins**, and gallotanins) as the second most abundant class, and minor constituents such as **phenolic acids** (hydroxybenzoic acids and hydroxycinnamic acids) together with condensed tannins (proanthocyanidins) (Giampieri et al., 2012a; Mazzoni et al., 2013, 2016).

Flavonoids are a large family of compounds with a common chemical structure, being all in possess of the same C15 (C6 -C3 -C6) flavone nucleus: two benzene rings linked through an oxygen containing pyran or pyrone ring (Figure 17).

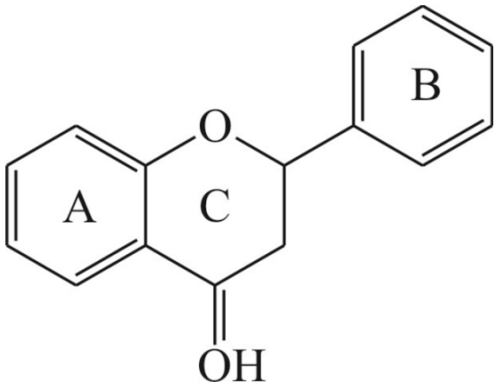
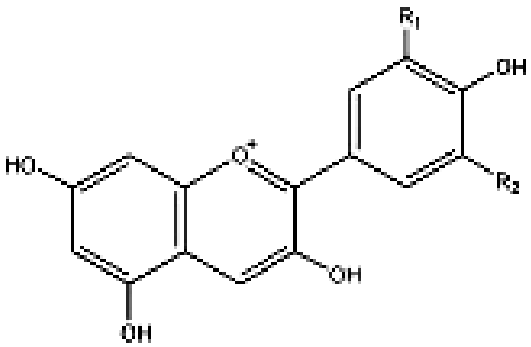


Figure 17. Basic chemical structure of flavonoids

They may be further divided into subclasses that, in their turn, covered into two main categories depending to the structure of the central heterocyclic ring: unsaturated (anthocyanidins, flavonols, flavones and isoflavones) or not (flavanones, flavanols). *Anthocyanins* are the glycosylated form of anthocyanidins, which are polyhydroxyl and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salt (Figure 18)

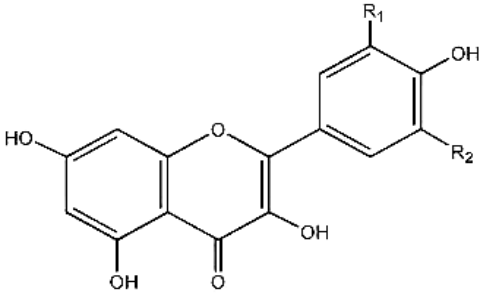


R_1 = H;	R_2 = H;	Pelagonidin
R_1 = OH;	R_2 = H;	Cyanidin
R_1 = OH;	R_2 = OH;	Delphinidin
R_1 = OCH_3;	R_2 = OH;	Peturnidin
R_1 = OCH_3;	R_2 = OCH_3;	Malvidin

Figure 18. Chemical structure of some anthocyanins

They represent the most relevant water-soluble pigments in plant, since their daily uptake through diet is notable and much higher than the total intake estimated for other flavonoids. Anthocyanins, the flavonoids that provide much of the flavor and color to strawberries, represent the best known

polyphenolic compounds and quantitatively the most important present in this berry (Clifford, 2000; Lopes-da-Silva et al., 2002; Kähkönen et al., 2003). Several hundreds of anthocyanins are known, and they differ from each other in: (i) the basic anthocyanidin skeleton; (ii) the identity, number and positions at which sugars are attached to the skeleton; (iii) the identity of eventual acylating agents and the extent of sugar acylation. To date, in strawberry fruits from different varietes and selections, more than 25 different anthocyanin pigments have been described (Lopes-da-Silva et al., 2007). The major representative compounds are pelargonidin-3-glucoside (Pg-3-glc) and in smaller proportion cyanidin-3-glucoside (Cy-3-glc): their presence seems constant in all varietes, but a qualitative and quantitative variability among cultivars can be observed. Glucose is the most usual substituting sugar, but in some cultivars is possible to find rutinose, arabinose and rhamnose, as well as various acylated anthocyanins with a range of aliphatic acids. Furthermore, anthocyanin concentrations may also strongly differ among the same variety, depending of the degree of ripeness, climatic factors and post-harvest storage. Finally, the presence of small amounts of anthocyanin-derived pigments (also called condensed pigments), such as condensed products containing C-C linked anthocyanin and flavanol residues, has been recently detected in small quantities (Fossen et al., 2004; González-Paramás et al., 2005). During the past decade, the interest in anthocyanins has widely increased (Wang and Stoner, 2008). In earlier studies, it has been shown that anthocyanin and other phenolic compounds may reduce the risk of cardiovascular diseases and cancers, through their antioxidant, antiinflammatory and chemoprotective properties (Mazza, 2007; Wang and Stoner, 2008). These compounds have also been reported to scavenge ROS, inhibit *in vitro* low-density lipoprotein oxidation, prevent platelet aggregation and decrease serum lipids (Dell'Agli et al., 2004; Han et al., 2007; Liu et al., 2008). Even if present in strawberry in a smaller extent than anthocyanins, the *flavonol* content and composition have been object of numerous studies (Häkkinen et al., 1999) (Figure 19).



R_1 = H;	R_2 = H:	Kaempferol
R_1 = OH;	R_2 = H:	Quercetin
R_1 = OH;	R_2 OH:	Myricetin
R_1 = OCH_3;	R_2 = H:	Isorhamnetin

Figure 19. Chemical structure of some flavonols.

Flavonols occur naturally in glycosilated forms where the associated sugar moiety is very often glucose or rhamnose, although other substituents such as glucuronic acid, galactose, arabinose and xylose may also be present. The flavonols detected in strawberries are derivatives of quercetin and kaempferol, being quercetin-3-glucuronide the major flavonol. Also acylated form of flavonols such as quercetin- and kaempferol-3-malonylglucoside, and kaempferol-coumaroylglucoside have been found in some cultivars (Aaby et al., 2007).

The interest on the flavonol composition in strawberry is related to the confirmation of the presence of these molecules in human plasma and urine, in far higher concentrations than anthocyanins. Human (Hollman et al., 1995; Manach et al., 2005) and animal studies (Gee et al., 2000; Sesink et al., 2003) have investigated the mechanisms of passive and active transport of quercetin aglycones and glucosides through the epithelia of the gastrointestinal tract, and the potential bioefficacies of the bioavailable metabolites are under investigation.

Flavanols are the only class of flavonoids that do not occur naturally as glycosides (Figure 20).

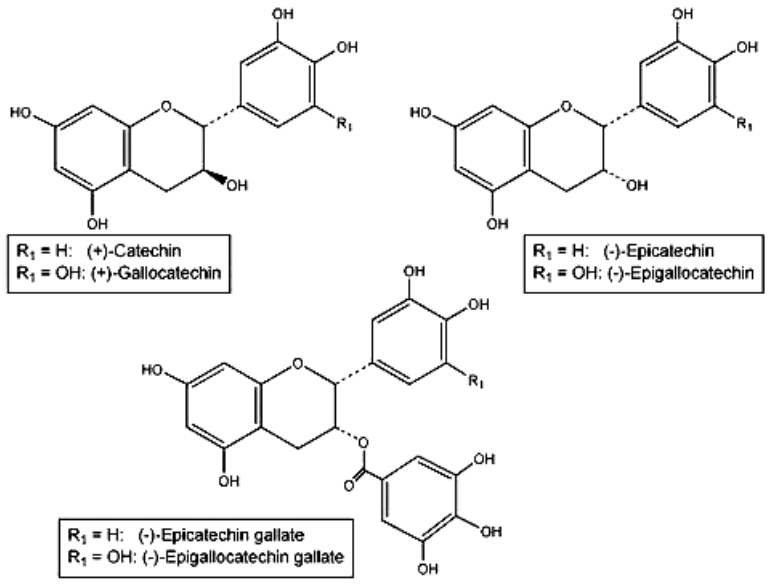


Figure 20. Chemical structure of some flavanols.

They are present in strawberries in both monomeric (catechins) and polymeric forms called condensed tannins or procyanidins. Procyanidins, one subclass of proanthocyanidins, are mixtures of dimers, oligomers and polymers of catechins that are bound together mainly through C4→C8 or C4→C6 bonds (B-type linkages). These flavan-3-ol units can be doubly linked by a C4→C8 bond and an additional ether bond between O7→C2 or O5→C2 (A-type linkages) (Manach et al., 2004).

Procyanidins are present in strawberry flesh and achenes. The interest in the proanthocyanidins/procyanidins content is strongly increased, due to the variety of physiological activities they directly and indirectly possess (such as antioxidant, antimicrobial, anti-allergy, anti-hypertensive, and inhibition of the activities of some physiological enzymes and receptors) (Santos-Buelga and Scalbert, 2000). Nevertheless, the complications in isolating pure higher polymers and the lack of efficient analytical methods make difficult to estimate their content in berries and other foods.

The *ellagitannins*, along with anthocyanins, are the most abundant phenolic compounds in strawberries. Ellagitannins are esters of glucose and hexahydroxydiphenic acid, and may occur in different structures: as monomers (such as ellagic acid glycosides), as oligomers (sanguiin H-6, the most common ellagitannin in strawberry) and as complex polymers (Figure 21).

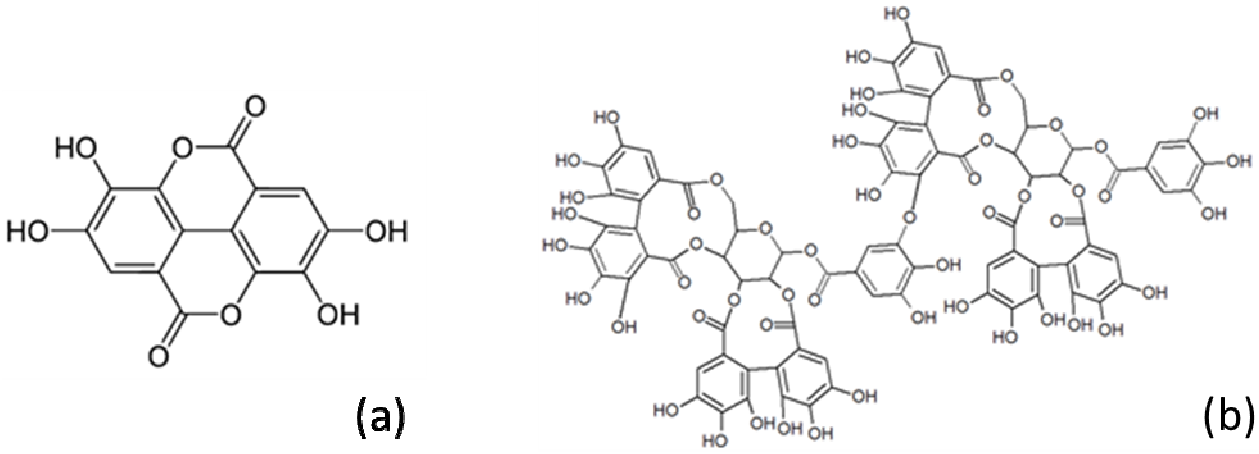


Figure 21. Chemical structure of ellagic acid (a) and sanguiin H-6 (b).

The content of ellagitannins in strawberry is between 25 and 59 mg/100 g FW (Mazzoni et al., 2013, 2016). The ellagitannins, together with gallotannins, constitute the group of **hydrolysable tannins**; following the hydrolysis reaction, these compounds release *ellagic acid* allowing the formation of different metabolites. Strawberries, raspberries and blackberries contain by far the most ellagic acid concentration among the commonly consumed foods (in strawberry, representing about 50% of the total phenolic content) (Koponen et al., 2007). Thanks to its antioxidant properties, in some countries ellagic acid is used as a food additive. The biological and pharmacological properties of ellagic acid are due, at least in part, to the ability to interact with biomolecules such as proteins and DNA. A diet rich in foods that contain high concentrations of

ellagic acid is definitely recommended, even though the doses to which ellagic acid should be taken for exerting its real benefits are still unknown (Freedman et al., 2008).

Finally, strawberries also contain a variety of ***phenolic acids***, which occur as derivatives of hydroxycinnamic acid (i.e. caffeic acid) and hydroxybenzoic acid (i.e. gallic acid). (Giampieri et al., 2013). Soluble hydroxycinnamic acid are more common than the hydroxybenzoic acid, consist chiefly of p-coumaric, caffeic and ferulic acids and are generally found as glycoside and as esters with sugars or quinic acids (such as p-coumaroylhexose, p-coumaroylhexose- 4-O-hexoside, or ferulic acid hrxose derivatives) (Määttä-Riihinen et al., 2004; Mattila et al., 2006; Aaby et al., 2007) (Figure 22).

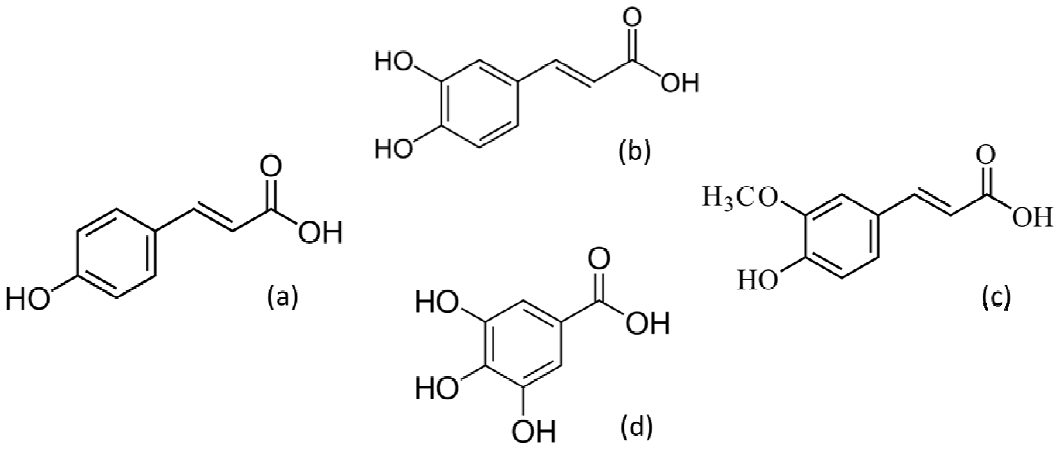


Figure 22. Chemical structure of p-coumaric (a), caffeic (b), ferulic (c) and gallic acid (d).

The concentration of p-coumaric in strawberries can reach values close to 6 mg/100 g of FW, with average values between 0.9 and 4.1 mg/100 g of FW (Mattila et al., 2006).

3.7.2. Strawberry bioactive compounds and human health

The attention for the potential health effects of polyphenols, that represent the most abundant antioxidants ingested through the diet, has raised only recently: research on flavonoids and other phenolics, their antioxidant activities and their potential effects in disease prevention have considerably developed only in the last 20 years. In this context, several experimental data report the potential role of bioactive compounds-rich strawberries in health promotion (Giampieri et al., 2012a).

The strawberries have been widely appreciated for their therapeutic properties. The energy provided by these fruits is extremely low, since they are constituted for the 90% only by water. On the other side, their excellent nutritional profile is linked to their high concentrations of minerals, vitamins and to the variety of phenolic compounds that they contain, such as flavonoids (catechin, quercetin, kaempferol), anthocyanins and other polyphenols, like ellagic acid and stilbenes (resveratrol) (Wang et al., 2007).

Several studies suggest that strawberry phenolics show huge biological potentialities in humans, from antioxidant capacity to antiinflammatory, anti-hypertensive and anti-proliferative abilities, exerting a prevention role in the development of many chronic diseases, such as oxidative stress, CVD, certain types of cancers, type 2 diabetes, obesity and inflammation (Forbes-Hernandez e al., 2015).

In the past few years much attention has been paid to the total antioxidant capacity of fruit as an eligible parameter for quality and as an indicator of bioactive compounds present in foodstuffs and, therefore, of their healthfulness. In this context, several *in vitro* and *in vivo* investigations have been recently performed to outline the antioxidant activity of the strawberry bioactive compounds in different experimental models (Forbes-Hernandez e al., 2015). In skin and liver cell model lines, the protective effects of different strawberry polyphenols in counteracting oxidative stress have been deeply analysed (Giampieri et al., 2012b; Giampieri et al., 2014a; Kim et al., 2013a; Giampieri et al., 2014b; Forbes-Hernandez et al., 2014; Lee et al., 2014). At the same time, phenol supplementation has shown to be effective against an oxidative stress condition in animal (Alvarez-Suarez et al., 2011; Benedetti et al., 2012; Kim et al., 2013b; Diamanti et al., 2014; Giampieri et al., 2015b) and human models (Henning et al., 2010; Romandini et al., 2013; Prymont-Przyminska et al., 2014).

Certainly, the antioxidant capacity remains an essential instrument, but it is not sufficient to explain the beneficial effects of strawberry polyphenols alone. Therefore, more complex mechanisms, that interact and supplement with the antioxidant capacity, begin to be investigated in order to clarify the healthy effects of strawberry polyphenols against the most common chronic diseases (Giampieri et al., 2014c).

The dietary strawberry consumption can positively affect risk factors for CVD (represented by obesity, type 2 diabetes mellitus and the metabolic syndrome) through the inhibition of platelet aggregation, the improvement of endothelial function and plasma lipid profile, the modulation of eicosanoid metabolism and the amelioration of LDL resistance to oxidation (Youdim et al., 2000; Mazza, 2007; Basu et al., 2010; Edirisinghe et al., 2011).

There are several mechanisms by which strawberries exert these capacities even if they are not yet fully understood.

Strawberry supplementation significantly decreases oxidative stress, reducing malondialdehyde formation, preserving LDL from oxidation and protecting mononuclear blood cells against increased DNA damage (Azzini et al., 2010; Tulipani et al., 2011a). Moreover the relatively long-term consumption of moderate amounts of mixed berries increases HDL cholesterol, reduces blood pressure and resultes in positive changes in platelet function, indicating that some of the constituents of berries, alone or sinergisitically, play a role in the reduction of CVD risks at normal levels (Erlund et al., 2008; Giongo et al., 2010; Alvarez-Suarez et al., 2014). Moreover, the strawberry has been recently studied for its potential contribution to the dietary management of hyperglycemia linked to type 2 diabetes and to the related complications of hypertension (Cassidy et al., 2011; Torronen et al., 2012; Kurotobi et al., 2012; Torronen et al., 2013; Tulipani et al., 2014)

Regarding cancer prevention, the principal chemopreventive agents present in berries include vitamins (vitamins A, C and E and folic acid), minerals such as calcium and selenium, dietary fibre, carotenoids, phytosterols such as sitosterol and stigmasterol, triterpene esters and phenolic compounds such as anthocyanins, flavonols, flavanols, proanthocyanidins, ellagitannins, and phenolic acids (Duthie 2007; Seeram 2008). Several studies showed that strawberry phenolics may suppress mutagenesis through genoprotective and antioxidative properties (Xue et al., 2001; Giampieri et al., 2015a). Strawberry extracts is also able to modulate cell signalling in cancer cells: (i) inhibiting cell proliferation of several type of cancers (Zhang et al., 2008), (ii) inducing cell cycle arrest and apoptosis (Seeram et al., 2006; Boivin et al., 2007; Somasagara et al., 2012), and (iii) suppressing tumour angiogenesis (Atalay et al., 2003).

Finally, the neuroprotective effect of strawberry in animal and human models have been also investigated in the last few years (Maher et al., 2011; Devore et al., 2012; Zhen et al., 2012), highlighting that strawberries could represent a promising powerful disease-fighting food, involved in the prevention of chronic degenerative pathologies or in support of traditional therapies for the best achievement of therapeutic goals.

3.7.3. Strawberry and inflammation

Dietary polyphenols are known to act as both anti-inflammatory and antioxidants molecules, thereby exerting a protective role against development of inflammation-related chronic diseases (Giampieri et al., 2012a; Giampieri et al., 2014c; Joseph et al., 2014; Giampieri et al., 2015a). In this context, the control of the inflammatory status, through the modulation of pro- and anti-inflammatory cytokine expression in immune cells by potential food components, could represent

an essential instrument to counteract immune disorders and to maintain a state of health and wellbeing (Liu and Lin, 2012; Dia et al., 2014). Only in last years, several *in vitro* and *in vivo* studies considering the effects of strawberries on inflammatory status have been published, in order to investigate their possible role in the inhibition on pro-inflammatory cytokines production.

In LPS-treated mouse macrophages and murine primary splenocytes and peritoneal macrophages, for example, the strawberry supplementation leads to the downregulation of NF-κB and mitogen-activated protein kinases (MAPK) signalling pathways, consequently reducing cytokine levels (TNF-α, IL-1β, IL-6), NO production and iNOS expressions (Liu and Lin, 2012; Liu and Lin, 2013; Lee et al., 2014; Dia et al., 2014) (Figure 23).

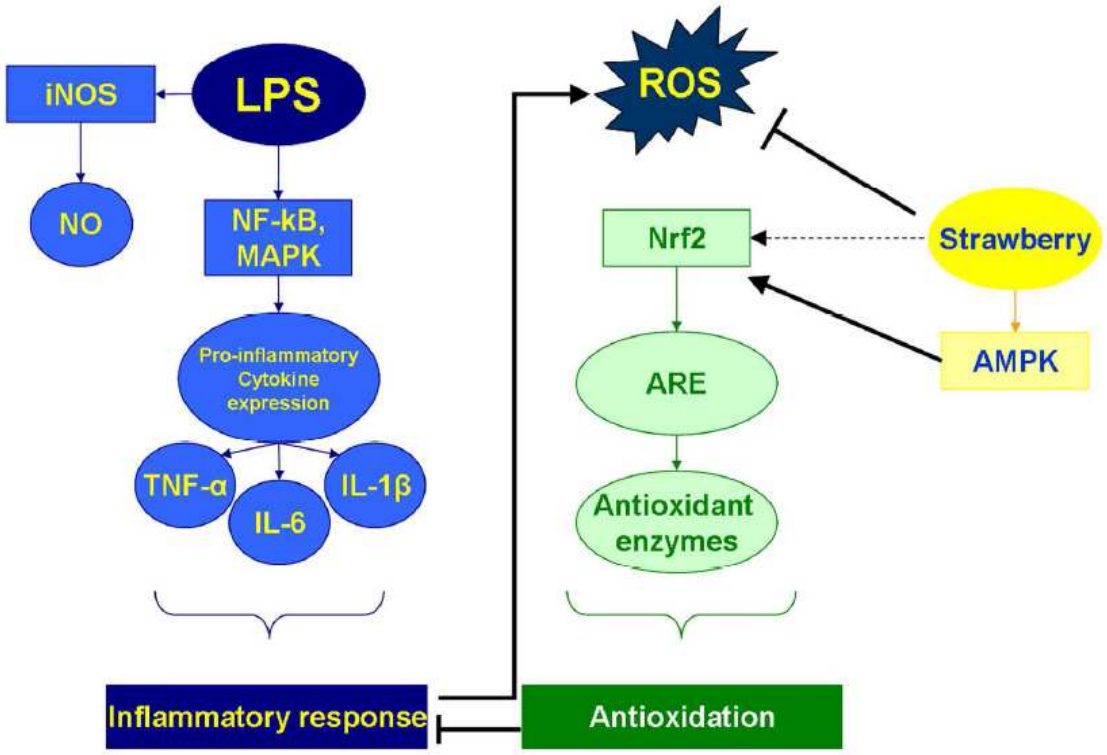


Figure 23. Proposed mechanism of the crosstalk between different molecular pathways induced by strawberries and LPS-stimulated inflammation. (taken from Frobes-Hernandez et al., 2015).

Similarly, strawberries appear to exert positive effects on the expression of anti-inflammatory cytokines: a significant increase in IL-10 levels has been found after strawberry treatment in the above mentioned cells (Liu and Lin, 2012; Liu and Lin, 2013).

In vitro anti-inflammatory effects have been also tested for isolated polyphenols present in strawberry. Specificlly, in LPS-stimulated RAW 264.7 cells (Kim et al., 2012) and in LPS-stressed mouse and human phagocytes and N9 microglial cells (Gelderblom et al., 2012), fisetin exerts anti-inflammatory activities suppressing pro-inflammatory intracellular pathways (activator protein-1 and NF-kB), with the subsequent reduction of pro-inflammatory cytokines (IL-6, TNF-α), pro-inflammatory mediators (iNOS and cyclooxygenase-2 (COX2)) and NO formation. These important findings demonstrate that strawberries, or their isolated compounds, are able to counteract an anti-inflammatory status by modulating the cellular pro-/anti-inflammatory cytokine secretion ratios.

Several *in vivo* studies taking into account the relationship between berry consumption and inflammation have been recently published (Joseph et al., 2014), but very little literature data investigated the involvement of strawberries in inflammation and in its related diseases in animal and human models (Giampieri et al., 2015a; Forbes-Hernandez et al., 2015)

In a mouse model of diet-induced obesity, regular strawberries intake contributes to the maintenance of blood glucose and is beneficial in regulating many aspects of inflammation and inflammatory-mediated dysfunction, as highlighted by the reduction of some systemic markers as C reactive protein (CRP), plasminogen activator inhibitor, IL-6 and TNF-α (Parelman et al., 2012).

The protective effect of strawberries has been also tested on atherosclerosis and thrombus formation. In mice models, strawberry consumption inhibits platelet aggregation decreasing the production of platelet pro-inflammatory molecules, that seem to be directly involved in the long-term atherosclerosis process and in triggering and propagation of acute coronary syndromes. This protective effect on thromboembolic-related disorders may be considered a novel anti-inflammatory effect of this berry (Alarcon et al., 2015).

Moreover strawberry consumption is able to reduce inflammation and pro-oxidant load in critical regions of the brain of rats exposed to 1.5Gy irradiation of ^{56}Fe particles (Shukitt-Hale et al., 2013; Poulose et al., 2014). Strawberry counteracts the neurochemical changes induced by these particles through the reduction of ROS production, induced by iNOS and NADPH oxidase 2 activation, (Poulose et al., 2014) and antagonizing the effects of oxidative and inflammatory signals, such as COX-2 and NF-kB (Shukitt-Hale et al., 2013).

Finally, in a mouse model of stroke, fisetin shows neuroprotective effects in cerebral ischemia through the inhibition of the infiltration of macrophages and dendritic cells into the ischemic hemisphere and suppressing the intracerebral immune cell activation as measured by intracellular TNFα production (Gelderblom et al., 2012).

In the same way, in a human cross-over design, the effect of strawberry antioxidants has been evaluated in a milk-based beverage form on meal-induced postprandial inflammatory and insulin responses. The postprandial test was conducted on overweight adults who consumed a high-carbohydrate, moderate-fat meal to induce acute oxidative and inflammatory stress, accompanied by a single serving of strawberries, for 6 weeks. The reduction in CRP, IL-6 and IL-1β levels and in postprandial insulin response provides evidence for the positive effect of an acute and medium strawberry consumption on postprandial inflammatory response and insulin sensitivity (Edirisinghe et al., 2011; Ellis et al., 2011).

Another chronic feeding study was performed in obese individuals subjected to an intervention with strawberry freeze-dried powder, for 3 weeks (Zunino et al., 2012). In this case, it was no observed any reduction in the different inflammatory markers (IL-1 β, IL-6, IL-8, TNF-α and CRP), even if a decrease in plasma concentrations of cholesterol and small HDL-cholesterol particles, and an increase of low density lipoprotein particle size was registered, suggesting a potential role of strawberries as a dietary instrument to reduce obesity-related disease (Zunino et al., 2012).

3.7.4. The influence of genotype on the nutritional quality of strawberry

Once it has become clear that the micronutrients and phytochemical compounds of the strawberries are responsible of their health-promoting effects, both biotechnological and breeding aspects were taken into account to increase the levels of these substances in the fruit, thus improving its nutritional quality.

The current knowledge of the molecular tools that are necessary to improve the biosynthesis and accumulation of antioxidant phytochemicals in strawberries is still poor, so that the biotech approach is only an integrative option to ameliorate the strawberry nutritional quality.

However, it is already known that the micronutrient and phytochemical composition of the fruit varies significantly depending on several pre- and post-harvest factors, such as:

- genetic factors**:** the genetic background has a very important role in the determination of nutritional quality in strawberries, since the content of micronutrients and phytochemicals can significantly vary between different cultivars (Scalzo et al., 2005a; Tulipani et al., 2008b; Diamanti et al., 2012; Mazzoni et al., 2013, 2016);
- enviromental factors: the nutritional value of strawberries may also be affected by the climatic conditions of cultivation, such as soil type, exposure to sunlight and moisture levels, which can determine the amount of micronutrients and phytochemicals (Lopes da Silva et al., 2007);
- agronomic techniques: different culture system could negatively affect the quality of the fruit, some examples are represented by (i) the nitrogen fertilization, that is capable to decrease the

vitamin C in fruits and also increase the amount of foliage in the plant reducing the light intensity and the accumulation of AA in the shadowed parts (Nagy, 1980), or (ii) the cultivation under tunnels covered with plastic film that is negatively correlated to the presence of antioxidants, which are produced by the plant mainly as a defense against pests or any kind of stress (related to the climate, the environment, etc.);

- ripening degree: the polyphenolic composition of strawberries varies according to the ripening degree and the harvesting period which is considered (Tulipani et al., 2011b; Mazzoni et al., 2013, 2016);
- storage: the short-term storage greatly influences both the nutritional quality and the profiles of strawberries phytochemical compounds and the storage temperature seems to be one of the key factors in the modification of the stability of phenolic antioxidants in fruits during post-harvest. (Kalt et al., 1999; Ayala-Zavala et al., 2004; Tulipani et al., 2008c; Piljac-Zegarac and Samec, 2011; Jin et al., 2011).

For all these reasons, the current strawberry genetic improvement programs (breeding), in addition to the priorities already set in the last decades (improvement of specific agronomic, qualitative, sensorial characteristics, improvement of yield, disease and pest resistance, plant adaptability and strawberry shelf life), is a very useful tool for the production of fruits that possess certain qualitative and quantitative characteristics. (Diamanti et al., 2012).

Nowadays the breeding process is involved not only in improving the nutritional quality of the strawberry, but it can be considered an important instrument for the meeting of the needs of consumers, which are always more demanding. So the genetic improvement today not only aims to select plants with high productivity, adequate resistance to diseases and insects, and perfect flower, but also tries to get a good texture, size and color of the fruit that is consistent with market demands, respecting qualitative and organoleptic parameters that satisfy the consumer, and providing a high nutritional value to the final product.

4. EXPERIMENTAL DESIGN

4.1. Part I: Evaluation of the nutritional quality of strawberry fruits

4.1.1. Objective

The first aim of this study was to assess and compare the nutritional and phytochemical quality of strawberry fruit extracts of different commercially available cultivars/varieties, obtained through

specific breeding programs (Adria, Alba, Cristina, Romina and Sveva), with the purpose to evaluate the influence of genetic background on these parameters. All genotypes were evaluated for their nutritional value, by measuring the Total Antioxidant Capacity through the Ferric Reducing Antioxidant Power (FRAP), the Trolox Equivalent Antioxidant Capacity (TEAC) and the 2,2-DiPhenyl-1-PicrylHydrazyl free radical method (DPPH), the Total Phenolic Content (TPC), the Total Flavonoid Content (TFC) and the Total Anthocyanin Content (ACY) of strawberry extracts. In addition to these common general assays, vitamin C amount was quantified in strawberry extracts by HPLC analysis. Moreover, identification and quantification of anthocyanins in the extracts were carried out in a diode array spectrophotometer (DAD) and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

Finally, folate contents were measured by a new HPLC methodology that was developed in order to classify and quantify which kind of folic acid-derivatives were present in the analyzed extracts. On the basis of the results obtained, Alba cultivar was chosen for the second part of the study, because it demonstrated the best nutritional profile among the different cultivars.

4.1.2. Materials & methods

All chemicals and reagents were bought from Sigma–Aldrich Chemical Company (Sigma-Aldrich, St. Luis, MO).

4.1.2.1. Strawberry material

For the study, the evaluation of strawberry fruit nutritional value was tested by analyzing fruits of 5 different varieties available in the experimental collections of Agricultural Faculty of Università Politecnica Marche (UNIVPM) located in Agugliano (AN), in central east of Italy.

All the fruits destined to analysis were harvested from plants grown in the experimental center, were hand-picked at the same day-time in different days, corresponding to the ripening times of the selected clones, from the second to the fourth picking, in the 2012 and 2013 cultivation years. Fruit samples were selected for homogenous fruit, avoiding wounded, shriveled or unripe fruits. The selected cultivars were 4 from Università Politecnica delle Marche license (Adria, Cristina, Romina and Sveva) and 1 from New Fruits license (Alba). Within 2 h after harvest, whole fruits were stored at -20°C before analyses. All the samples have been analyzed at UNIVPM laboratory.

4.1.2.2. Extraction method

According to the analysis to be performed, the compounds extraction was carried out *via* homogenization or *via* sonication.

For the evaluation of antioxidant capacity, TPC, ACY and TFC, the methanolic extract was prepared *via* homogenization.

Frozen strawberries were thawed for 60 min at 4°C. 10 grams aliquots of the fruits were added to 100 ml of the extraction solution, consisting of methanol/milliQ water/concentrated formic acid (FA) (80:20:0.1 v/v), and fruits were homogenized using an Ultraturrax T25 homogeniser (Janke & Kunkel, IKA Labortechnik) at medium-high speed for 2 min. Extraction was maximized by stirring the suspension for 2 h in the dark at room temperature, then tubes were centrifuged at 3500 rpm for 15 min in two sequential times, to sediment solids. Supernatants were filtered through a 0.45 μm Minisart filter (PBI International), transferred to 5.0 mL amber glass vials and stored at –20°C until analysis.

Anthocyanin extraction for HPLC-MS analysis was performed as previously described (Alvarez Suarez et al., 2011). Frozen strawberries (50 g) were homogenized in methanol containing 0.1% HCl, kept overnight (~14 h) at 3–5 °C and later filtered through a Büchner funnel under vacuum.

The solid product was exhaustively washed with methanol; the filtrates obtained were centrifuged (4000 x g, 15 min, 21 °C) and further submitted to the same process for the number of times necessary to complete color extraction. To remove liposoluble substances, the aqueous extract obtained was washed with n-hexane and then an aliquot (2 ml) of the aqueous phase was carefully deposited onto a C-18 SepPaks Vac 6cc cartridge (Waters). Sugars and more polar substances were removed by passing 15 ml of ultrapure water and anthocyanin pigments further eluted with 5 ml of methanol:0.1% trifluoroacetic acid (95:5). The methanolic extract was concentrated in a rotary evaporator at temperature < 30 °C, after adding water. The aqueous extract was collected, its volume completed to 2 ml with ultrapure water and filtered through a Minisart filter of 45 μm (PBI International, Milan, Italy) for HPLC analysis. For each strawberry cultivar, three independent extracts were prepared and analyzed.

For folate extraction, 5 g FW frozen strawberries were added to 15 ml of extraction buffer (0.1 M phosphate buffer containing 1.0% of L(+)-ascorbic acid (w/v) and 0.1% 2,3-Dimercapto-1-propanol (v/v) at pH 6.5, freshly prepared) in a 50 ml plastic centrifuge tube, and homogenized using an Ultraturrax T25 homogenizer (Janke & Kunkel, IKA Labortechnik) at medium-high speed for 2 min. The capped tube was then placed on a water bath at 100°C for 10 min, and then rapid cooled on ice. Tubes were then centrifuged at 4696 g for 20 min at 4°C. The supernatants were filled to an exact volume in 25 ml volumetric flasks with extraction buffer. For deconjugation of polyglutamylated folates, 175 μl of folate conjugase from rat serum was added to the 5 ml of extraction solution to another centrifuge tube, which was then incubated on a shaking oven at 37°C for 2 h. Rat serum (10 ml) for folate conjugase was dialyzed in three steps (40 min each) by using

800 ml of 50 mM phosphate buffer, pH 6.1, containing 0.1% 2,3-Dimercapto-1-propanol in each step. The dialysis was performed with stirring at 4 °C. Folate conjugase activity was checked using PteGlu3 as substrate in 0.1 M phosphate buffer, pH 6.1, containing 1% sodium ascorbate at 37 °C as described by Pfeiffer et al. (Pfeiffer et al., 1997). Concentrations of PteGlu3 and produced folic acid were measured by means of UV detection at 290 nm. The dialyzed rat serum was stored at -80 °C. It was frozen in small portions (0.5 ml) to elude the possible adverse effects of refreezing and rethawing on enzyme activity, that was always checked prior to use (Patring et al., 2005). Then, an additional treatment of 5 min at 100°C was carried out to inactivate the enzyme, again followed by cooling on ice. The samples were then centrifuged again at 4696g for 20 min at 4°C. The final surnatant was then filtered through 0.45 μm filter pore size, 25 mm inner diameter, nylon disposable syringe filters, and the filtrates were purificated through solid-phase extraction (SPE) on strong anion-exchange (SAX) Isolute cartridges (3 ml/500 mg of quaternary amine N+, counter ion Cl-, Supelco, Bellefonte, PA) as described by Jastrebova et al. and Iniesta et al. (Jastrebova et al., 2003; Iniesta et al., 2009). The cartridges were conditioned by washing with methanol (2.5 ml, 2 times) and water (2.5 ml, 2 times), followed by purification buffer (0.01 M dibasic potassium phosphate containing 1% L(+)-ascorbic acid (w/v) at pH 7.0, 2.5 mL, 2 times). Aliquots (2.5 ml) of the samples were applied to the cartridges and passed slowly with a flow rate not exceeding 1 drop/s. The elution of retained folates was performed slowly (flow rate not exceeding 1 drop/s) with 0.1M sodium acetate containing 10% sodium chloride (w/v), 1% L(+)-ascorbic acid (w/v), and 0.1% 2,3-Dimercapto-1-propanol (v/v). The first portion (0.7 ml) of eluate was discarded, and the second portion (3.8 ml) was collected and injected in HPLC (Shohag et al., 2011).

Finally, for vit C quantification, the extracting solution consisted in MilliQ water containing 5% meta-phosphoric acid and 1 mM EDTA. Vit C was extracted by sonication of 1 g of freeze strawberries in 4 ml of extracting solution, for 5 minutes, after a previous homogenization using an Ultraturrax T25 homogenizer (Janke & Kunkel, IKA Labortechnik) at medium-high speed for 2 min. After the extraction, the cell walls and proteins were precipitated by centrifugation at 2500 rpm for 10 min at 4°C, the surnatant was filtered through a 0.45 μm PTFE filter into 1.8 mL HPLC vials, and immediately analysed.

4.1.2.3. Measurement of total antioxidant capacity

Three methods were used for the determination of the TAC of strawberry extracts: the FRAP, the TEAC and the DPPH assays.

4.1.2.3.1. FRAP

The FRAP assay was conducted according to the protocol set by Deighton and co-workers (Deighton et al., 2000), with minor modifications respect to the original method proposed in 1996 by Benzie and Stain (Benzie and Strain, 1996). The antioxidant capacity of the sample is determined through its ability to reduce ferric (Fe^{3+}) to ferrous ion (Fe^{2+}). When iron is complexed with 2,4,6-tripyridyl-s-trizine (TPTZ) in sodium acetate solution at an acidic pH, its reduction determines a colour change of the solution, from pale rust to blue. The absorbance of the solution at 593 nm is directly proportional to the extent of reduction. The reducing power may be compared to that of the ferrous sulphate or Trolox, another aqueous antioxidant, often used as alternative standard. The FRAP reagent solution was prepared immediately before to procedure, by combining ten volumes of sodium acetate (300 mM, pH 3.6) with one volume of TPTZ (10 mM in HCl 40 mM) and one volume of ferric chloride (20 mM) aqueous solution. The sodium acetate and TPTZ solutions could be prepared in advance and stored at room temperature in (dark) glass bottles, whereas the ferric chloride solution must be freshly prepared the day of analysis; once prepared the FRAP reagent is stable for at least 2 hours at room temperature. Blank to set the spectrophotometer consisted in milliQ water, since interference from the extraction solvent was not supposed. Briefly, 100 µL of blank, Trolox or ferrous sulphate standard or 10-fold milliQ water diluted strawberry extract were added to 900 µL FRAP reagent into 1.5 ml eppendorf. The mix was then quickly vortexed for 15 seconds and after exactly 4 min following the addition of the sample to the FRAP reagent, the absorbance of the solution was read at 593 nm (Beckman spectrophotometer, DU644 model) against blank. Trolox or ferrous sulphate aqueous dilutions were used for calibration. All samples were analyzed in eight replicates and FRAP results were expressed as micromoles of Trolox (or Ferric Reducing) equivalents per gram of fresh weight of strawberry (µmol TxEq/g FW or µmol FeEq/g FW). Data were generally reported as a mean value ± standard deviation (SD).

4.1.2.3.2. TEAC

The TEAC assay was realized according to the method modified by Re and co-workers (Re et al., 1999).

This method is based on the ability of antioxidant bioactive compounds to quench the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation ($ABTS^{\cdot+}$) and reduce this radical form, which possesses maximal absorption in the visible range at 734 nm (Figure 24), into the colourless neutral form.

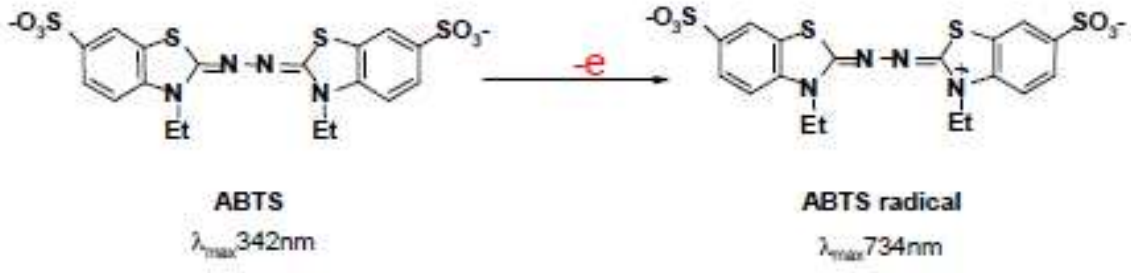


Figure 24. Reaction of radicalization of ABTS.

When biological or chemical samples, containing antioxidant species, react with $ABTS^{\cdot+}$, they scavenge the radical, detrmining an inhibition of absorbance.

The $ABTS^{\cdot+}$ solution was produced by reacting 7 mM of ABTS stock solution with 2.45 mM potassium persulfate, and maintained in the dark for 12 h before use. In this form, the $ABTS^{\cdot+}$ form resulted stable for at least two days, if appropriately stored at 25°C, in the dark. Immediately before analysis, 1 ml of the $ABTS^{\cdot+}$ radical solution (properly diluited 1:50 with PBS buffer at pH 7.4) is added to 10 µl of reagent, that is: (i) ethanol for the control of the standard curve, (ii) Trolox solution for the standard curve (Re et al., 1999; Pellegrini et al., 1999), (iii) methanol:water 80:20 for the control of the strawberry extracts or (iv) strawberry extract for the analysis. Blank to set the spectrophotometer consisted in milliQ water. After this step, the analysis solution must be vortexed for 20 seconds, and after 1-3 minutes the absorbance value must be read in the spectrophotometer at 734 nm. The % of color inhibition must be calculated following this formula:

$$\%\ \text{inhibition}_{734nm} = [(\text{control Abs}_{734nm} - \text{sample Abs}_{734nm})/\text{control Abs}_{734nm}] \times 100$$

TEAC value are determined comparing the % color inhibition with the standard calibration curve obtained with Trolox. Each samples were analyzed in eight replicates and the TEAC results were expressed as micromoles of Trolox equivalents per gram of fresh weight of strawberry (µmoles TxEq/g FW). Data were generally reported as a mean value ± SD.

4.1.2.3.3. DPPH

This method is based on the ability of DPPH to react with the phenolic compounds present in strawberry extracts. The DPPH radical is a persistent molecule, characterized by a violet color, which presents an absorption peak at 515 nm. As in the case of ABTS, when the radical species are

added to the sample, a reaction of reduction is carried out proportionally to the antioxidant species present; for this reason, also in this assay, a decoloration proportional to the antioxidant activity of the sample will be detected and the percentage of absorbance decrease at 515 nm of DPPH, is calculated by the equation:

$$\%\ \text{inhibition}_{515nm} = [(\text{control Abs}_{515nm} - \text{sample Abs}_{515nm})/\text{control Abs}_{515nm}] \times 100$$

The preparation of the solution of DPPH is required fresh made. To prepare 5 ml of 3 mM DPPH solution in methanol, it is necessary to weight 5,91 mg of DPPH. The obtained solution is stirred with a vortex and is kept in the dark until the moment of its use. To perform the assay it is necessary to dilute the concentrated solution of DPPH with methanol, in a way that gives an absorbance from 0.6 to 0.7.

The working solution was prepared in a test tube, putting 1.450 mL of DPPH diluted solution and 50 µl of sample, that could be water (for the control), strawberry extract, or Trolox (for the standards). Blank to set the spectrophotometer consisted in milliQ water. All the prepared solutions are stirred by vortexing and left in the dark for 1 hour; after that the samples absorbance is read at 515 nm. The evaluation of the antioxidant activity is always made through the construction of a calibration curve with Trolox: comparing the samples results (expressed as % inhibition) with the calibration line, it is possible to go back to their unknown antioxidant ability and quantify the strawberry antioxidant capacity. Each samples were analyzed in eight replicates and the results were finally expressed as micromoles of Trolox equivalents per gram of fresh weight of strawberry (µmoles TxEq/g FW). Data were generally reported as a mean value ± SD.

4.1.2.4. Measurement of Total Phenolic Content

The TPC of the strawberry extracts was determined using the Folin-Ciocalteu colorimetric method, as reported by Slinkard & Singleton (Slinkard and Singleton, 1977). Briefly, 100 µl of alternatively milliQ water for blank (to zeroing the spectrophotometer), water diluted strawberry extracts or gallic acid standard solutions (1/10) were added to 500 µl of Folin-Ciocalteau reagent previously water diluted (1/10) and kept in the dark, at 4°C. The mixture was incubated for about 5 min at room temperature, then 400 µl of 0.7 M sodium carbonate (Na_2CO_3) were added and the solution vortexed well. The final product was incubated for 2 h at room temperature (~23°C), in the dark, then the specific absorbance was read at 760 nm. As standard, a methanol:water (80:20, v/v) solution of 6 mM gallic acid (GA) was prepared, and stored at 4°C for at maximum one week.

Serial standard dilutions were daily prepared from the stock solution, for quantifications and for the realization of a calibration curve. Each sample was analyzed in eight replicates and the final results were expressed as milligrams of gallic acid equivalents per gram of fresh weight of strawberry (mg GAEq/g FW). Data were generally reported as a mean value ± SD.

4.1.2.5. Measurement of Total Anthocyanin Content

The ACY of the hydroalcoholic strawberry extracts was determined using a pH differential method previously described (Giusti and Wrolstad, 2001), with some modifications. The method depends on the reversible structural transformations of the anthocyanin chromophore in function of the pH: these changes appear through strikingly different absorbance spectra and thus can be measured using optical spectroscopy (Figure 25).

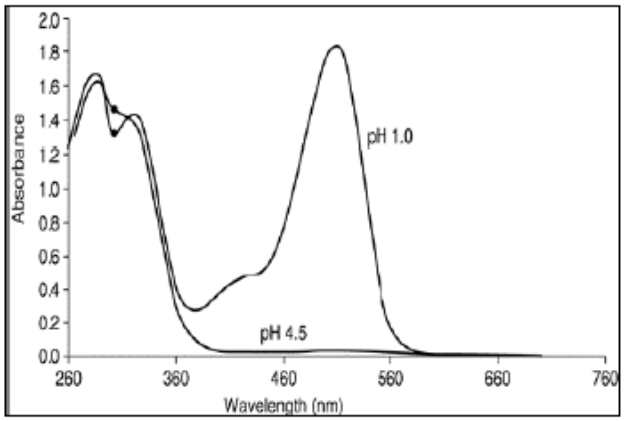


Figure 25. Spectral characteristics of purified radish anthocyanins (acylated pelargonidin-3-sophoroside-5-glucoside derivatives) in pH 1.0 and pH 4.5 buffers (taken by Giusti and Wrolstad, 2001).

The pH-differential method takes into account only the anthocyanin-specific reactions, and permits accurate and rapid measurement of the total anthocyanins, even in the presence of polymerized degraded pigments and other interfering compounds.

Briefly, 0.025 M potassium chloride (KCl) buffer, pH 1.0 and pH 4.5 buffer (buffer 1 and 2, respectively), and 0.4 M sodium acetate buffer (CH_3CO_2Na) were prepared. After that, two dilutions (1/10 v/v) of the strawberry extracts and of the standard solutions were prepared, one with buffer 1 and the other with buffer 2. These dilutions were let to rest for 15 min, before measuring the absorbance of each extract at 500 nm and at 700 nm to correct for haze, against a blank

represented by milliQ water (used to zeroing the spectrophotometer). The final absorbance of the diluted samples was calculated as follows:

$Abs = (Abs_{500nm} - Abs_{700nm})\ pH\ 1.0 - (Abs_{500nm} - Abs_{700nm})\ pH\ 4.5$

A Pg-3-glc stock solution (pH= 4) was used as standard, and was prepared by diluting 0.002 g of Pg-3-glc into 10 ml methanol:water 80:20, v/v (0.2 mg/ml): then it was aliquoted in amber glass vials and stored at –80°C until the analysis.
Absorbance values were converted to a concentration value through a calibration curve, obtained by plotting known concentrations of Pg-3-glc serial dilutions versus the corresponding absorbance calculated. Each sample was analyzed in eight replicates and the final results were expressed as milligrams of Pg-3-glc equivalents per gram of fresh weight of strawberry (mg PgEq/g FW). Data were generally reported as a mean value ± SD.

4.1.2.6. Identification and quantification of anthocyanins in "Alba" cultivar by HPLC-DAD-MS analysis

Analyses were performed in a Hewlett-Packard 1100 series liquid chromatograph. Separation was obtained on a 5 μm AQUA® C 18 150 mm x 4.6 mm column (Phenomenex, Torrance, CA) thermostated at 35 °C. Solvents used were: (A) 0.1% trifluoroacetic acid in water, and (B) HPLC grade acetonitrile, and the following isocratic gradient was established: 10 % B for 5 min, 10–15 % B over 15 min, 15 % B for 5 min, 15–18 % B over 5 min, and 18–35 % B over 20 min, using a flow rate of 0.5 ml/min. Double on-line detection was carried out in a DAD, using 520 nm as the selected wavelength, and a MS connected to the HPLC system via the DAD cell outlet. The mass spectrometer was a Finnigan LCQ (San Jose, CA) equipped with an ESI source and an ion trap mass analyzer, which was controlled by the LCQ Xcalibur software. Nitrogen was used as both auxiliary and sheath gas at flow rates of 6 and 1.2 L/min, respectively. The capillary voltage was 4 V and the capillary temperature 195 °C. Spectra were recorded in positive ion mode between *m/z* 150 and 1500. The MS detector was programmed to complete a series of three consecutive scans: (i) a full scan, (ii) a zoom scan of the most abundant ion in the first scan and (iii) a MS–MS scan of the most abundant ion, using a normalized collision energy of 45%. The anthocyanins amount were quantified from the areas of chromatographic peaks recorded at 520 nm by comparison with calibration curves obtained with external standards of Cyanidin-3-glucoside and of Pelargonidin 3-glucoside, for cyanidin-based and pelargonidin-based anthocyanins, respectively. Strawberry

extracts were analysed in triplicate and the results were expressed as mg/kg of FW. Data were generally reported as a mean value ± SD.

4.1.2.7. Measurement of Vitamin C Content

Vitamin C (ascorbic acid) content was evaluated as indicated by Helsper and co-workers (Helsper et al., 2003). Strawberry extracts were injected to HPLC system immediately after the sonication-extraction procedure. The HPLC equipment comprised a Waters 600 controller, a Waters 996 Photodiode array (PDA) detector set at absorbances of 262 and 244 nm, and a column incubator at 30 °C. The HPLC column used was a YMC Pack Pro 150x4.6 mm. The elution was isocratic with 50 mM potassium phosphate (KH_2PO_4) in MQ water, reaching the pH value of 3.2 (below the pKa of the ascorbic acid) by adding orthophosphoric acid. The analysis consisted in a 10 minutes run, at the end of the process the column was washed with 50% acetonitrile. Vit C eluted at RT ≈ 5.3 min. Quantification of the vitamin C content was realized through a calibration curve prepared by running standard concentrations of vitamin C similarly prepared in respect to the extracts, and measured in duplicate at the beginning and the end of the analysis. Each sample was analyzed in three replicates and the final results were expressed as mg vitamin C per gram of fresh weight of strawberry (mg vit C/g FW). Data were generally reported as a mean value ± SD.

4.1.2.8. Measurement of Total Flavonoid Content

TFC was determined by using a colorimetric method described previously (Zhishen et al., 1999; Dewanto et al., 2002). Briefly, 250 µl of alternatively water for blank, strawberry hydrophylic extract or (+)-Catechin standard solution was mixed to 1.25 ml of MilliQ water, following by addition of 75 µl of a 5% sodium nitrate ($NaNO_2$) solution. After 6 min, 150 µl of a 10% aluminium chloride hexahydrate ($AlCl_3$ *$6H_2O$) solution was added to the mix, and allowed to stand for 5 min. Finally, 500 µl of 1 M sodium hydroxide (NaOH) were added, the mixture was brought to 2,5 mL with MilliQ water and mixed well, and the absorbance was read immediately at 510 nm against blank (used to zeroing the spectrophotometer). For quantitative results, from a methanol:water (80:20, v/v) stock solution of (+)-Catechin, serial dilutions were prepared, and their known concentrations versus the corresponding absorbance were plotted. Each sample was analyzed in eight replicates and the final results were expressed as mg of catechin equivalents per gram of fresh weight of strawberry (mg CatEq/g FW). Data were generally reported as a mean value ± SD.

4.1.2.9. Folates identification and quantification

The chromatographic separation method described by Patring et al. and Jastrebova et al. (Patring et al. 2005; Jastrebova et al. 2003) was used, with slight modification, to determine individual folate from the strawberry folate extract. An HPLC system (Jasco PU-2089 Plus) was used, composed by a gradient binary pump, an UV detector (Jasco UV-2070 Plus), a fluorescence detector (FLD) (Jasco FP-2020 Plus), and a computer running ChromNAV software. The separation of folates was performed at room temperature, on a Mediterranea Sea18 250x4.6mm. The flow rate was 0.4 ml/min. The injection volume was 20 µl, with a total running time of 42 min for each sample. For the detection and quantification of folates, a FLD (excitation/emission = 290/360 nm for reduced folates and 360/460 nm for 10-HCO-folic acid) and an UV detector were used (290 nm). Peak purity and identity were confirmed by a comparison of relative peak areas in both detectors. The mobile phase was a binary gradient mixture of 30 mM potassium phosphate buffer at pH 2.3 and acetonitrile. The gradient started at 6% (v/v) acetonitrile for the first 5 min, afte that the acetonitrile content was raised linearly to 25% within 20 min and was kept constant for 2 min. Finally, it was decreased linearly to 6% acetonitrile for 1 min and was applied for 14 min to re-equilibrate the column. Quantification was based on an external standard method, in which the peak area was plotted against the concentration and the least-squares regression analysis was used to fit lines to the data. Each sample was analyzed in three replicates and the results were expressed as mg of each folate per gram of fresh weight of strawberry (mg folate/g FW). Data were generally reported as a mean value ± SD.

4.1.2.10. Statistical analysis

Statistical analyses were performed using STATISTICA software (Statsoft Inc., Tulsa, OK, USA). Data were subjected to one-way analysis of variance for mean comparison, and intergenotypes significant differences were calculated according to HSD Tukey's multiple range test.

Data were reported as mean ± SD. Correlations were calculated on a genotype mean basis, according to Pearson's Test. Differences at $p < 0.05$ were considered statistically significant.

4.1.3. Results

Genetic background seems to significantly affect all the studied parameters of fruit quality of the five strawberry varieties analyzed. Significant differences among cultivars were outlined both by using more approximate general assays and HPLC fine determinations.

4.1.3.1. Total antioxidant capacity

TAC has been measured through three different methodologies: FRAP, TEAC and DPPH tests. For TEAC and DPPH assays, Alba showed the higher antioxidant capacity, with values of 22.64 and 7.71 µmol TxEq/g FW, respectively. In the case of FRAP, the cultivar Sveva present the highest value (14.94 µmol TxEq/g FW) but Alba is not statistically different from it, with a value of 14.43 µmol TxEq/g FW. The lowest TAC values were found in the cultivars Adria (for TEAC and DPPH assays, with values of 14.07 and 3.74 µmol TxEq/g FW, respectively) and Cristina (for FRAP assay, with 8.23 µmol TxEq/g FW) (Figure 26).

Cultivar	*Antioxidant Capacity*			
	FRAP (µmol TxEq / g FW)	FRAP (µmol FeEq / g FW)	TEAC (µmol TxEq / g FW)	DPPH (µmol TxEq / g FW)
Alba	14.43 ± 0.27 ^{a}	22.85 ± 0.39 ^{a}	22.64 ± 0.49 ^{a}	7.71 ± 0.32 ^{a}
Cristina	8.23 ± 0.08 ^{b}	13.91 ± 0.12 ^{b}	16.07 ± 0.81 ^{b}	4.45 ± 0.05 ^{c}
Sveva	14.94 ± 0.38 ^{a}	23.59 ± 0.57 ^{a}	22.59 ± 0.90 ^{a}	7.68 ± 0.31 ^{a}
Romina	14.23 ± 0.17 ^{a}	22.56 ± 0.25 ^{a}	21.23 ± 1.00 ^{a}	6.61 ± 0.40 ^{b}
Adria	8.66 ± 0.06 ^{b}	14.41 ± 0.09 ^{b}	14.07 ± 0.57 ^{b}	3.74 ± 0.16 ^{d}

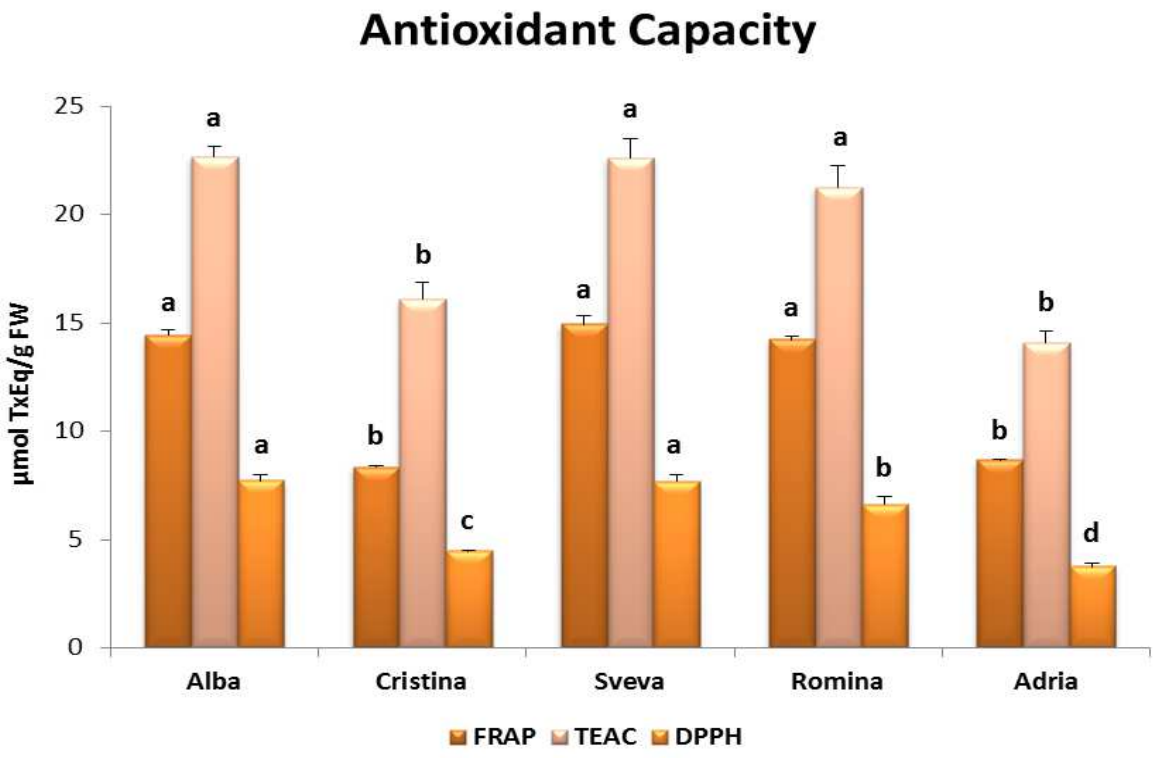


Figure 26. Total antioxidant capacity, measured by FRAP, TEAC and DPPH assays, of the five strawberry genotypes tested. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.1.3.2. Total Phenolic Content

TPC measured in the five strawberry cultivars showed that Alba contained the higher amount, with a value of 2.52 mg GAEq/g FW. Sveva and Romina possessed intermediate values (2.19 and 2.18 mg GAEq/g FW respectively), while the lower TPC was detected in Adria (1.48 mg GAEq/g FW) and Cristina (1.45 mg GAEq/g FW) (Figure X).

4.1.3.3. Total Anthocyanin Content

Regarding ACY content, Romina showed the higher values, with 0.51 mg PgEq/g FW. Following, Alba and Adria presented an amount of 0.38 and 0.29 mg PgEq/g FW, respectively. Finally, Sveva, with a value of 0.22 mg PgEq/g FW and Cristina, with 0.18 mg PgEq/g FW, showed the lower ACY value among the tested cultivars (Figure X).

4.1.3.4. Vitamin C Content

Vitamin C content represents a fundamental parameter to assess the nutritional quality of strawberry. Among the five evaluated varieties, the highest value for vitamin C was measured in cultivars Alba and Cristina, with 0.58 and 0.57 mg vitC/g FW, respectively. Lower and statistically different values of vitamin C were detected in the cultivar Sveva (0.48 mg vitC/g FW) and Romina (0.45 mg vitC/g FW). The lowest amount of vitamin C was found in Adria cultivar, which possessed 0.40 mg vitC/g FW (Figure X).

4.1.3.5. Total Flavonoid Content

Concerning TFC, the cultivar Sveva showed the higher value, 0.70 mg CatEq/g FW, statistically higher with respect to all the other cultivars. After Sveva, the cultivars that possesssed the highest TFC were Alba and Romina, with 0.66 and 0.61 mg CatEq/g FW, respectively. Statistically lower than Alba and Romina was Cristina, with 0.40 mg CatEq/g FW. Finally, Adria variety registered the lowest value of TFC, 0.35 mg CatEq/g FW, statistically different from all the other measures (Figure 27).

Cultivar	*Bioactive Compounds*			
	TPC (mg GAEq / g FW)	ACY (mg PgEq / g FW)	vit C (mg vitC / g FW)	TFC (mg CatEq / g FW)
Alba	2.52 ± 0.01^{a}	0.38 ± 0.01^{b}	0.58 ± 0.02^{a}	0.66 ± 0.02^{b}
Cristina	1.45 ± 0.04^{c}	0.18 ± 0.01^{d}	0.57 ± 0.04^{a}	0.40 ± 0.01^{d}
Sveva	2.19 ± 0.09^{b}	0.22 ± 0.02^{d}	0.48 ± 0.04^{b}	0.70 ± 0.02^{a}
Romina	2.18 ± 0.03^{b}	0.51 ± 0.03^{a}	0.45 ± 0.02^{b}	0.61 ± 0.02^{c}
Adria	1.48 ± 0.03^{c}	0.29 ± 0.04^{c}	0.40 ± 0.03^{c}	0.35 ± 0.01^{e}

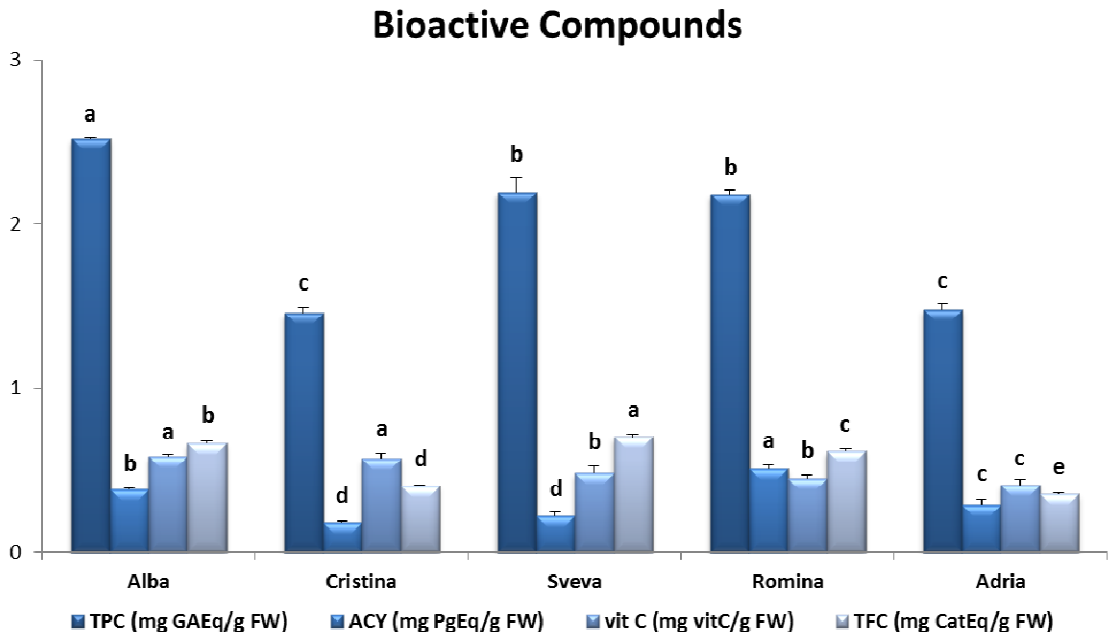


Figure 27. Total bioactive compounds content, consisting of TPC, ACY, vitC and TFC of the five strawberry cultivars tested. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.1.3.6. HPLC-MS Analysis of anthocyanins in "Alba" cultivar

In Alba cultivar eight anthocyanin pigments were identified. The compounds were detected on the basis of their UV-Vis and mass spectra obtained by HPLC-DAD-ESI/MS in positive mode, as well as their chromatographic behavior compared to external standards. In Figure 28 a representative HPLC chromatograms of anthocyanin profiles in Alba cultivar is showed.

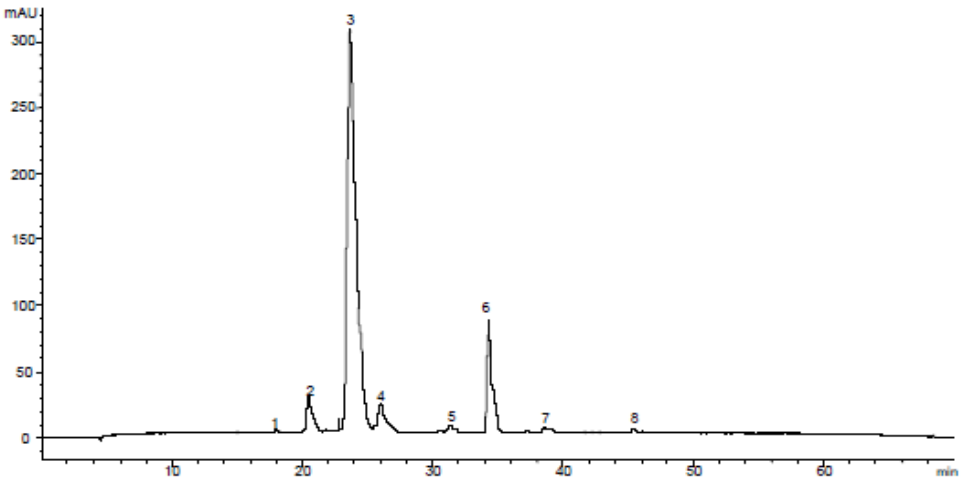


Figure 28. Representative HPLC-DAD chromatograms recorded at 520 nm showing the anthocyanin profiles of Alba strawberry. Peaks: 1, (Epi)afzelechin-(4→8)Pg 3-glucoside; 2, Cy-3-glucoside; 3, Pg 3-glucoside; 4, Pg 3-rutinoside; 5, Cy 3-malonylglucoside; 6, Pg 3-malonylglucoside; 7, Pg 3-acetylglucoside, 8, Pg 3-succinylarabinose.

Moreover, peak data obtained in HPLC-DAD-MS analysis (RT in the HPLC system, λmax in the visible region, molecular ion and main fragments observed in MS^2) are summarized in (Table 4).

Peak	Rt (min)	λmax (min)	[M⁺] (*m/z*)	Molecular Ion MS^2 (*m/z*)	Tentative identification	Total anthocyanin content (mg/Kg of FW)
1	21.7	515	705	543, 407, 313, 271	(Epi)afzelechin-(4→8)Pg 3	4.65 ± 0.21
2	23.2	515	449	287	Cy-3-glucoside	31.11 ± 0.28
3	27.3	502	433	271	Pg-3-glucoside	397.40 ± 1.3
4	29.3	503	579	433, 271	Pg-3-rutinoside	38.67 ± 1.6
5	33.7	n.a.	535	287	Cy 3-malonylglucoside	9.05 ± 0.28
6	37.7	504	519	271	Pg 3-malonylglucoside	66.94 ± 0.44
7	40	504	475	271	Pg 3-acetylglucoside	3.87 ± 0.04
8	41.8	508	533	271	Pg 3-succinylarabinose	5.25 ± 0.3

Table 4. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, tentative identification and total anthocyanin content in Alba cultivar. Values expressed as means (mg/kg of FW) ± SD.

In addition to the compounds indicated in the table, other minor pigments were also detected, although no good absorption or mass spectra were obtained to confirm their identity.

Pelargonidin shows a characteristic UV-vis spectrum peak with λmax in the region of visible at lower wavelengths than other common anthocyanins and an additional maximum about at 430 (Lopes da Silva et al., 2002; Lopes da Silva et al., 2007; Buendia et al., 2010). Based on this spectral profile, the peaks corresponding to Pg-derived anthocyanins could be easily identified in the chromatograms. The presence of Pg as anthocyanin in those peaks was also confirmed by their mass spectra, which showed MS^2 signal at *m/z* $[M]^+$ 271. The major peak in the HPLC chromatograms corresponded to Pg-3-glc, firstly identified by Robinson and Robinson (Robinson and Robinson, 1931) and which represent the most known and reported anthocyanin in strawberry (Lopes da Silva et al., 2002; Lopes da Silva et al., 2007; Buendia et al., 2010). In addition to this, up to five peaks could be assigned to Pg derivates (peaks 3, 4, 6, 7, 8). Moreover, another important

compound previously reported in several strawberries varieties (Lopes da Silva et al., 2002; Lopes da Silva et al., 2007;Buendia et al., 2010) was detected in the sample. It is Pg 3-malonylglucoside which presents characteristic UV-vis (λmax at 504 nm) and mass spectra molecular ion at *m/z* 519 (releasing a unique MS^2 fragment at *m/z* 271 $[M+H]^+$ corresponding to Pg).

Pg 3-acetylrutinoside has been also identified in the extract, and showed a molecular ion $[M+H]^+$ at *m/z* 579, releasing a major MS^2 fragment at *m/z* 271 (Lopes da Silva et al., 2002).

Moreover, condensed pigments containing C–C linked anthocyanin (Pg) and flavanol (afzelechin) residues were detected (peak 1). This compound showed a molecular ion $[M+H]^+$ at *m/z* 705, releasing major MS^2 fragments at *m/z* 543, 407, 313 and 271. Its UV-vis, mass spectrum and retention time were consistent with the (Epi)afzelechin-(4→8)-Pg 3-glucoside.

Finally, cyanidin (Cy) derivative compounds were identified (peaks 2 and 5), based on the presence of a signal at *m/z* $[M]^+$ 287 in their MS^2 spectra (Lopes da Silva et al., 2002; Alvarez-Suarez et al., 2011; Giampieri et al., 2012b).

4.1.3.7. Folates identification and quantification

After the injection of folate extracts in HPLC, three peaks were detected in HPLC, through the FLD. Two of these peaks were recognized and quantified as 5-methyltetrahydrofolic acid and Folinic acid calcium salt hydrate, through the injection of pure standards in HPLC, while the third peak remained unknown (Figure 29).

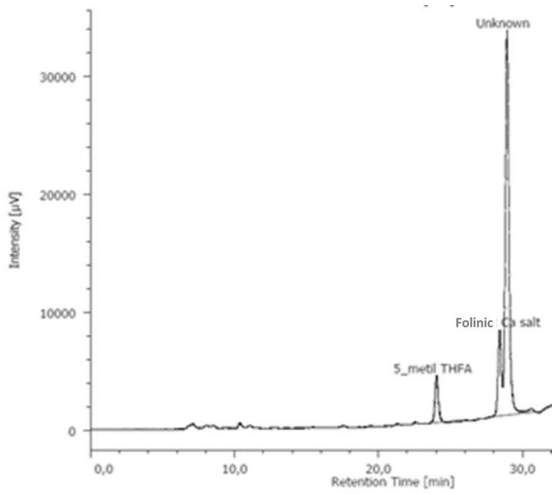


Figure 29. HPLC chromatogram of folate detection. The first two peaks indicate the 5-methyltetrahydrofolic acid and the Folinic acid calcium salt hydrate, respectively. The third peak represent the unknown folate to be identified.

Further analysis are necessary to recognize the nature of this peak and also quantify the concentration of this folate. Regarding the known peaks, Sveva possessed the highest concentration of 5-methyltetrahydrofolic acid, with a value of 7.8 µg/100g FW. Romina and Alba were statistically similar to Sveva, but with a lower amount (6.84 and 6.2 µg/100g FW respectively). The lowest value was detected for Cristina and Adria, with values of 5.89 and 5.29 µg/100g FW respectively (Figure X). For the folinic acid calcium salt hydrate, instead, the highest value was detected for Cristina (2.37 µg/g FW), that was statistically higher than all the others cultivars. Romina, Sveva and Alba were statistically different from Cristina and showed levels of Folinic acid calcium salt hydrate of 1.22, 1.06 and 0.99 µg/g FW respectively. The lowest value was detected for Adria, with 0.23 µg/g FW (Figure 30).

Cultivar	*Folates Compounds*	
	5-methyltetraidrofolic acid (µg / 100g FW)	folicin acid calcium salt idrate (µg / g FW)
Alba	6.20 ± 0.62^{ab}	0.99 ± 0.09^{b}
Cristina	5.89 ± 0.59^{b}	2.37 ± 0.23^{a}
Sveva	7.80 ± 0.78^{a}	1.06 ± 0.10^{b}
Romina	6.84 ± 0.68^{ab}	1.22 ± 0.12^{b}
Adria	5.29 ± 0.53^{b}	0.23 ± 0.02^{c}

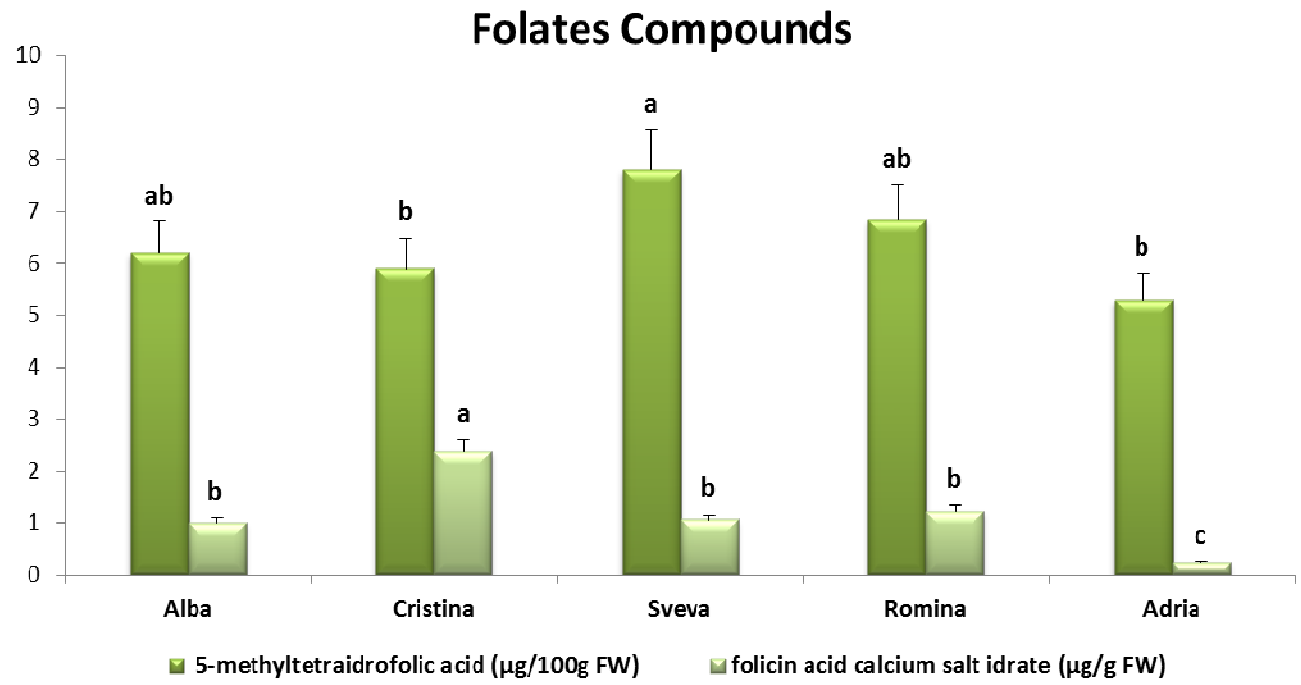


Figure 30. Known folates content, consisting of 5-methyltetrahydrofolic acid and folinic acid calcium salt hydrate of the five strawberry genotypes tested. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.1.3.8. Interrelationship among bioactive compounds

To evaluate whether the contents of different compounds co-varied, linear correlations were calculated for the whole material, paying particular attention on total antioxidant capacity (Table 5).

	FRAP	TEAC	DPPH	TPC	ACY	vit C
TEAC	0.971 *					
DPPH	0.966 *	0.994 **				
TPC	0.950 *	0.952 *	0.956 *			
ACY	0.513 ns	0.409 ns	0.352 ns	0.554 ns		
vit C	0.900 ns	0.815 ns	0.684 ns	0.622 ns	-0.235 ns	
TFC	0.981 *	0.992 **	0.993 **	0.936 *	0.365 ns	0.642 ns

Table 5. Pearson's correlation coefficients for quantitative determinations in the selected strawberry cultivars. 95% confidence interval. ns, non-significant; *, significant at $p < 0.05$; **, significant at $p < 0.001$.

First of all, it is important to highlight that the assays for the evaluation of TAC significantly correlated among them. This result is very interesting because, even if the final results of each assay produced different values, the means of the results were the same. In particular, the correlation between TEAC and DPPH is $r = 0.994$, $p = 0.001$, between FRAP and TEAC is $r = 0.971$, $p = 0.006$ and between DPPH and FRAP is $r = 0.966$, $p = 0.007$.

The concentration of total phenolic compounds significantly correlated with all the different methods used to evaluate TAC ($r= 0.950$, $p= 0.013$ with FRAP; $r= 0.952$, $p= 0.012$ with TEAC and $r= 0.956$, $p= 0.011$ with DPPH) and with flavonoids concentration ($r= 0.936$. $p= 0.019$). On the contrary, TPC is not correlated with other bioactive compounds as ACY and vitamin C, with $r= 0.554$, $p= 0.333$ and $r= 0.622$, $p= 0.256$, respectively.

Similarly, and in agreement with previous studies (Prior et al., 1998; Deighton et al., 2000), the association between anthocyanins content and the TAC of the fruit, is not evident in strawberry, since that no significance correlations were found by the Pearson's test between ACY and FRAP (r= 0.513, p= 0.377), TEAC (r= 0.409, p= 0.494) and DPPH (r= 0.352, p= 0.561).

These findings suggested that, even though individual anthocyanins have remarkable antioxidant properties, several other phenolic compounds seem to give a more important contribution to the TAC of the fruits. Moreover, as previously observed (Tulipani et al., 2008b), no significant relationships were found between ACY and vitamin C (r= -0.235, p = 0.703). These results should be related to the different chemical reactions, principles and reference standard compounds to which the methods are based on.

Also for vitamin C, the association between this compound and TAC was not detected, since that the values were r= 0.900, p= 0.086 with FRAP, r= 0.815, p=0.160 with TEAC and r= 0.684, p= 0.211 with DPPH. Furthermore, as observed by Tulipani et al. (Tulipani et al., 2008b), the association between vitamin C and TFC is not present, since no significant correlation was found (r= 0.642, p= 0.213).

Finally, flavonoids did not show an association with ACY (r= 0.365, p= 0.545), but significantly correlated with FRAP, TEAC and DPPH (r= 0.981, p= 0.003; r= 0.992, p= 0.001 and r= 0.993, p= 0.001, respectively), suggesting that these antioxidants provide an important contribute to the TAC of the fruit, and confirming precedent similar findings for strawberries and several species of berries (Deighton et al., 2000; Olsson et al., 2004).

4.1.4. Discussion

The first aim of this PhD project was to evaluate and compare the nutritional and phytochemical quality of strawberry fruit extracts of different commercially available varieties in order to investigate the influence of genetic background on these parameters.

Strawberries (*Fragaria x ananassa*) were chosen as the fruit model for this study due to their high content of antioxidants and bioactive componds, since they are important berries both for fresh consumption and for the food industry (Giampieri et al., 2012a).

Many published studies showed that strawberries possess high antioxidant capacity (Wang et al., 2002; Scalzo et al., 2005a; Scalzo et al., 2005b; Tulipani et al., 2008b) and contain a significant amount of ascorbic acid, anthocyanins, phenolic compounds and folates (Olsson et al., 2004a; Tulipani et al., 2008b).

In the last decades, particular attention has been payed to the antioxidant power of fruit as an eligible parameter for quality and as an indicator of beneficial bioactive compounds present in strawberries and, therefore, of their healthfulness.

This parameter is strictly correlated to the presence of efficient oxigen radical scavengers, such as vitamin C and polyphenols, especially anthocyanin and ellagic acid, which represent highly reactive species that act in plants as antioxidants and protective agents against several sources of damage (UV, pathogens, etc.).

Moreover, it should be kept in mind that only recently it has been demonstrated that the high TAC of strawberries is strongly influenced by species and cultivar (Capocasa et al., 2008b; Tulipani et al., 2008a; Tulipani et al., 2008b; Tulipani et al., 2009b; Tulipani et al., 2011b) and, on this basis, particular breeding programs have been applied in order to create new varieties with the purpose of increase the content of specific health-related compounds in fruits (Battino and Mezzetti, 2006; Diamanti et al., 2010; Scalzo et al., 2005a).

In this context, Adria, Cristina, Romina and Sveva represent the results of such genetic improvement program of Università Politecnica delle Marche.

Furthermore, many studies also suggest that strawberry phenolics show a wide range of biological activities in the prevention of inflammation, oxidative stress, cardiovascular disease, certain types of cancers, type 2 diabetes and obesity (Carlton et al., 2001; Pajk et al., 2006; Mazza, 2007; Pinto Mda et al., 2010, Basu et al., 2010), even if the mechanisms by which this berry exerts these capacities are several and not yet completely understood.

Many of the biological actions of phytochemicals have been attributed to the antioxidant capacity of strawberries, that represents one possible and relevant mechanism directly involved in the reduction of oxidative stress, by decreasing malondialdehyde formation, protecting LDL from oxidation and protecting mononuclear blood cells against increased DNA damage (Azzini et al., 2010; Tulipani et al., 2011a).

Therefore, the antioxidant capacity of fruit or individual components represents a useful parameter to couple with other measurements.

The results of the FRAP, TEAC and DPPH assays for TAC obtained in the present work were slightly different but with closely similar trends, suggesting that the three assays are almost comparable and inter-changeable in case of strawberry, as highlighted by the strong significant correlation that was previously observed (Table 5). Among these tests, the TEAC assay gave higher values than the corresponding FRAP values, while the DPPH assay, that highlighted more inter-genotypes significant differences among the analyzed varieties than the other tests, showed the lower results. On the basis of the obtained results, Alba and Sveva cultivars resulted the best

varieties in term of TAC; on the contrary Adria and Cristina presented, in all the tests applied, the lower values. The evaluation of the antioxidant capacity of strawberry genotypes contributed to give an overview on their capacity and potential to reduce oxidative reactions that can cause negative effects on human health.

Strawberries are important for their extremely high content of vitamin C (even higher than citrus fruit), which makes them an important source of this vitamin for human nutrition. Vitamin C is an essential compound, with several biological functions: different observational as well as case-control studies indicate that higher intakes of vitamin C (from diet or supplements) are often correlated with a lower incidence of cardio and cerebrovascular diseases (Carr et al., 1999a; Yokoyama et al., 2000), most types of cancers (Steinmetz and Potter, 1996), lead toxicity-health problems (Houston and Johnson, 2000), and seem to be a beneficial aid to conventional therapies for individuals with atherosclerosis, hypertension and diabetes mellitus (Duffy et al., 1999; Dawson et al., 1999). Moreover, it is generally accepted that this compound is one of the most important free radical scavengers in plants, animals and humans (Giampieri et al., 2012a).

When evaluating vitamin C content in strawberry fruits, it is important to consider that the molecule is very labile and under adverse conditions undergoes oxidation, depending on several factors such as temperature, water and pH (Giampieri et al., 2012a; Sapei and Hwa, 2014).

However, the methodology applied in this work to extract and analyze vitamin C content in the fruits was particularly optimized to limit and prevent any degradation process. In any case, the oxidation of the active form of the vitamin, L-ascorbic acid, to dehydroascorbic acid (DHA) does not determine any loss in the vitamin C biological activity, due to the rapid reconversione of DHA into L-ascorbic acid. The results obtained in this work showed that vitamin C content varied among the five analyzed strawberry cultivars, with the higher values registered for Alba and Cristina, followed by Sveva, Romina and Adria. It is interesting to note that vitamin C concentration is not strongly related to the antioxidant capacity of the analyzed strawberries, even if the cultivar that possessed the higher vitamin C concentration (Alba) was the same which showed the higher antioxidant capacity.

Recently, strawberry bioactive phytochemicals with high antioxidant properties such as many phenolic compounds, together with vitamin C, have received a wide attention especially for their potential benefits on human health. Numerous biological activities, ranging from anticancer to anti-inflammatory, neurodegenerative and antioxidant effects, have been proved, stimulating a detailed phytochemical investigation to identify the phenolic compounds present in strawberries.

Flavonoids, represent the main phenolic representative class in strawberry and the most abundant compounds belonging to the subgroups of anthocyanins, flavonols quercetin and kaempferol, and

flavanols. In the present study TFC showed a high correlation with all the antioxidant activity assays, in particular with TEAC and DPPH.

Among phenolics, anthocyanins in strawberry are the best known compounds and quantitatively the most important (Giampieri et al., 2012a), so that more than 25 different anthocyanin pigments have been described from different varieties and selections (Lopes da Silva et al., 2002).

In this work 8 anthocyanin pigments were identified, of which Pelargonidine derivatives were predominant, followed by the Cyanidin derivatives. These compounds have been previously described in strawberries by many authors, considering anthocyanins, together with ascorbic acid, folates and ellagitannins, as one of the most important phenolics in strawberries being responsible for different beneficial actions in human health (Robinson et al., 1931; Lopes da Silva et al., 2002; Lopes da Silva et al., 2007; Buendia et al., 2010).

Among the different analyzed cultivars, Romina and Alba showed the highest content of ACY. Alba showed also the higher content of TFC and of TPC: this could explained the high antioxidant capacity registered for Alba with respect to the other cultivars.

Together with ascorbic acid, phenols and ellagitannins, folates represent one of the most important compounds in strawberries being responsible for several beneficial actions in human health, as previously described by many authors (Lopes da Silva et al., 2002, 2007; Buendia et al., 2010). Folates represent a group of essential dietary compounds necessary for cell replication and for their important role in the prevention of possibly coronary heart diseases (Graham et al., 1997; Rimm et al., 1998; Brouwer et al., 1999) and neural tube defects (Czeizel and Dudas, 1992; Berry et al., 1999). Furthermore, some studies also suggested that a correct folate intake is positive for cognitive functions (Seshadri et al., 2002) and for the prevention of certain forms of cancer (Giovannucci et al. 1995). In the present work, the evaluation of the folates content was assessed with a novel HPLC method previously utilized only in spinach, and so of great novelty for the application on strawberry. Among the five analyzed cultivars, Sveva, followed by Romina and Alba, showed the highest content of 5-methyltetrahydrofolic acid. On the contrary, Cristina possessed the higher content of folinic acid calcium salt hydrate. These results seem to confirm that the genotypes deriving from Università Politecnica delle Marche breeding program, in particular Sveva, Romina and Cristina, showed all good performances in terms of folate concentrations even if a more in-depth analysis on folate identification and quantification must be performed, , and further research are on the way to better characterize the complete folates profile of strawberries.

On the basis of the above findings , the strawberry cultivar chosen for the second part of the study was Alba, for several reasons. First of all, Alba was one of the cultivar with the highest TAC and with very high concentrations of vitamin C, ACY and TFC. Regarding folates, Alba presented a

medium-high concentration of all the folates and no one of the other cultivars possessed a clearly highest folate concentration with respect to all the other cultivars. Finally, another important reason for the choice of Alba to be tested in cells is that our research team has recently obtained very interesting results with the use of this cultivar, both *in vitro* (Giampieri et al., 2014a; Giampieri et al., 2014b) and *in vivo* studies (Romandini et al., 2013; Alvarez-Suarez et al., 2014).

4.2. Part II: : The role of strawberry in the modulation of inflammatory response induced by LPS

4.2.1. Objective

The second aim of this PhD project was to evaluate the effects of methanolic purified extracts from Alba cultivar on inflammatory status induced by *E.Coli* LPS on two different cell lines, human dermal fibroblast (HDF) and RAW 264.7 macrophages.

In order to assess the effect of strawberry treatment on these two cell lines, in presence or absence of LPS, the cell viability (MTT assay), apoptosis rate and ROS intracellular production assays were performed. On the basis of the obtained results, specific concentration values of strawberry methanolic extracts and LPS were chosen to estimate the principal biomarkers related to inflammatory (TNF-α, IL-1β, IL-6, IL-10) and oxidative stress (NO, TBARS and carbonyl groups). The activity of the principal antioxidant enzymes (catalase, SOD, GSH, GPx, GR, GST) was also tested.

Protein expression was analysed to clarify the principal molecular pathways involved in LPS and strawberry mechanisms of action, such as NFkB/IκBα, iNOS, Nrf2 and AMPK and its related pathway (SIRT1 and PGC1-α). The level of some antioxidant enzymes (catalase, SOD and heme-oxygenase 1 (HO-1)) and the possible DNA damage, through OGG1 expression, were also evaluated.

Finally, the oxygen consumption rate (OCR) related to mitochondria functionality were tested after strawberry and LPS treatments.

4.2.2. Material and methods

All chemicals and reagents were bought from Sigma–Aldrich Chemical Company (Sigma-Aldrich, St. Luis, MO).

4.2.2.1. Cell culture

HDF were human dermal fibroblasts isolated from adult skin provided by GIBCO® Invitrogen cell culture-Life Technologies Corporation.

RAW 264.7 murine macrophage cell line was purchased from American Type Culture Collection (ATCC) and was derived from a murine tumor induced by Abelson leukemia virus.

Both HDF and RAW macrophages cell lines were plated into a T-75 flasks and cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, stable glutamine and sodium pyruvate, supplemented with 10% fetal bovine serum (FBS) heat-inactivated (65°C for 20 min) and 1% penicillin-streptomycin antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) (Nicolin et al., 2005; Giampieri et al., 2012b; Norowski et al., 2012; Giampieri et al., 2014b; Gasparrini et al., 2015). Cells were manteined in HeraCell CO_2 incubator at 37°C with 5% CO_2 and the medium was changed every 2–3 days. All the test and the different pellet preparations were conducted on cells between the 4^{th} and the 6^{th} passage.

4.2.2.2. Strawberry for cell treatment

Methanolic extract of Alba cultivar was dried with the miVac Duo Concentrator, a centrifugal vacuum concentrator suitable for use with a wide range of solvents, from volatile organic solvents through water. The dry strawberry extract obtained was weighted and then directly dissolved in the cell cultivation medium (DMEM) accordingly to the test to perform.

4.2.2.3. MTT viability assay

The MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole)) assay represent a quick and comprehensive colorimetric method used for assessing cell viability: it is based on the ability of viable cells to reduce a soluble MTT to blue formazan crystals (Lyu and Park, 2012).

The test was peformed according to what was previously described by Moongkarndi et al. (Moongkarndi et al., 2004), with some modifications.

Briefly, HDF and RAW macrophages cell lines were seeded into 96-well plates at a density of 5×10^3 cells/well and let them adhere for 16-18 h.

After the cells were adherent, they were incubated with (i) strawberry, (ii) LPS or (iii) strawberry + LPS, in order to evaluate the effect of the different treatments, along or in combination, on the cell viability rate.

Both HDF and RAW macrophages cell lines were incubated for 24, 48 and 72 h with 200 µl of different concentrations of dried strawberry extract (25, 50, 100, 250, 500 µg/ml and 1, 2.5, 5, 7.5, 10 mg/ml), weighted and then directly dissolved in the cell cultivation medium.

To test the effect of LPS on cell viability the HDF were incubated with 200 µl of 0.1, 1, 5, 10 µg/ml of LPS, previously dissolved in PBS, for 6, 12 and 24 h. In this case the concentration values and

the exposure time applied were chosen accordingly to the available letterature (Hong and Lyu, 2011; Wheater et al., 2012; Rizzo et al., 2012). In the case of RAW macrophages, the cells were incubated with 200 µl of 20, 50, 100, 500 ng/ml and 1, 5, 10 µg/ml of LPS, for 6, 12 and 24 h. Also in this case the different values of concentrations and exposure time of LPS treatment were chosen on the basis of previous studies (Ben et al., 2011; Liew et al., 2011; Zhang et al., 2012; Mo et al., 2014; Lee et al., 2014; Oliveira et al., 2014).

Finally, the combination of strawberry + LPS treatment on HDF cell viability (strawberry at 25, 50, 100, 250, 500 µg/ml and 1, 2.5, 5, 7.5, 10 mg/ml, for 24 h + LPS at 10 µg/ml, for 12 and 24 h) and RAW macrophages cell viability (strawberry at 25, 50, 100, 250, 500 µg/ml and 1, 2.5, 5, 7.5, 10 mg/ml, for 24 h + LPS at 1 µg/ml, for 24 h) was also evaluated, taking into account the results obtained in the two previous sets of MTT assays (i and ii). In this case, at the end of the pre-treatment with strawberry, the medium was removed and a fresh DMEM with LPS was placed in each well (Figure 31).

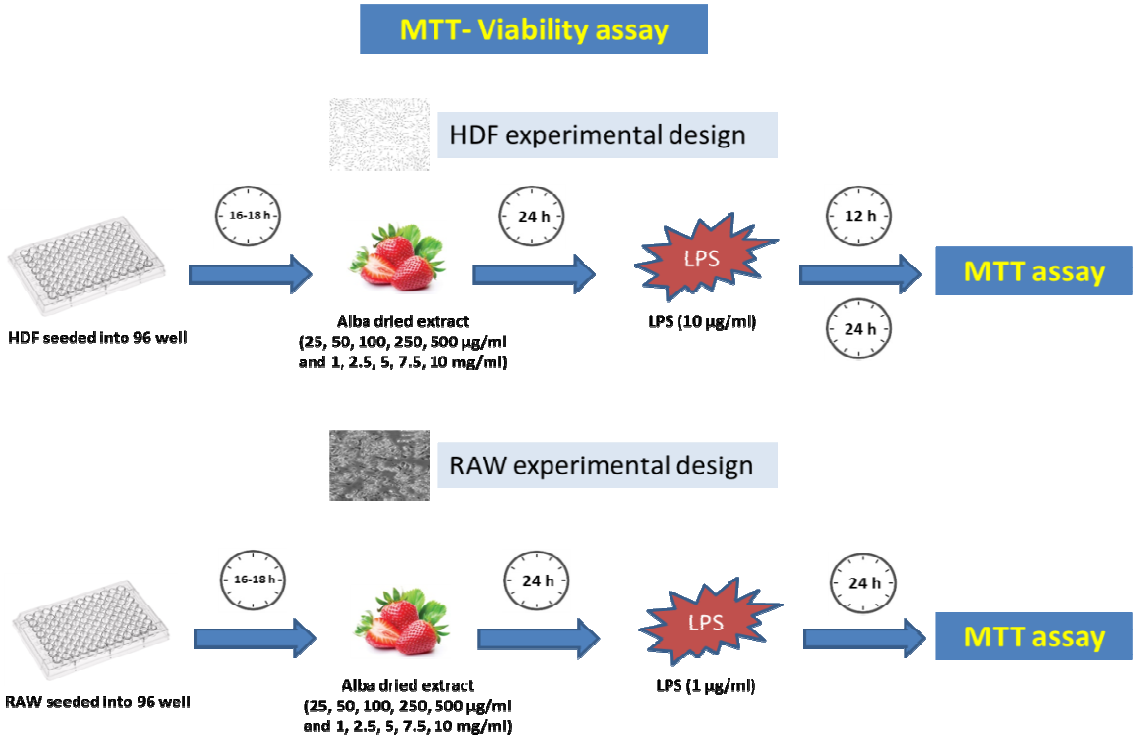


Figure 31. Combination of strawberry dried extract and LPS treatment on HDF and RAW macrophages cell lines, in MTT viability assay.

At the end of the different incubations (i, ii or iii), 30 µl of RPMI medium containing 2 mg/ml MTT were added into each well, and cells were incubated for other 2 h at 37°C in 5% CO_2 incubator.
The content of each well (DMEM + MTT solution) was then removed and 100 µl of dimethyl sulfoxide (DMSO) were added into each well to dissolve insoluble formazan crystal. The solutions were then mixed at room temperature for complete solubilization. The level of colored formazan derivative was analysed on a microplate reader (ThermoScientific Multiskan EX) at a wavelength of 590 nm (Moongkarndi et al. 1991).
The percentage of cell viability was calculated according to the following equation:

$$\% \text{ viability} = (\text{treated cells Abs/control cells Abs}) \times 100$$

Each treatment was carried out in three replicates and the final results were expressed as % viability. Data were generally reported as a mean value ± SD.

4.2.2.4. TALI® ROS concentration assay

The TALI® ROS concentration assay was performed to evaluate the concentration of intracellular ROS in both cell lines after the treatments with Alba dried extracts and LPS.
The methodology used for the test was the same indicated by the manufacturer. Briefly, the first day of the assay 1.5 x 10^5 cells were seeded in a 6-well plate, and let them adhere for 16-18h.
The range of concentration and the time of strawberry dried extract and LPS treatment were chosen, for both cell lines, accordingly to the values founded with the MTT viability assay previously described.
In the case of HDF the day after the seeding, cells were treated with 2 ml of medium (DMEM) containg:
- i) strawberry dried extract at different concentrations (25, 50, 100, 250, 500 µg/ml and 1mg/ml), incubated for 24 h;
- ii) LPS at 10 µg/ml, incubated for 12 and 24 h;
- iii) strawberry dried extract at different concentrations (25, 50, 100, 250, 500 µg/ml and 1mg/ml, for 24 h) + LPS treatment (10 µg/ml, for 24 h). In this case, at the end of the pre-treatment with strawberry, the medium was removed and a new DMEM with LPS was placed in each well.

For RAW 264.7, the cells were treated, the day after the seeding, with 2 ml of medium containg:
- i) strawberry dried extract at 25, 50, 100, 250, 500 µg/ml and 1mg/ml, incubated for 24 h;
- ii) LPS at 1 µg/ml, incubated for 24 h;

- iii) strawberry dried extract at different concentrations (25, 50, 100, 250, 500 µg/ml and 1mg/ml, for 24 h) + LPS treatment (1 µg/ml, for 24 h). In this case, at the end of the pre-treatment with strawberry, the medium was removed and a new DMEM with LPS was placed in each well (Figure 32).

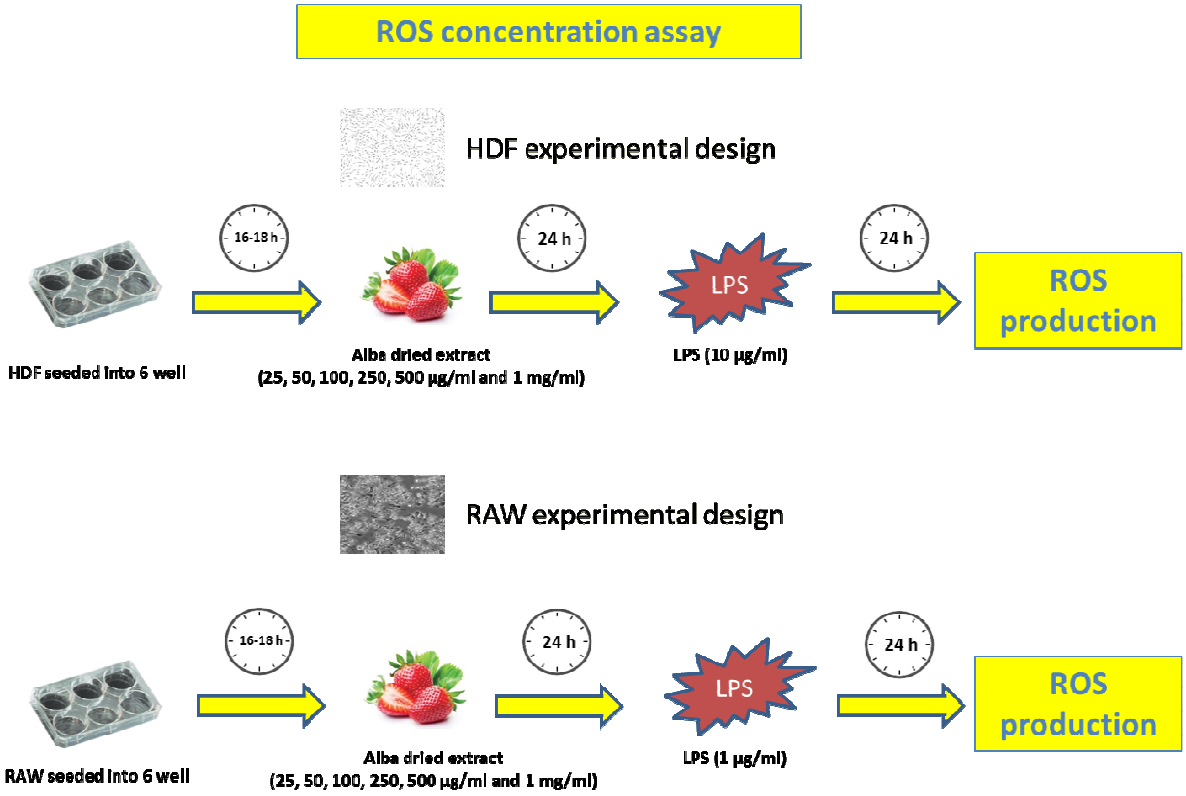


Figure 32. Combination of strawberry dried extract and LPS treatment on HDF and RAW macrophages cell lines, in ROS concentration assay.

In all cases, control wells were treated only with medium, without extracts. At the end of each treatment (i, ii and iii), the medium was removed and collected, cells were washed twice with 2 x 0.5 ml of phosphate-buffered saline (PBS), collecting also the PBS, and in the case of HDF cells, were detached by tripsynization with 0.5 ml for 2-5 minutes at 37°C in 5% CO_2 incubator. Then trypsin was neutralized with 1.5 ml of medium, and then collected. On the contrary, RAW macrophages were detached by scraping (after adding 0.5 ml of DMEM) through the use of a cell scraper, and the medium was collected. All the fractions were centrifuged at 1500 rpm for 10 min at 4°C, the surnatant was discarded and the pellet was resuspended in 1 ml of complete medium. Then CellROX® Orange Reagent was directly added to 1 ml of complete medium at a 1:500 dilution (2 µl dye to 1 ml cell solution). Samples were incubated for 30 minutes at 37°C, centrifuged once to

remove medium and excess dye, and then resuspended in PBS. After labeling with CellROX® Orange Reagent, 25 µl of the stained cells were loaded into a Tali™ Cellular Analysis Slide by pipetting the sample at an angle of approximately 80° into the half-moon shaped sample loading area. The sample was loaded into the chamber through capillary action, taking care to avoid forming bubbles in the sample or to cause back splatter. In this assay, "RFP fluorescence" (530 nm EX/580 nm EM) represented the fluorescence signal from CellROX® Orange Reagent. Untreated cells, which were also labeled with CellROX® Orange Reagent, were used to determine baseline levels of oxidative activity. The Tali™ Image-Based Cytometer automatically captured and analyzed the images of the samples, and presented the results of the analysis as % of intracellular ROS detected in cells.

Each treatment was carried out in three replicates and the final results were expressed as fold increase respect to control. Data were generally reported as a mean value ± SD.

4.2.2.5. TALI® apoptosis assay

The TALI® apoptosis assay was performed to evaluate the response of HDF cell line regarding the live, the dead and the apoptotic rate after the treatment with Alba dried extracts and LPS.

The apoptosis assay was realized only on HDF, since that the treatment with strawberry and LPS in RAW macrophages didn't showed any inhibition of viability, as demonstrated through the MTT test.

The methodology used for the evaluation of cell apoptosis was the same indicated by the manufacturer. Briefly, the first day of assay 1.5 x 10^5 cells were seeded in a 6-well plate, and let them adhere for 16-18h.

As for the ROS assay, the range of concentration and the time of strawberry dried extract and LPS treatment were chosen, for HDF cell line, accordingly to the values founded with the MTT viability assay previously described.

The day after the seeding, cells were treated with 2 ml of medium (DMEM) containg:

- i) strawberry dried extract at different concentrations (50, 100 µg/ml and 1 mg/ml), incubated for 24 h;
- ii) LPS at 10 µg/ml, incubated for 24 h;
- iii) strawberry dried extract at different concentrations (50, 100 µg/ml and 1mg/ml, for 24 h) + LPS treatment (10 µg/ml, for 24 h). In this case, at the end of the pre-treatment with strawberry, the medium was removed and a new DMEM with LPS was placed in each well (Figure 33).

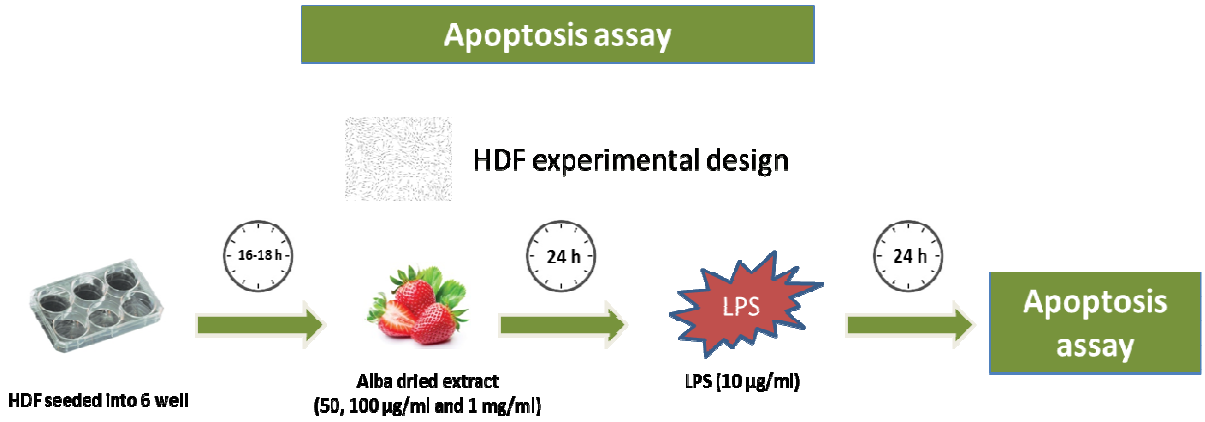


Figure 33. Combination of strawberry dried extract and LPS treatment on HDF cell line, in apoptosis assay.

In all cases, control wells were treated only with medium, without extracts. At the end of each treatment (i, ii and iii), the medium was removed and collected, cells were washed twice with 2 x 0.5 ml of PBS, collecting also the PBS, and then were detached by tripsynization with 0.5 ml for 2-5 minutes at 37°C in 5% CO_2 incubator. Then trypsin was neutralized with 1.5 ml of medium and collected. All the collected fractions were centrifuged at 1500 rpm for 10 min at 4°C, and then the surnatant was discarded and the pellet was resuspended in 100 µl of Annexin binding buffer (ABB, Component C), and transferred into a 1.5 ml microcentrifuge tube. To each 100 µl of sample, 5 µl of Annexin V Alexa Fluor® 488 (Component A) were added and mix well. The cell-Annexin V Alexa Fluor® 488 mixture was incubated at room temperature in the dark for 20 minutes, then centrifuged and resuspended again in 100 µl of ABB. 1 µl of Tali™ Propidium Iodide (PI, component B) was added to each 100 µl of sample and mixed well. The samples were incubated at room temperature in the dark for 1–5 minutes. 25 µl of the stained cells were loaded into a Tali™ Cellular Analysis Slide by pipetting the sample at an angle of approximately 80° into the half-moon shaped sample loading area. The sample was loaded into the chamber through capillary action, taking care to avoid forming bubbles in the sample or to cause back splatter. The Tali™ Image-Based Cytometer automatically captured and analyzed the images of the samples, and presented the results of the analysis as % of live, dead and apoptotic cells. The instrument works at different excitation/emission wavelength in this assay: 458/495 nm for Annexin V and 530/580 nm for the Propidium Iodide. Each treatment was carried out in three replicates and the final results were expressed as fold increase respect to control. Data were generally reported as a mean value ± SD.

4.2.2.6. Mitochondrial functionality with Seahorse XF24 Analyzer®: respiratory capacity assay

OCR in mitochondria of both HDF and RAW macrophages cell lines, after the treatment with Alba dried extracts and LPS, was measured with a XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica MA, USA). The assay is based upon fluorimetric detection of O_2 levels via solid state probes on a sensor cartridge that lowers to within 200 microns of the well bottom during a measurement cycle, creating a transient micro chamber. After a measurement cycle, the sensor cartridge raises, and the medium becomes re-oxygenated through mechanical mixing, thus allowing repeated measurements of O_2 over time. The sensor cartridge is equipped with four reagent delivery chambers per well for injecting compounds into the wells during the assay (Rogers et al., 2011).

After a preventive determination of the optimal cell seeding density and the optimization of all 4 inhibitors (oligomycin, 2,4-dinitrophenol, antimycin and rotenone) for both the cell lines, a respiratory capacity assay was started as indicated by the manufacturer. Briefly, the first day of assay 3.0 x 10^4 cells, for HDF and 5.0 x 10^4 cells, in the case of RAW macrophages, were respectively seeded in a 24-well plate, except for 4-blank wells, and let them adhere for 16-18 h.

The range of concentration and the time of strawberry dried extract and LPS treatment were chosen, for both cell lines, accordingly to the values founded with the ROS production assay previously described. In particular, these values represent the range of concentration which provided a significant difference in ROS reduction, in function of the time and the dose of LPS applied, compared to the control.

In the case of HDF the day after the seeding, cells were treated with 0.5 ml of medium (DMEM) containg:

- i) strawberry dried extract at different concentrations (50, 100 μg/ml and 1mg/ml), incubated for 24 h;
- ii) LPS at 10 μg/ml, incubated for 24 h;
- iii) strawberry dried extract at different concentrations (50, 100 μg/ml and 1 mg/ml, for 24 h) + LPS treatment (10 μg/ml, for 24 h). In this case, at the end of the pre-treatment with strawberry, the medium was removed and a fresh DMEM with LPS was placed in each well.

For RAW 264.7, the cells were treated, the day after the seeding, with 0.5 ml of medium containg:

- i) strawberry dried extract at 50, 100 μg/ml and 1 mg/ml, incubated for 24 h;
- ii) LPS at 1 μg/ml, incubated for 24 h;
- iii) strawberry dried extract at different concentrations (50, 100 μg/ml and 1mg/ml, for 24 h) + LPS treatment (1 μg/ml, for 24 h). Also in this case, at the end of the pre-treatment with strawberry, the medium was removed and a fresh DMEM with LPS was placed in each well (Figure 34).

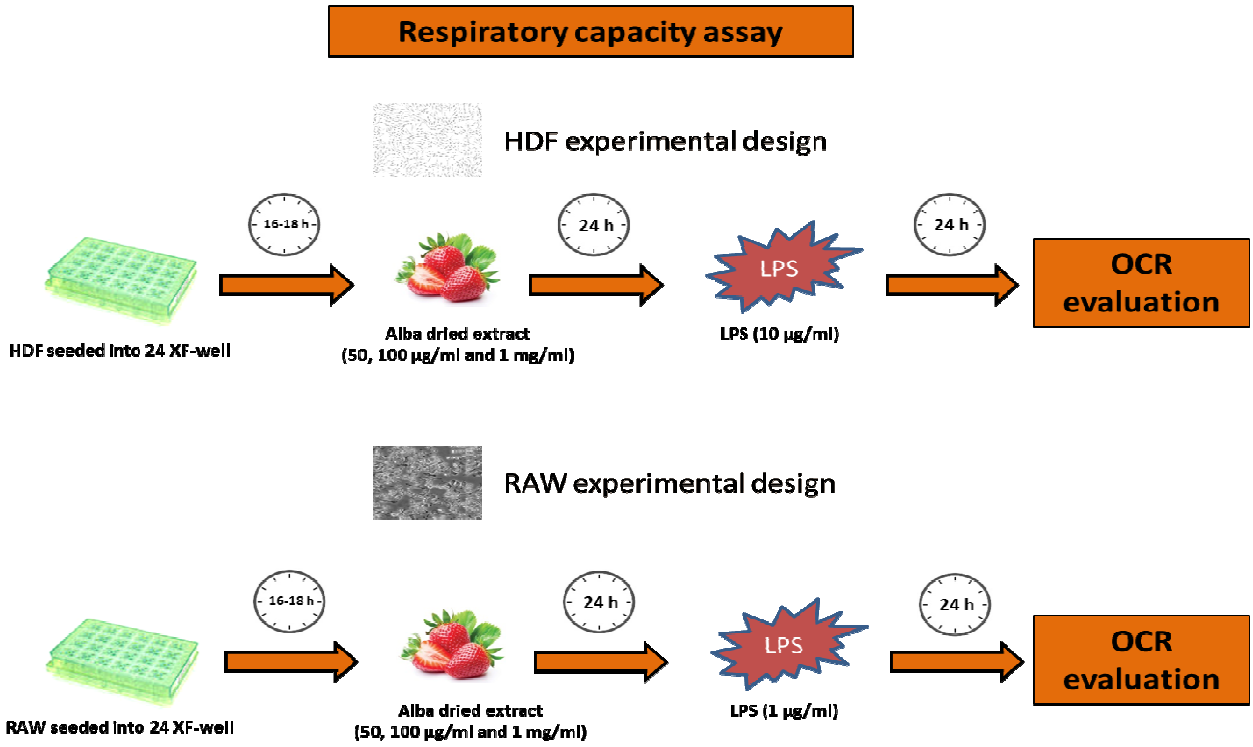


Figure 34. Combination of strawberry dried extract and LPS treatment on HDF and RAW macrophages cell lines, in respiratory capacity assay.

In all cases, control wells were treated only with medium, without extracts. The day before the assay, a calibration plate of 24-wells was hydrated with 1 ml each well with the calibrating solution, and incubated overnight in the XF Prep Station, at 37°C without CO_2.

At the end of each treatment (i, ii and iii), the cell culture media was changed, one hour before the starting of the experiment in the XF Analyzer.

The medium added to the cells was composed of DMEM with high glucose (25mM) and sodium pyruvate (1mM), pH 7.4. In this step, the culture medium was removed, leaving only 50 µl in each well, and washing one time with the new medium. Then 450 µl of the new medium was added to each well, in order to have a final volume of 500 µl. This plate was incubated in the XF Prep Station at 37°C without CO_2 for 1 hour, in order to allow to the cells to equilibrate with the medium. In the meantime, compounds to be injected were loaded into the injection ports of the calibration plate at the following volumes: Port A, 55 µl; Port B, 61 µl; Port C, 68 µl; just before starting the analysis, the calibration plate was placed in the XF Analyzer for the calibration of the instrument, before the

loading of the plate containing cells. After the OCR baseline measurement, 55 µl of oligomycin (2.5 µg/ml final, for HDF and 1 µg/ml final, for RAW, respectively), 61 µl of 2,4-Dinitrophenol (1 mM final, for both cell lines) and 68 µl of antimycin A / rotenone (10 µM / 1 µM final, for both cell lines) were sequentially added to each well. Each treatment was carried out in three replicates and the final results (normalized per 10^5 cells) were expressed as pmol of O_2 consumed per minute per 10^5 cells. Data were generally reported as a mean value ± SD.

Moreover the Maximal Respiratory Capacity value of each treatment was calculated with the following equation:

Maximal Respiratory Capacity = (2,4-dinitrophenol $_{\text{OCR value}}$ - antimycin A / rotenone $_{\text{OCR value}}$)

Also in this case, the final results of the three replicate were expressed as pmol of O_2 consumed per minute per 10^5 cells. Data were generally reported as a mean value ± SD.

4.2.2.7. Determination of nitrite production

Nitric oxide production was assayed by quantification of the stable end product of nitric oxide oxidation, nitrite (NO_2^-). Nitrite accumulation in cell culture media was determined by Griess method (D'Agostino et al., 2001; Souza et al., 2006; Kim et al., 2007; Koide et al., 2009; Pekarova et al., 2009). Griess first described a colorimetric assay to measure the levels of nitrite in aqueous solutions over 100 years ago (Griess, 1879). "Griess Reaction" is based on two-step diazotization reaction in which acidified NO_2^- produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm.

Briefly, 1 x 10^6 cells were seeded in a T75 flask, and let adhere overnight. The range of concentration and the time of strawberry dried extract and LPS treatment were chosen, for both cell lines, accordingly to the values founded with the ROS production assay previously described. In particular, these values represent the range of concentration which provided a significant difference in ROS reduction, in function of the time and the dose of LPS applied, compared to the control.

In the case of HDF the day after the seeding, cells were treated with 10 ml of medium (DMEM) containg:

- i) strawberry dried extract at different concentrations (50, 100 µg/ml and 1 mg/ml), incubated for 24 h;
- ii) LPS at 10 µg/ml, incubated for 24 h;

- iii) strawberry dried extract at different concentrations (50, 100 μg/ml and 1mg/ml, for 24 h) + LPS treatment (10 μg/ml, for 24 h). In this case, at the end of the pre-treatment with strawberry, the medium was removed and a fresh DMEM with LPS was placed in each well.

For RAW 264.7, the cells were treated, the day after the seeding, with 10 ml of medium containg:

- i) strawberry dried extract at 50, 100 μg/ml and 1mg/ml, incubated for 24 h;
- ii) LPS at 1 μg/ml, incubated for 24 h;
- iii) strawberry dried extract at different concentrations (50, 100 μg/ml and 1mg/ml, for 24 h) + LPS treatment (1 μg/ml, for 24 h). Also in this case, at the end of the pre-treatment with strawberry, the medium was removed and a fresh DMEM with LPS was placed in each well (Figure X).

In the case of control group, cells were treated only with medium. At the end of the different incubations (i, ii and iii) in HeraCell CO_2 incubator at 37°C with 5% CO_2, the different cell supernatants were collected, the samples (1 ml) were mixed with equal volume of Griess reagent (1 ml of 1:1 0.1% naphthyl-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) in a tube, and incubated in dark for 10 min at room temperature. Then the absorbance of the reaction mixture was measured at 540 nm on a microplate reader (ThermoScientific Multiskan EX).

The concentration of nitrite in the sample was determined using a sodium nitrite ($NaNO_2$) standard curve (working range: 0.1-6.25 μM).

Each treatment was carried out in three replicates and the final results were expressed as nmol nitrite/mg protein. Data were generally reported as a mean value ± SD.

4.2.2.8. Cell RIPA preparation

To perform enzymatic activity assays (GPx, glutathione reductase (GR), glutathione trasfrase (GST), SOD and catalase) and to evaluate the level of lipid and protein oxidation (TBARS, GSH and protein carbonyl content), a cell RIPA (Radio-ImmunoPrecipitation Assay) preparation was realized, accordingly to the method previously described by Dokotrovova et al. (Dokotrovova et al., 2014), with some modifications.

Briefly, 1 x 10^6 cells were seeded in a T75 flask, and let adhere overnight. The range of concentrations and the time of strawberry dried extract and LPS treatment were chosen, for both cell lines, accordingly to the values founded with the ROS production assay previously described. In particular, these values represent the range of concentration which provided a significant difference in ROS reduction, in function of the time and the dose of LPS applied, compared to the control.

In the case of HDF the day after the seeding, cells were treated with 10 ml of medium (DMEM) containg:

- i) strawberry dried extract at different concentrations (50, 100 μg/ml and 1mg/ml), incubated for 24 h;
- ii) LPS at 10 μg/ml, incubated for 24 h;
- iii) strawberry dried extract at different concentrations (50, 100 μg/ml and 1mg/ml, for 24 h) + LPS treatment (10 μg/ml, for 24 h). In this case, at the end of the pre-treatment with strawberry, the medium was removed and a fresh DMEM with LPS was placed in each well.

For RAW 264.7, the cells were treated, the day after the seeding, with 10 ml of medium containg:
- i) strawberry dried extract at 50, 100 μg/ml and 1mg/ml, incubated for 24 h;
- ii) LPS at 1 μg/ml, incubated for 24 h;
- iii) strawberry dried extract at different concentrations (50, 100 μg/ml and 1mg/ml, for 24 h) + LPS treatment (1 μg/ml, for 24 h). Also in this case, at the end of the pre-treatment with strawberry, the medium was removed and a new DMEM with LPS was placed in each well (Figure X).

In the case of control group, cells were treated only with medium. At the end of the different incubations (i, ii and iii) in HeraCell CO_2 incubator at 37°C with 5% CO_2, medium was discarded and both HDF and RAW macrophages cell lines were washed twice with PBS. After that, 1 ml of RIPA buffer (Sigma-Aldrich, St. Luis, MO) was added in each flask, and cells were incubated at 4°C for 5 minutes. Then, cells were detached with a cell scraper and collected in an Eppendorf tube, and stored at -80°C until the day of analysis. In the analysis day, cells were thawed and centrifuged at 8000 x g for 10 minutes at 4°C for the precipitation of cells debris. Then, the surnatant was collected, representing "the cell RIPA preparation": its protein content were measured using Bradford assay (Bradford, 1976) and bovine serum albumin (BSA) as standard for calibration (working range: 0.5-8 mg/ml). Protein quantity was determined evaluating the absorbance at 590 nm.

4.2.2.9. Cell pellets for Western Blot analysis

For Western Blot analysis, proteins were extracted from cell pellets. Briefly, 1 x 10^6 cells were seeded in a T75 flask, and let adhere overnight.

The range of concentration and the time of strawberry dried extract and LPS treatment were chosen, for both cell lines, accordingly to the values founded with the ROS production assay previously described. In particular, these values represent the range of concentration which provided a significant difference in ROS reduction, in function of the time and the dose of LPS applied, compared to the control.

In the case of HDF the day after the seeding, cells were treated with 10 ml of medium (DMEM) containing:

- i) strawberry dried extract at different concentrations (50, 100 µg/ml and 1mg/ml), incubated for 24 h;

- ii) LPS at 10 µg/ml, incubated for 24 h;

- iii) strawberry dried extract at different concentrations (50, 100 µg/ml and 1mg/ml, for 24 h) + LPS treatment (10 µg/ml, for 24 h). In this case, at the end of the pre-treatment with strawberry, the medium was removed and a fresh DMEM with LPS was placed in each well.

For RAW 264.7, the cells were treated, the day after the seeding, with 10 ml of medium containg:

- i) strawberry dried extract at 50, 100 µg/ml and 1mg/ml, incubated for 24 h;

- ii) LPS at 1 µg/ml, incubated for 24 h;

- iii) strawberry dried extract at different concentrations (50, 100 µg/ml and 1mg/ml, for 24 h) + LPS treatment (1 µg/ml, for 24 h). Also in this case, at the end of the pre-treatment with strawberry, the medium was removed and a new DMEM with LPS was placed in each well (Figure 35).

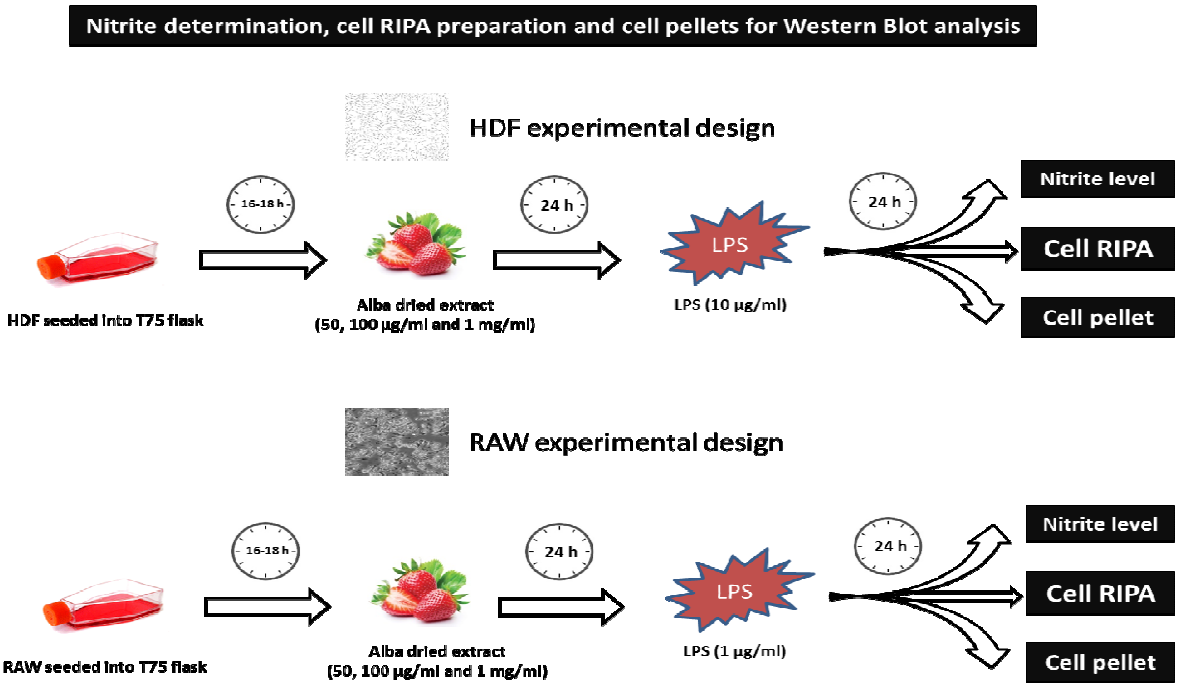


Figure 35. Combination of strawberry dried extract and LPS treatment on HDF and RAW macrophages cell lines, for nitrite determination, cell RIPA preparations and cell pellets for Western Blot analysis.

In the case of control group, cells were treated only with medium. After different incubation time (i, ii and iii) in HeraCell CO_2 incubator at 37°C with 5% CO_2, medium was discarded and both HDF and RAW macrophages cell lines were washed twice with PBS.

After that, HDF cells were detached through trypsinization, and 7 ml of medium were added to each flask to neutralize the 3 ml of trypsin added before. On the contrary, RAW macrophages were deteached by scraping (after adding 10 ml of DMEM) through the use of a cell scraper, and the medium was collected.

Then each cell suspension was transferred from the T75 flask to a 15 ml centrifuge tube, and centrifuged at 1500 rpm for 10 minutes. After that, surnatant was discarded and the cell pellet was resuspended in 1 ml of PBS, and transferred into an Eppendorf tube and centrifuged for 10 minutes at 2000 rpm. Then the surnatant was discarded and the cell pellet was kept at -80°C until the day of analysis.

4.2.2.10. Enzymatic activity assays

4.2.2.10.1. Glutathione Peroxidase (GPx)

GPx, (EC. 1.11.1.9) activity was analyzed by the method of Sies et al. (Sies et al., 1979), with slight modification. The method is based on the ability of glutathione peroxidase to remove H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione (GSH). Briefly, the assay mixture contained 795 µl of phosphate PBS-EDTA buffer (50 mM – 0.40 mM, pH 7.4), 25 µl of glutathione (40 mM), 25 µl of glutathione reductase (5 U/ml), 50 µl of sodium azide (1 mM), 50 µl NADPH (2 mM) and 50 µl of the different "cell RIPA preparation" (i, ii and iii) of both HDF and RAW macrophages cell lines, or 50 µl of PBS-EDTA buffer, in the case of blank. The reaction was started with the addition of 5 µl of H_2O_2 (0.25 mM) (Brown and Stuart, 2007).

GPx activity was determined by the disappearance of NADPH in the samples, measured immediately at 340 nm against blank at 10 s intervals for 3 minutes, in a Beckman DU-640 spectrophotometer (Dokotrovova et al., 2014).

The unit of GPx activity was expressed as nmole of NADPH oxidized per min per mg protein (Park et al., 2005; Park et al., 2011; Choi et al., 2015).

Each sample was analyzed in three replicates and the final data were generally reported as a mean value ± SD.

4.2.2.10.2. Glutathione Reductase (GR)

GR, (EC. 1.6.4.2) activity was assayed by the modified method of Carlberg and Mannervik (Carlberg and Mannervik, 1985). The method is based on the capacity of glutathione reductase to reduce oxidized glutathione (GSSG) back to reduced glutathione (GSH). Briefly, the assay mixture contained 800 µl of phosphate buffer PBS (100 mM, pH 7.6), 50 µl of EDTA (0.5 mM), 50 µl of

NADPH (2 mM), 50 µl of glutathione disulfide (20 mM) and 50 µl of of the different "cell RIPA preparation" (i, ii and iii) of both HDF and RAW macrophages cell lines, or 50 µl of PBS, in the case of blank.

GR activity was measured following the NADPH oxidation at 340 nm against blank at 10 s intervals for 3 minutes, in a Beckman DU-640 spectrophotometer (Dokotrovova et al., 2014).

The unit of GR activity was expressed as nmole of NADPH oxidized per min per mg protein (Yen and Lai, 2002; Park et al., 2005; Park et al., 2011).

Each sample was analyzed in three replicates and the final data were generally reported as a mean value ± SD.

4.2.2.10.3. Glutathione Transferase (GST)

GST, (EC. 2.5.1.18) activity was detected by the method previously described by Habig et al. (Habig et al., 1974), with minor modifications. The assay measures the capacity of GST to conjugate 1-chloro-2,4-dinitro benzene (CDNB) with reduced glutathione, producing a dinitrophenyl thioether which can be detected at 340 nm. Briefly, the assay mixture consisted of 1.475 ml of phosphate buffer PBS (100 mM, pH 6.8), 200 µl of reduced glutathione (1 mM), 25 µl di CDNB (1 mM) and 300 µl of the different "cell RIPA preparation" (i, ii and iii) of both HDF and RAW macrophages cell lines, or 300 µl of PBS, in the case of blank.

The formation of CDNB-GSH conjugate was measured immediately at 340 nm against blank for 1 minute, in a a Beckman DU-640 spectrophotometer (Dokotrovova et al., 2014).

The unit of GST activity was defined as the amount of enzyme producing 1 mmol of CDNB-GSH conjugate per min per mg protein (Noeman et al., 2011).

Each sample was analyzed in three replicates and the final data were generally reported as a mean value ± SD.

4.2.2.10.4. Superoxide Dismutase (SOD)

SOD, (EC. 1.15.1.6) activity was performed according to the method of Kakkar et al. (Kakkar et al., 1984), with slight modification. The method is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon by SOD after xanthine conversion by xanthine oxidase (Paya et al., 1992; Feng et al., 2013).

The assay mixture (total volume 2.8 ml) contained 1 ml of water, 1.2 ml of phosphate buffer PBS (25 mM, pH 8.3), 0.3 ml of nitroblue tetrazolium (300 µM), 0.1 ml of phenazine methosulphate (186 µM), and 200 µl of the different "cell RIPA preparation" (i, ii and iii) of both HDF and RAW macrophages cell lines, or 200 µl of PBS, in the case of blank. The reaction was initiated by the

addition of 200 µl of NADH. The mixture was incubated at 30°C for 90 seconds and arrested by the addition of 1 ml of glacial acetic acid. After adding 4 ml of n-butanol, the reaction mixture was allowed to stand for 10 minutes and centrifuged at 1500 rpm for 1 minute. The intensity of the chromogen in the butanol layer was measured at 540 nm in a microplate reader (Thermo Scientific Microplate Reader, Multiskan® EX, USA) coupled to an Ascent software (Thermo LabSystems Oy, Version 2.6), against blank. A serial standard dilution of SOD (working range: 50 – 500 U/ml) was treated and read together with the samples, to realize a standard curve.

One unit of SOD activity is defined as the amount of enzyme that gave 50% inhibition of nitroblue tetrazolium reduction per minute per mg protein (Camera et al., 2009; Gerbaud et al., 2005; Dokotrovova et al., 2014). Each sample was analyzed in three replicates and the final data were generally reported as a mean value ± SD.

4.2.2.10.5. Catalase

Catalase activity assay (EC. 1.11.1.6) was conducted by following the decomposition of hydrogen peroxide as described by the method of Aebi (Aebi, 1984). Briefly, the assay mixture consisted of 990 µl of buffer (39% of NaH_2PO_4 50 mM + 61% of Na_2HPO_4 57 mM; pH 7.0), 600 µl of H_2O_2 (30%) and 10 µl of the different "cell RIPA preparation" (i, ii and iii) of both HDF and RAW macrophages cell lines, or 10 µl of buffer, in the case of blank. Decrease in absorbance due to H_2O_2 degradations was monitored at 240 nm against blank for 70 seconds, in a Beckman DU-640 spectrophotometer. One unit of catalase was defined as the amount of enzyme that decomposed 1 µmol of H_2O_2 per minute per mg protein (Yen et al., 2002; Camera et al., 2009; Briganti et al., 2014).

Each sample was analyzed in three replicates and the final data were generally reported as a mean value ± SD.

4.2.2.11. Lipid and protein oxidation level

4.2.2.11.1. Lipid peroxidation (TBARS) level

Lipid peroxidation was measured by the assay of thiobarbituric acid-reactive substances (TBARS) production, according to a standardized method (Ohkawa et al., 1979), with minor modification. Briefly, 600 µl of the different "cell RIPA preparation" (i, ii and iii) of both HDF and RAW macrophages cell lines, or 600 µl of water, in the case of blank, were mixed with 1.4 ml of thiobarbituric acid reagent (0.37% in 0.2 M HCl) and 15 % of trichloroacetic acid, and heated at 95 °C for 20 min. The samples were then cooled, centrifuged at 1200 x g for 15 min at 4°C and the

supernatant was measured for absorbance at 532 nm in a Beckman DU-640 spectrophotometer, against blank. The amount of malondialdehyde (MDA) formed was calculated by using 1,1,3,3-tetraethoxypropane as the standard (working range: 0.078-2.5 µM) and expressed as nmoles of MDA/100 mg of proteins (Park et al., 2005; Briganti et al., 2014).
Each sample was analyzed in three replicates and the final data were generally reported as a mean value ± SD.

4.2.2.11.2. Reduced glutathione (GSH) determination

The method is based on the reduction of 5,5 dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione (GSH) to produce a yellow compound (Ellman, 1959; Griffith, 1980). Briefly, 100 µl of the different "cell RIPA preparation" (i, ii and iii) of both HDF and RAW macrophages cell lines, or 100 µl of buffer, in the case of blank, were added at 1.375 ml of phosphate buffer (5.3% of NaH_2PO_4 0.023 mM + 94.7% of Na_2HPO_4 2 mM; pH 8.0) and 25 µl of DTNB (0.1 mM). The solution was mixed for approximately 15 seconds and the absorbance was measured at 412 nm in a Beckman DU-640 spectrophotometer, against blank. A calibration curve was prepared using GSH as a standard (working range: 10-100 µg/ml). The results were expressed as nmol GSH/mg of protein (Emonet et al., 1997; Yen and Lai, 2002; Gimeno et al., 2004; Muller, 2011).
Each sample was analyzed in three replicates and the final data were generally reported as a mean value ± SD.

4.2.2.11.3. Determination of protein carbonyl content

Protein carbonyl content was determined by the dinitrophenylhydrazine (DNPH) method as described by Levine et al. (Levine et al., 1994) with some modifications. This assay is based on the reaction between DNPH and protein carbonyl, forming a Schiff base to produce the corresponding hydrazone that can be detected spectrophotometrically. Briefly, two Eppendorf tube containing 25 µl of the different "cell RIPA preparation" (i, ii and iii) of both HDF and RAW macrophages cell lines, were precipitated with 25 µl of trichloroacetic acid (20%) followed by centrifugation at 2800 g for 10 min at 4 °C. One of it represented the sample, the other one the control. The sample precipitates were incubated with 750 µl of DNPH (10 mM), while 250 µl of HCl (2.5 M) were added in the case of the control. Both the reaction mixtures were allowed to stand for 1 hour in the dark at room temperature with stirring at 15 min interval. After the incubation, 750 µl of trichloroacetic acid (10%) were added and both the solution (sample and control) were left for 15 min at room temperature. After that, the mixtures were centrifuged at 3400 g for 10 min at 4 °C. The sample precipitates were washed with 500 µl of an ethanol–ethylacetate (1:1) mixture twice (to

remove DNPH in excess) and the final precipitates were dissolved in 625 µl of guanidine hydrochloride (6 M). Finally, the samples were centrifuged at 3400 g for 10 min at 4 °C to remove any left over debris. The absorbance of sample supernatants was measured at 370 nm in a microplate reader (Thermo Scientific Microplate Reader, Multiskan® EX, USA), against the control. The carbonyl content was expressed as nmole per mg protein (Augustyniak et al., 2015).
Each sample was analyzed in three replicates and the final data were generally reported as a mean value ± SD.

4.2.2.12. Gene expression analysis with Western Blot

The cell pellets previously prepared (i, ii and iii) of both HDF and RAW macrophages cell lines were homogenized in 200 µl lysis buffer (containing Tris HCl 20 mM, 0.9% NaCl, 0.2% Triton and 1% of Protease Inhibitor Cocktail). After centrifugation at 10000 rpm for 5 minutes at 4°C, the supernatant containing the protein extract was collected. Protein quantification of the lysates was performed by Bradford's method (Bradford, 1976) and BSA as standard for calibration (working range: 0.5-8 mg/ml). Protein quantity was determined evaluating the absorbance at 590 nm. Samples were prepared in an eppendorf tube in order to have 80 µg of protein and 8 µl of tetrazolium blue, adjusting the volume with water until 40 µl.
Samples were then charged into a 12% sodium dodecyl sulfate-polyacrylamide running gel, consisted of 3.3 ml of H_2O, 4 ml of acrylamide mix (30%), 2.5 ml of Tris HCl (1.5 M, pH 8.8), 100 µl of sodium dodecyl sulfate (SDS, 10%), 100 µl of ammonium persulfate (10%) and 4 µl of TEMED. The stacking gel was composed by 3 ml of H_2O, 667 µl of acrylamide mix (30%), 1.25 ml of Tris HCl (1 M, pH 6.8), 50 µl of sodium dodecyl sulfate (10%), 50 µl of ammonium persulfate (10%) and 5 µl of TEMED. 5 µl of Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories, Inc., Hercules, CA) were used as marker of the electrophoresis running into the gel.
The electrophoresis was performed at 120V, in an elettrophoretic chamber using Tris-Glycine-SDS Buffer (1X).
After the electrophoresis run, proteins were transferred from the gel to nitrocellulose membranes, using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc., Hercules, CA) and a Transfer Buffer consisting of 50 ml Tris-Glycine Buffer (10X), 100 ml of methanol and 350 ml of H_2O. After regular washing, the membrnaes were incubated for 1 hour with 5% non fat dry milk (Blotting grade blocker, Bio-Rad Laboratories, Inc., Hercules, CA) dissolved in TTBS (50 ml of Tris HCl 200 mM + NaCl 1.5 M at pH 7.7, 450 ml of H_2O, 1.25 ml of Tween 20), for blocking.
At this point, the membranes were incubated overnight with rabbit polyclonal antibody against the proteins of interest, related to inflammatory status (NFkB, pIκBα, TNF-α, IL-1β, IL-6, IL-10 and

iNOS), antioxidant response (Nrf2, catalase, SOD and HO-1), the AMPK-related pathway (AMPK, p-AMPK, SIRT1 and PGC1α), the DNA damage level (OGG1) and apoptosis rate (caspase 3) (Figure 36). Finally GAPDH protein was used for the measurement of the amount of protein analyzed. All rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc., Dallas, Texas, and used at 1:500 dilution.

The following day, after regular washing, membranes were incubated with anti-rabbit IgG-Peroxidase antibody produced in goat (Sigma-Aldrich, St. Luis, MO) (1:80000 dilution) for 1 hour. Proteins of interest were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA), and the protein signals were detected using a Lycor C-Digit Blot Scanner. Finally, quantification of gene expression was made using the software provided by the manufacturer of the Blot Scanner (Image Studio 3.1) and data were expressed as fold increase respect to control. Each gene expression was performed in three replicates and the final data were generally reported as a mean value ± SD.

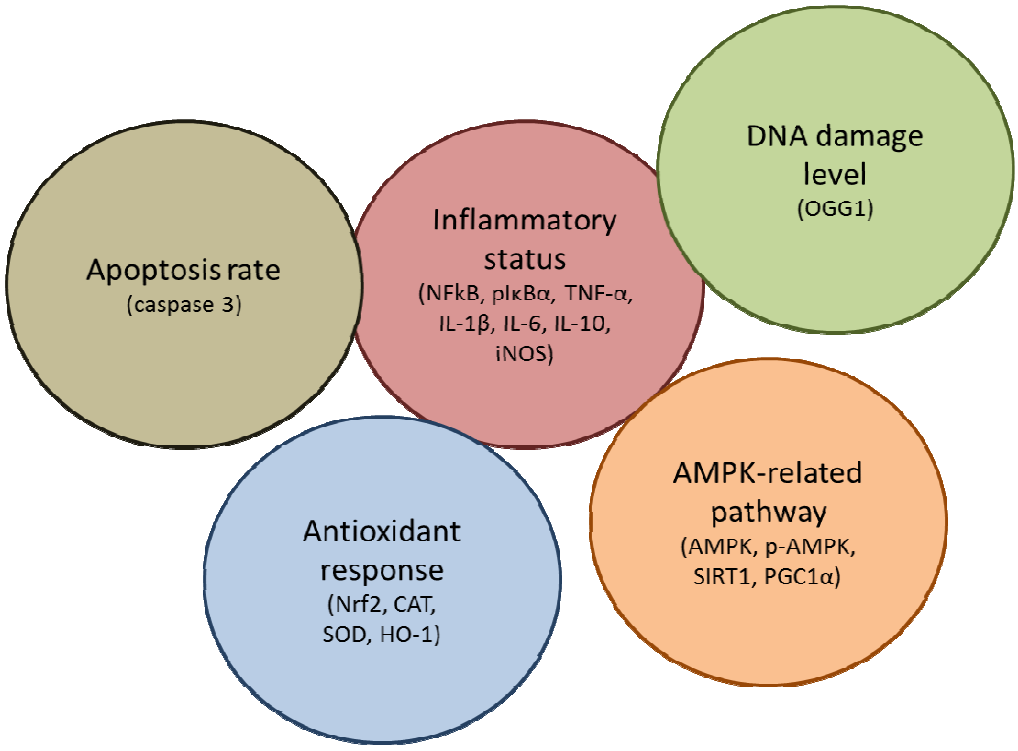


Figure 36. Schematic representation of the protein gene expression performed by Western Blot.

4.2.2.13. Statistical analysis

Statistical analyses were performed using STATISTICA software (Statsoft Inc., Tulsa, OK, USA). Data were subjected to one-way analysis of variance for mean comparison, and significant

differences among different treatments were calculated according to HSD Tukey's multiple range test. Data are reported as mean ± SD.

4.2.3. HDF results

4.2.3.1. MTT viability assay

HDF cells were incubated for 24, 48 and 72 h with 200 µl of different concentrations of dried strawberry extract (25, 50, 100, 250, 500 µg/ml and 1, 2.5, 5, 7.5, 10 mg/ml), in order to evaluate the possible cytotoxic effect of Alba solutions. At 24 h an increase in cell viability was observed in a dose-dependent manner (up to + 57% with 10 mg/ml of Alba). On the contrary, at 48 and 72 h, the viability started to decrease at 2.5 mg/ml (- 7%) and 250 µg/ml (- 10%), respectively (Figure 37). For this reason, 24 h of treatment with all the concentrations of strawberry applied were chosen to test the protective effect of these extracts against LPS action.

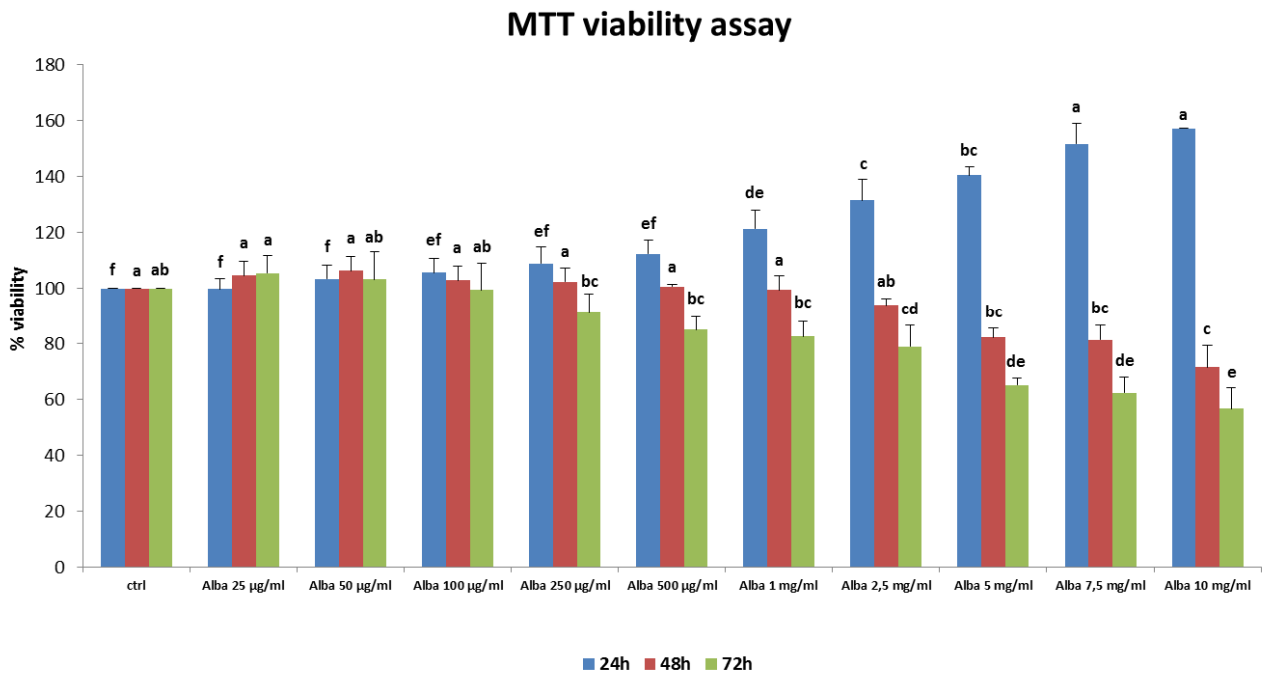


Figure 37. MTT assay for the determination of cell viability in HDF cells treated with different time-concentrations of strawberry extracts. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

To test the effect of LPS on cell viability, HDF cells were incubated with 200 µl of 0.1, 1, 5, 10 µg/ml of LPS, for 6, 12 and 24 h. Only 10 µg/ml of LPS applied for 12 and 24 h produced a significant reduction on viability (- 10% and - 15%, respectively), while all the other combinations of dose-time of LPS treatment did not produce any statistical difference respect to untreated cells (Figure 38). For this reason, 10 µg/ml, applied for 12 and 24 h, was chosen as LPS-dosage for the next steps.

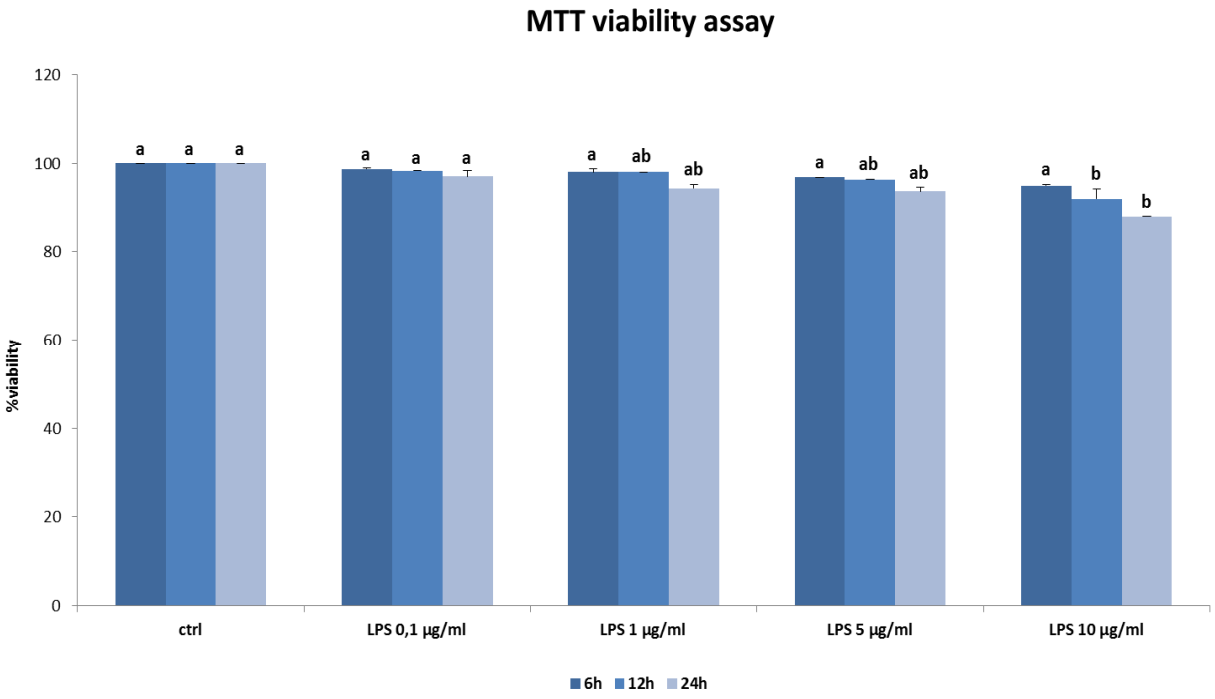


Figure 38. MTT assay for the determination of cell viability in HDF cells treated with different time-concentration of LPS. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

Finally, the effect of strawberry supplementation before the LPS treatment on HDF cell viability (strawberry at 25, 50, 100, 250, 500 µg/ml and 1, 2.5, 5, 7.5, 10 mg/ml, for 24 h + LPS at 10 µg/ml, for 12 and 24 h) was also evaluated, in order to highlight the possible protective role of strawberry pre-treatment against LPS effect. Alba extracts showed a protective role at all the different concentrations applied, in combination with both 12 or 24 h of LPS-treatment (except at 10 mg/ml of Alba, which determined a reduction of 13% of viability with 12 h of LPS incubation) (Figure 39). Further analysis were conducted using strawberry concentration up to 1 mg/ml, since that this

value provided the maximal cell viability (+30% and +40% with 12 and 24 h of LPS, respectively); in fact, at higher concentrations a drop of % of live cells was registered.

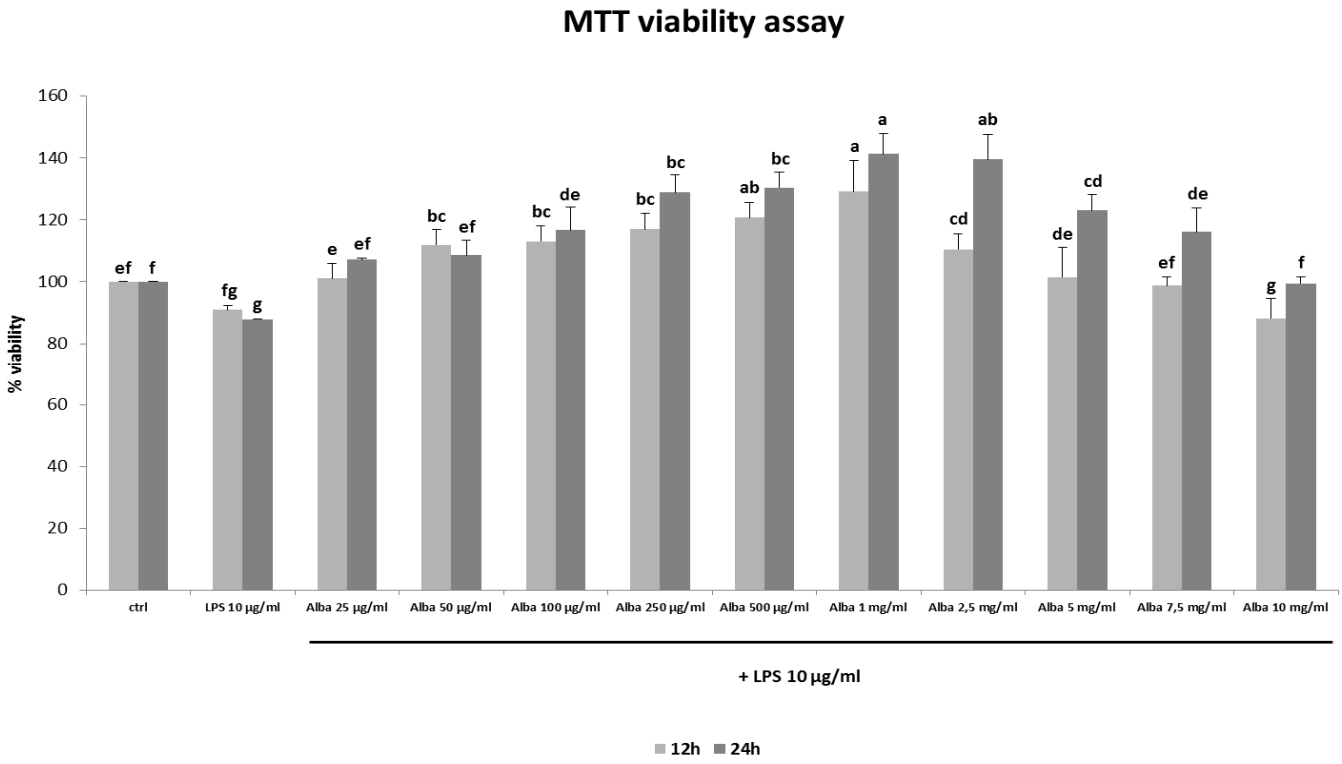


Figure 39. MTT assay for the determination of cell viability in HDF cells treated with different time-concentration of strawberry and LPS. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.2.3.2. TALI® ROS concentration assay

HDF cells were incubated for 12 and 24 h with LPS at 10 µg/ml, to test the effect of the endotoxin to ROS intracellular production. An increase in ROS concentration was found both after 12 (fold increase of 1.4) and 24 h (fold increase of 2.5) of treatment with LPS; but only at 24 h this effect produced a significantly difference respect to the control group (Figure 40a and 40b). For this reason 24 h of treatment with LPS (10 µg/ml) was chosen as LPS-dosage for the next step.

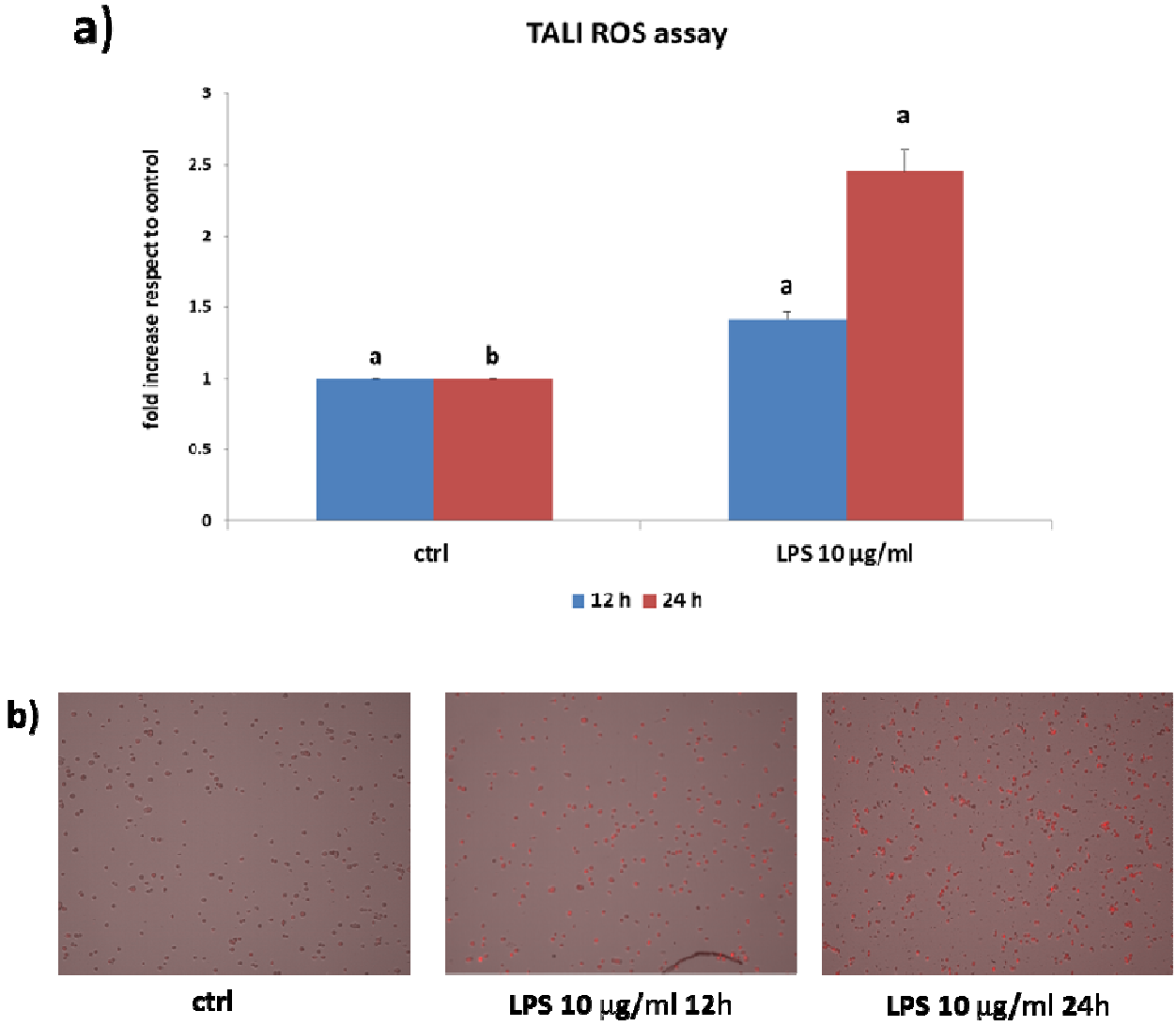


Figure 40. a) TALI ROS assay for the determination of cell ROS concentration in HDF cells treated with LPS. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$). **b)** TALI ROS images of ROS concentration in HDF cells treated with LPS. The amount of red point is proportional to the intracellular ROS present in the different samples.

To test the effect of strawberry pre-treatment on LPS-induced ROS production, HDF cells were treated with Alba dried extract at different concentrations (25, 50, 100, 250, 500 µg/ml and 1mg/ml, for 24 h) before the incubation with LPS (10 µg/ml for 24 h). In figure 41 (a and b) the effect of the strawberry extract on ROS concentration is shown: Alba did not stimulate ROS production at all the different concentrations applied. Moreover at dose of 1 mg/ml, a significant reduction of ROS level

(fold increase of 0.68) was registered, respect to untreated control group. In HDF cells pre-treated with different strawberry extracts and stressed wtith LPS a decrease of intracellular ROS was found in a dose-dependent manner. In particular a significant reduction in the amount of ROS respect to the control represented by only LPS (fold increase of 2.50), was obtained with Alba concentration of 50 µg/ml (fold increase of 2.03), 100 µg/ml (fold increase of 1.75) and 1 mg/ml (fold increase of 1.34). For this reason these doses of Alba extracts, in combination with LPS at 10 µg/ml, were used for all further analysis.

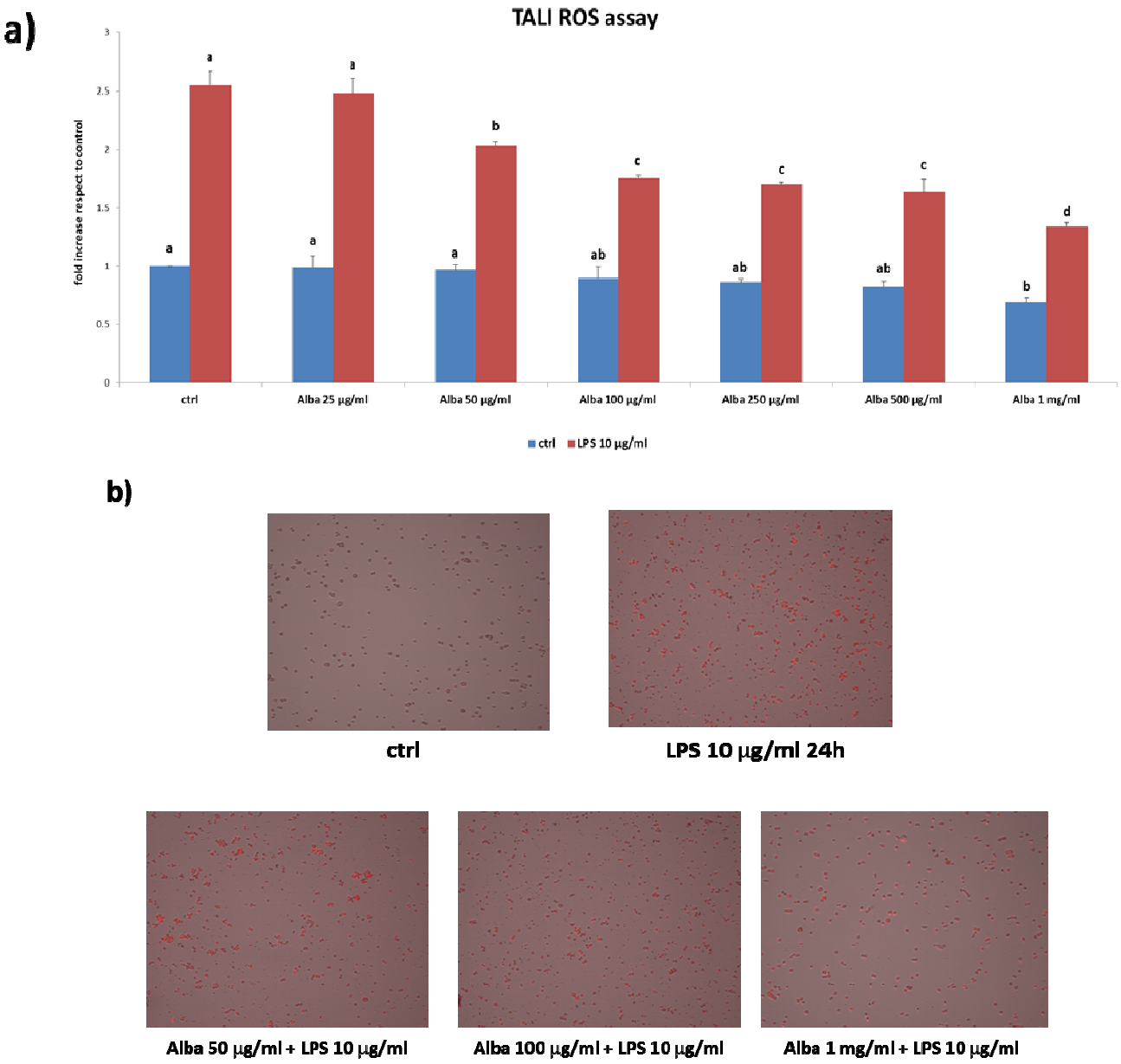


Figure 41. a) TALI ROS assay for the determination of cell ROS concentration in HDF cells treated with strawberry and LPS. Data are expressed as mean values ± SD. Columns belonging to

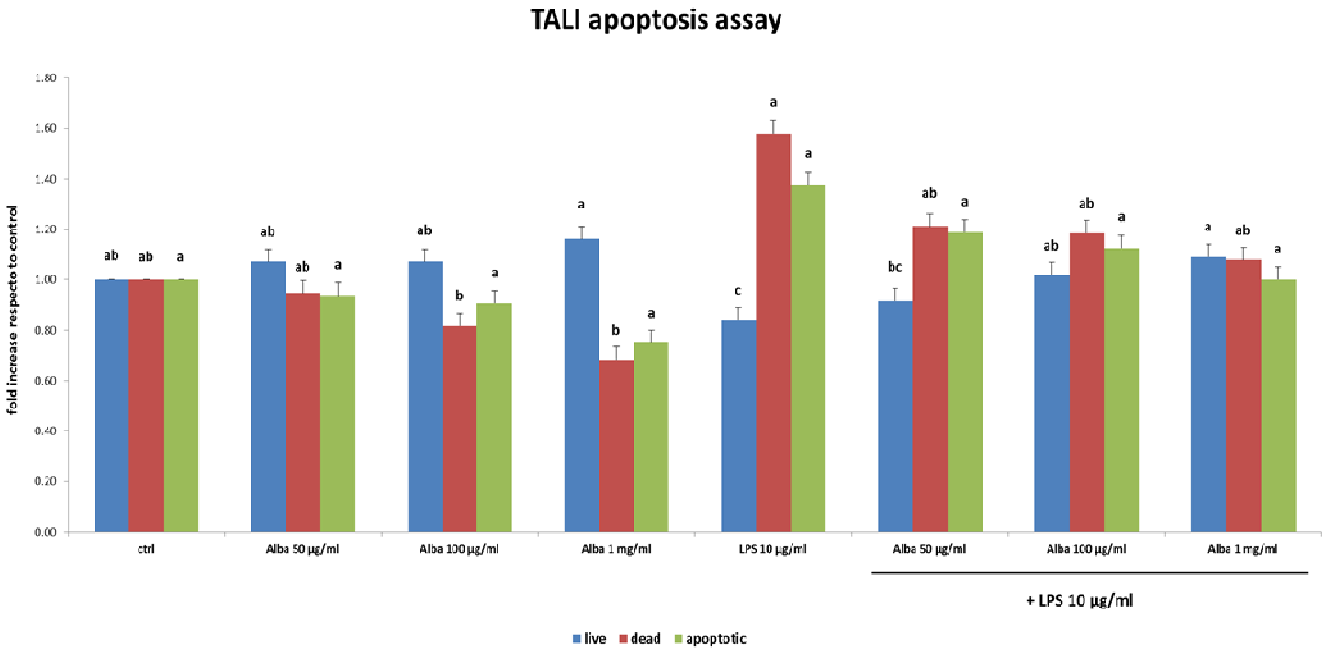
the same set of data with different superscript letters are significantly different ($p < 0.05$). **b)** TALI ROS images of ROS concentration in HDF cells treated with strawberry and LPS. The amount of red point is proportional to the intracellular ROS present in the different samples.

4.2.3.3. TALI® apoptosis assay

HDF cells were pre-treated with different strawberry concentrations (50, 100 µg/ml and 1 mg/ml, for 24 h) and subsequentally incubated with LPS (10 µg/ml, for 24 h), in order to evaluate the strawberry effect on the possible apoptosis rate induced by LPS. As highlighted by the MTT assay (chapter 4.2.3.1.), the strawberry treatment improved the HDF cell viability, increasing the number of live cells (blue bars) and reducing the amount of dead cells (red bars), in a dose-dependent manner. These effects occurred both in normal conditions and in presence of LPS (Figure 42a and 42b).

Regarding the apoptosis rate (green bars), the treatment with strawberry reduced the number of apoptotic cells in a dose-dependent manner, producing a maximal fold increase of 0.75 with Alba at 1 mg/ml. The incubation with LPS increased the apoptotic rate (fold increase of 1.38), which is counteracted by the Alba treatment, at all the different concentrations applied (fold increase of 1.19, 1.13 and 1.01 with 50, 100 µg/ml and 1 mg/ml, respectively). However, all the different treatments did not produce any significant difference respect to the control group (Figure 42a and 42b).

a)



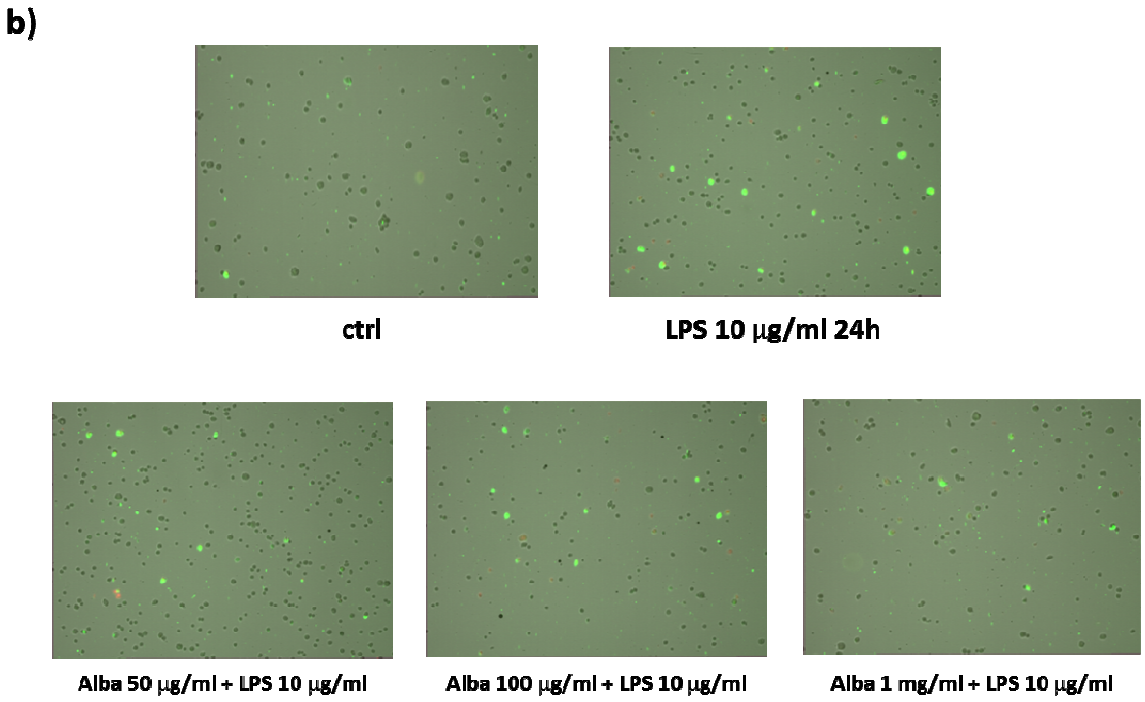


Figure 42. a) TALI apoptosis assay for the determination of cell apoptosis rate in HDF cells treated with strawberry and LPS. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$). **b)** TALI images of apoptosis rate in HDF cells treated with strawberry and LPS. The amount of green point is proportional to the apoptotic cell present in the samples.

4.2.3.4. XF24 Analyzer® respiratory capacity assay

To examine the potential protection of strawberry extracts on mitochondrial function against LPS stress, the respiratory capacity assay was performed on HDF cells treated with different strawberry concentrations (50, 100 µg/ml and 1 mg/ml, for 24 h) and subsequentally incubated with LPS (10 µg/ml, for 24 h). Figure 43 shows the trend of the different tested groups, in function of the different inhibitors applied (oligomycin, 2,4-DNP, antimycin A/rotenone). Starting from the baseline values of OCR, LPS treatment produced a considerable lowering of the oxygen consumption respect to the control group (- 50 pmol O_2/min/10^5 cells). On the contrary, the strawberry treatment improved the mitochondrial respiration increasing the OCR level, respect to control (197 pmol O_2/min/10^5 cells), of 20, 35 and 50 pmol O_2/min/10^5 cells, with 50, 100 µg/ml and 1 mg/ml, respectively. Moreover, Alba extracts were also able to counteract the depressive effect exerted by LPS, increasing the OCR value in a dose-dependent manner, and restoring the

oxygen consumption at the control level with Alba at 1 mg/ml (204 pmol O_2/min/10^5 cells). The response of the different tested groups to the inhibitors of the respiratory chain complex was the same: oligomycin produced a reduction of the respiration rate; 2,4-DNP strongly increased the OCR value and finally the antimycin A/rotenone injection determined a critical decrese of the oxygen consumption (Figure 43).

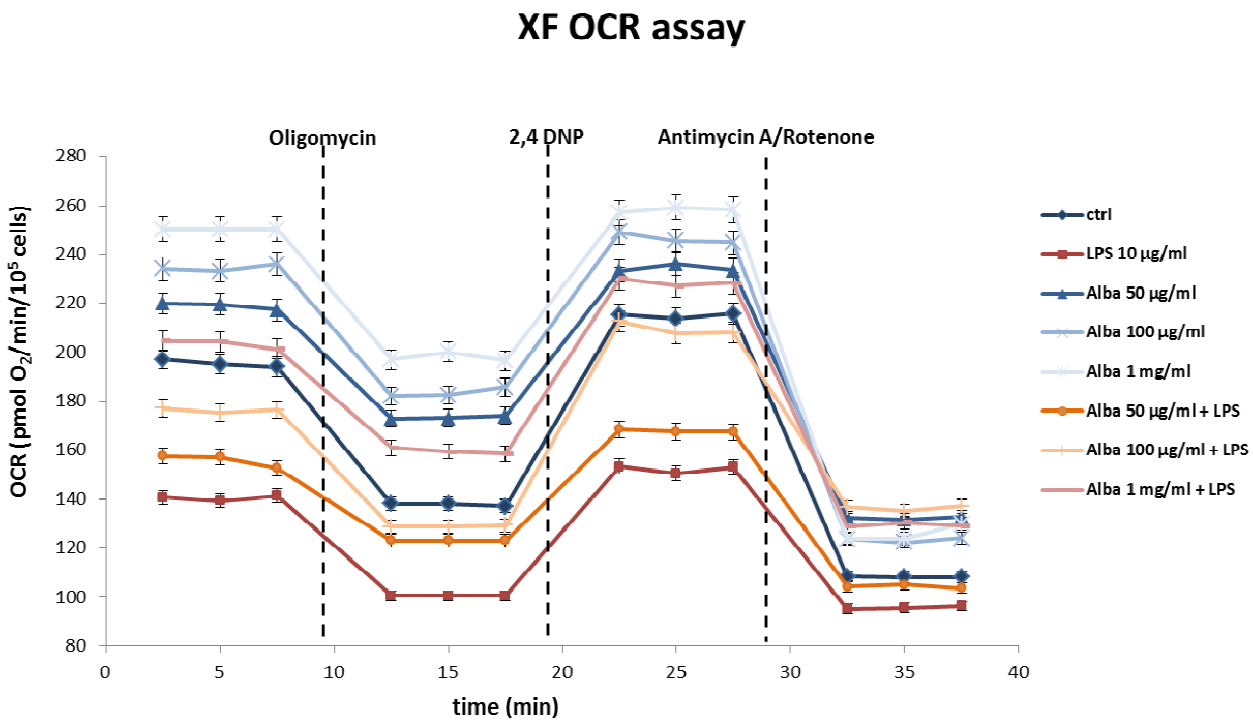


Figure 43. Effects of strawberry and LPS treatment on OCR in HDF cells. Mitochondria oxygen consumption was monitored with sequential injection of oligomycin, 2,4-DNP and antimycin A/rotenone at the indicated time points into each well, after baseline rate measurement. Data are expressed as mean values ± SD.

With regard to the maximal respiratory capacity, LPS-treated HDF cells showed the lower values, 58.12 pmol O_2/min/10^5 cells. Higher results were obtained in strawberry groups, with values of 100.96, 125.43 and 132.99 pmol O_2/min/10^5 cells obtained with 50, 100 µg/ml and 1 mg/ml of Alba, respectively. Finally, the pre-treatment with strawberry extracts before the LPS-incubation of cells, determined an improvement of the values of the maximal respiratory capacity: a data statistically similar to the control group (96.11 pmol O_2/min/10^5 cells) was obtained already with Alba at 100 µg/ml (84.64 pmol O_2/min/10^5 cells) (Figure 44).

	Maximal Respiratory Capacity (pmol O_2/min/10^5 cells)
ctrl	96.11 ± 4.81 ^{bc}
Alba 50 µg/ml	100.96 ± 5.05 ^{bc}
Alba 100 µg/ml	125.43 ± 6.27 ^{a}
Alba 1 mg/ml	132.99 ± 6.65 ^{a}
LPS 10 µg/ml	58.12 ± 2.91 ^{d}
Alba 50 µg/ml + LPS	64.16 ± 3.21 ^{d}
Alba 100 µg/ml + LPS	84.64 ± 4.23 ^{cd}
Alba 1 mg/ml + LPS	101.20 + 5.06 ^{bc}

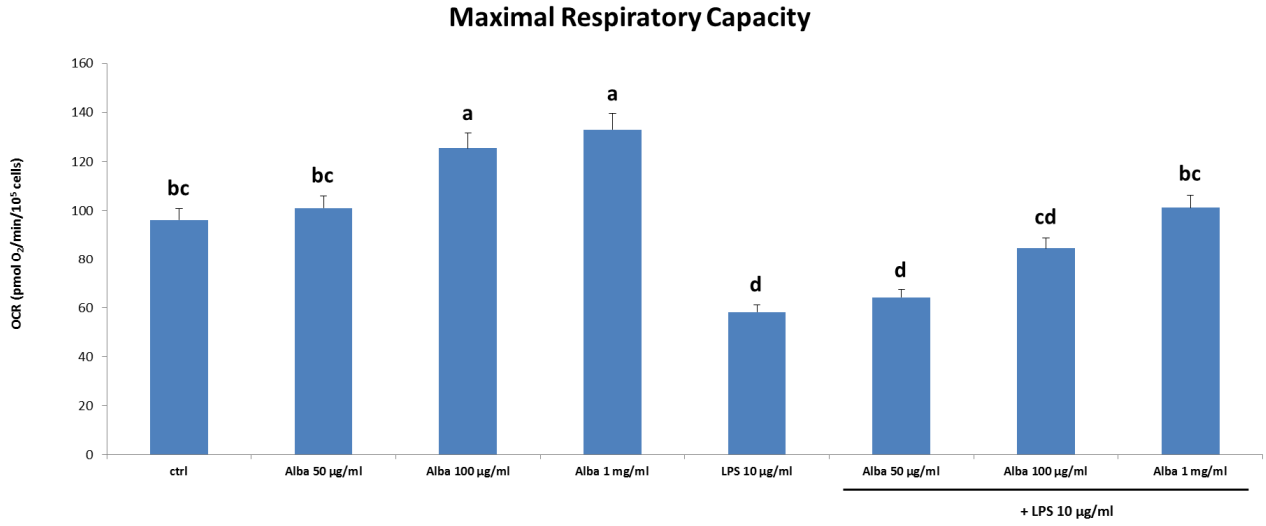


Figure 44. Effects of strawberry and LPS treatment on maximal respiratory capacity in HDF cells. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.2.3.5.Determination of nitrite production

HDF cells were pre-treated with different strawberry concentrations (50, 100 µg/ml and 1 mg/ml, for 24 h) and subsequentally incubated with LPS (10 µg/ml, for 24 h), in order to determine the nitric oxide production level of the different treatments.

As shown in Figure 45, Alba extracts were able to reduce the NO production in a dose-dependent manner (0.31, 0.28 and 0.21 nmol/mg prot, with 50, 100 µg/ml and 1 mg/ml of strawberry extracts, respectively), compared to the untreated HDF cells (0.33 nmol/mg prot). On the contrary the treatment with LPS significantly increased the NO value (0.62 nmol/mg prot), which was efficiently lowered with strawberry pre-treatment, and was restored to the control group value with Alba at dose of 100 µg/ml (0.33 nmol/mg prot).

	Nitrite levels (nmol/mg prot)
ctrl	0.33 ± 0.04^{bc}
Alba 50 µg/ml	0.31 ± 0.06^{bc}
Alba 100 µg/ml	0.28 ± 0.04^{bc}
Alba 1 mg/ml	0.21 ± 0.05^{c}
LPS 10 µg/ml	0.62 ± 0.08^{a}
Alba 50 µg/ml + LPS	0.51 ± 0.03^{a}
Alba 100 µg/ml + LPS	0.33 ± 0.03^{b}
Alba 1 mg/ml + LPS	0.25 ± 0.04^{bc}

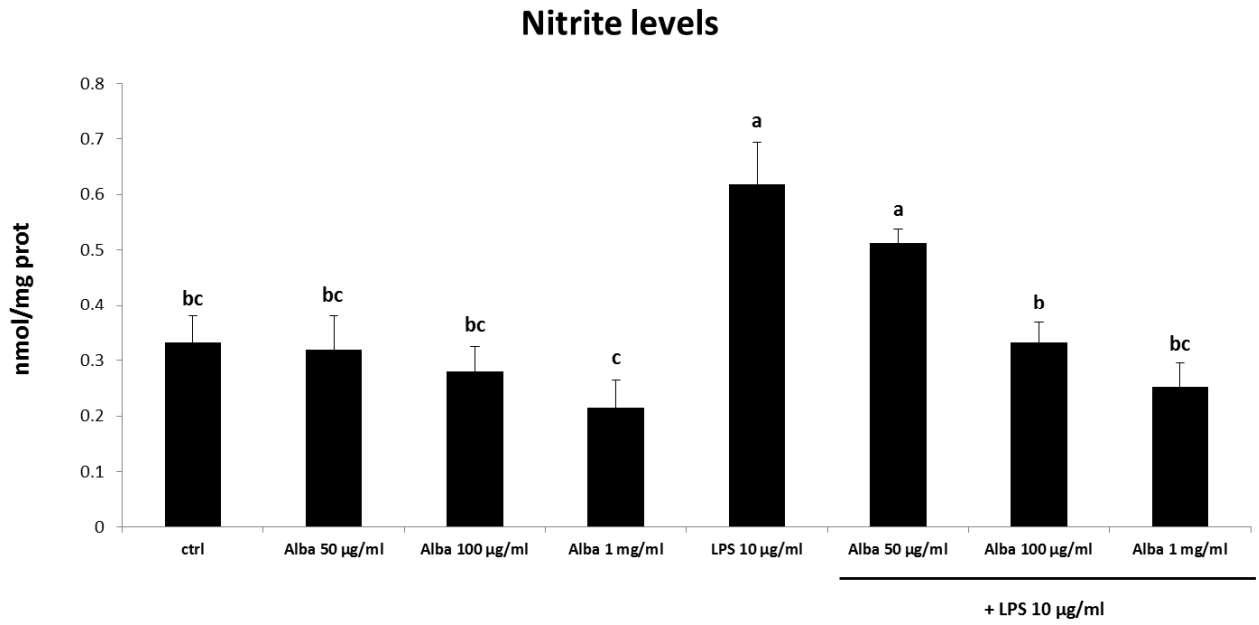


Figure 45. Effects of strawberry and LPS treatment on NO levels in HDF cells. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.2.3.6. Enzymatic activity assays

Cell RIPA preparation of HDF cells was used to evaluate the activity of different enzymes. As reported in Figure 46, a similar trend was obtained for GPx, GR and GST levels. In all cases Alba treatments were able to increase the enzymatic activity in a dose-dependent manner. The highest values were obtained with strawberry at concentration of 1 mg/ml (482.04, 373.55 and 535.93 nmol/min/mg prot for GPx, GR and GST, respectively); however a significative difference respect to the untreated groups was already obtained at dose of 100 µg/ml for GPx and GST (441.60 and 508.52 nmol/min/mg prot, respectively) and at 50 µg/ml for GR (340.99 nmol/min/mg prot). With LPS treatment, a significative reduction of enzymatic activity was registred for all the tested enzymes (230.22, 143.75 and 299.89 nmol/min/mg prot, for GPx, GR and GSR respectively). These

activities were improved with strawberry pre-treatment at the different concentrations applied: a value statistically similar to the control group (339.56, 241.12 and 430.43 nmol/min/mg prot, for GPx, GR and GST respectively) was obtained with Alba at 100 µg/ml in all the conducted assays (333.36 nmol/min/mg prot for GPx, 224.89 nmol/min/mg prot for GR and 422.37 nmol/min/mg prot for GST).

	GPx activity	GR activity	GST activity
	(nmol NADPH oxidized/min/mg prot)	(nmol NADPH oxidized/min/mg prot)	(nmol CDNB-GSH conjugate/min/mg prot)
ctrl	339.56 ± 2.75 ^{b}	241.12 ± 2.10 ^{b}	430.46 ± 24.99 ^{bc}
Alba 50 µg/ml	365.87 ± 20.11 ^{b}	321.53 ± 8.58 ^{a}	455.01 ± 50.01 ^{bc}
Alba 100 µg/ml	441.60 ± 23.51 ^{a}	340.99 ± 24.53 ^{a}	508.52 ± 30.88 ^{ab}
Alba 1 mg/ml	482.04 ± 33.43 ^{a}	373.55 ± 32.75 ^{a}	535.93 ± 10.76 ^{a}
LPS 10 µg/ml	230.22 ± 23.96 ^{c}	143.75 ± 13.75 ^{c}	299.89 ± 8.42 ^{d}
Alba 50 µg/ml + LPS	274.84 ± 10.71 ^{c}	158.50 ± 7.93 ^{c}	339.54 ± 16.98 ^{d}
Alba 100 µg/ml + LPS	333.36 ± 3.57 ^{b}	224.89 ± 11.24 ^{b}	422.37 ± 21.12 ^{c}
Alba 1 mg/ml + LPS	374.96 ± 9.12 ^{b}	275.59 ± 14.79 ^{b}	449.51 ± 44.29 ^{bc}

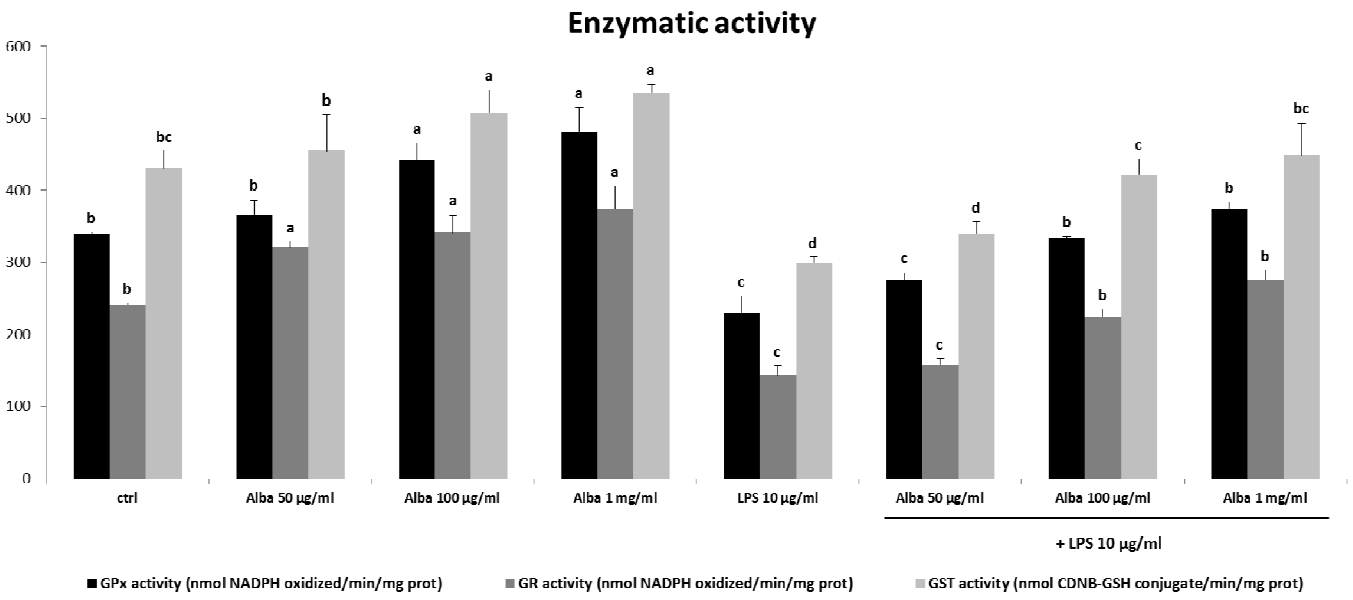


Figure 46. Effects of strawberry and LPS treatment on GPx, GR and GST activity in HDF cells. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

Similar results were obtained for SOD and catalase activities (Figure 47). Also in these cases strawberry treaments increased the enzymatic activities in a dose-dependent manner, and a significative differences respect to untreated cells (45.75 and 11.28 U/min/mg prot, for SOD and

catalase, respectively) were obtained with Alba at 50 µg/ml (68.46 U/min/mg prot) for SOD, and Alba at 100 µg/ml (14.91 U/min/mg prot) for catalase. However, the highest values were obtained with strawberry at dose of 1 mg/ml (97.90 and 18.70 U/min/mg prot, for SOD and catalase, respectively). The incubation with LPS determined a significative reduction of both enzymes activities (16.96 U/min/mg prot of SOD and 4.70 U/min/mg prot of catalase), that was efficiently counteracted by strawberry pre-treatment at concentration of 100 µg/ml in both SOD and catalase assays (38.59 and 11.01 U/min/mg prot, respectively), obtaining values similar to those of the control groups.

	SOD activity (U/min/mg prot)	Catalase activity (U/min/mg prot)
ctrl	45.75 ± 2.29^{c}	11.28 ± 0.56^{c}
Alba 50 µg/ml	68.46 ± 3.42^{b}	11.61 ± 0.58^{bc}
Alba 100 µg/ml	89.64 ± 4.48^{a}	14.91 ± 0.74^{ab}
Alba 1 mg/ml	97.90 ± 4.89^{a}	18.70 ± 0.93^{a}
LPS 10 µg/ml	16.96 ± 0.85^{c}	4.70 ± 0.24^{d}
Alba 50 µg/ml + LPS	23.97 ± 1.20^{c}	5.13 ± 0.26^{d}
Alba 100 µg/ml + LPS	38.59 ± 1.93^{c}	11.01 ± 0.55^{bc}
Alba 1 mg/ml + LPS	48.32 ± 2.42^{c}	15.89 ± 0.79^{ab}

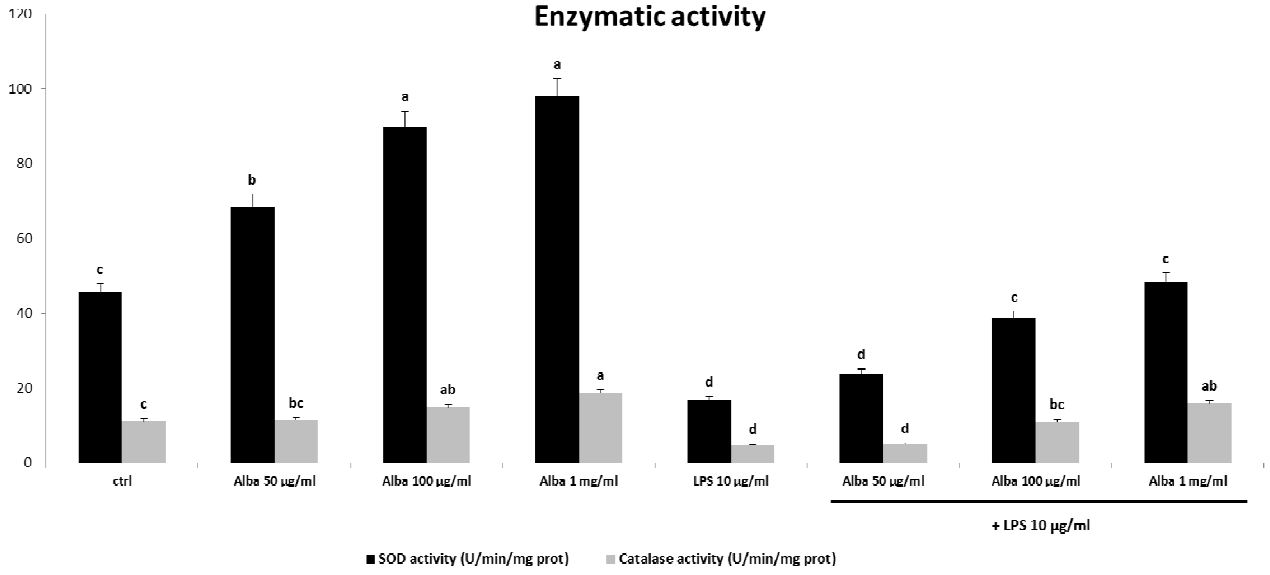


Figure 47. Effects of strawberry and LPS treatment on SOD and catalase activity in HDF cells. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.2.3.7. Lipid and protein oxidation level

In order to determine the level of lipid and protein damage, cell RIPA preparation of HDF cells was used to evaluate common markers of lipid (TBARS) and protein (GSH and protein carbonyl content) oxidation.

As shown in Figure 48, strawberry treatment significantly lowered the TBARS level respect to the control group (21.02 nmol/100 mg prot), expecially at doses of 100 µg/ml (14.56 nmol/100 mg prot) and 1 mg/ml (11.23 nmol/100 mg prot). LPS-treatment considerably increased TBARS level (33.25 nmol/100 mg prot); the oxidative damage was reduced by Alba pre-treatment, restoring values similar to the control group already with 100 µg/ml (23.85 nmol/100 mg prot).

	TBARS level (nmol /100 mg prot)	GSH level (nmol /mg prot)	Protein carbonyl content (nmol /mg prot)
ctrl	21.02 ± 2.12 ^{bc}	25.89 ± 1.29 ^{cd}	65.54 ± 1.63 ^{cd}
Alba 50 µg/ml	18.12 ± 1.39 ^{cd}	26.18 ± 1.31 ^{cd}	54.97 ± 4.92 ^{de}
Alba 100 µg/ml	14.56 ± 1.54 ^{de}	37.63 ± 1.88 ^{b}	41.15 ± 8.89 ^{ef}
Alba 1 mg/ml	11.23 ± 1.47 ^{e}	42.84 ± 2.14 ^{a}	31.55 ± 7.28 ^{f}
LPS 10 µg/ml	33.25 ± 2.89 ^{a}	9.72 ± 0.49 ^{f}	94.78 ± 4.74 ^{a}
Alba 50 µg/ml + LPS	31.66 ± 3.54 ^{a}	14.86 ± 0.74 ^{e}	93.28 ± 4.66 ^{a}
Alba 100 µg/ml + LPS	23.85 ± 1.58 ^{b}	22.42 ± 1.12 ^{d}	74.56 ± 3.72 ^{bc}
Alba 1 mg/ml + LPS	16.23 ± 1.77 ^{d}	31.89 ± 1.59 ^{bc}	62.24 ± 3.11 ^{cd}

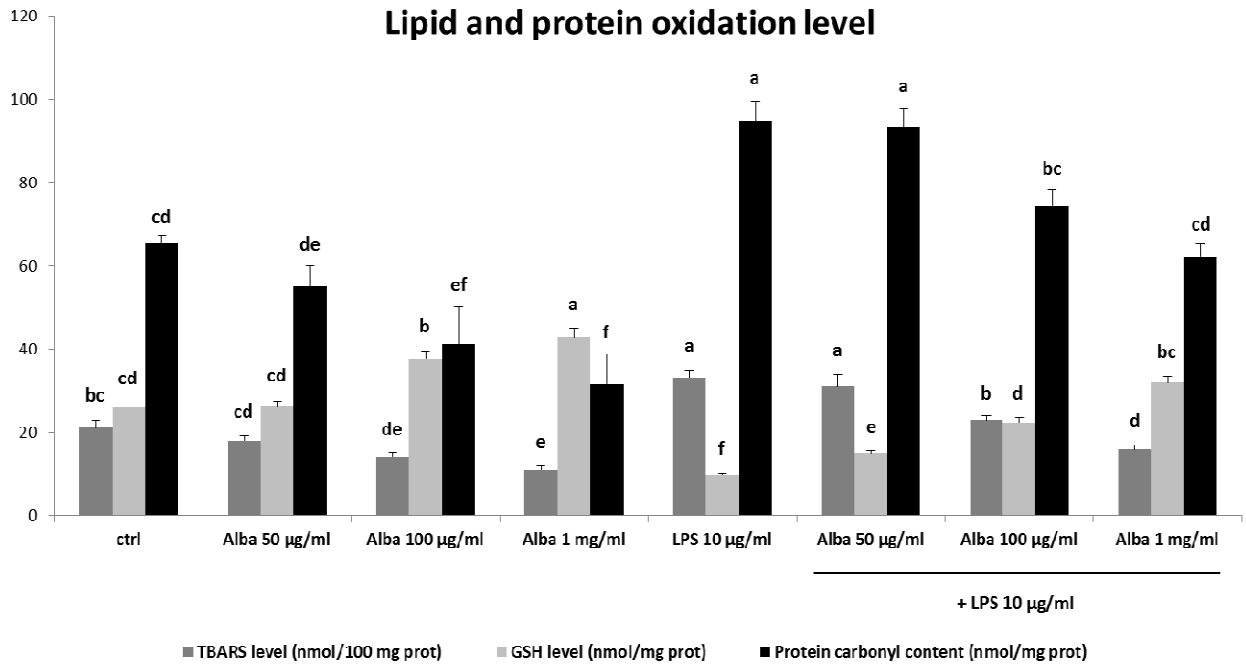
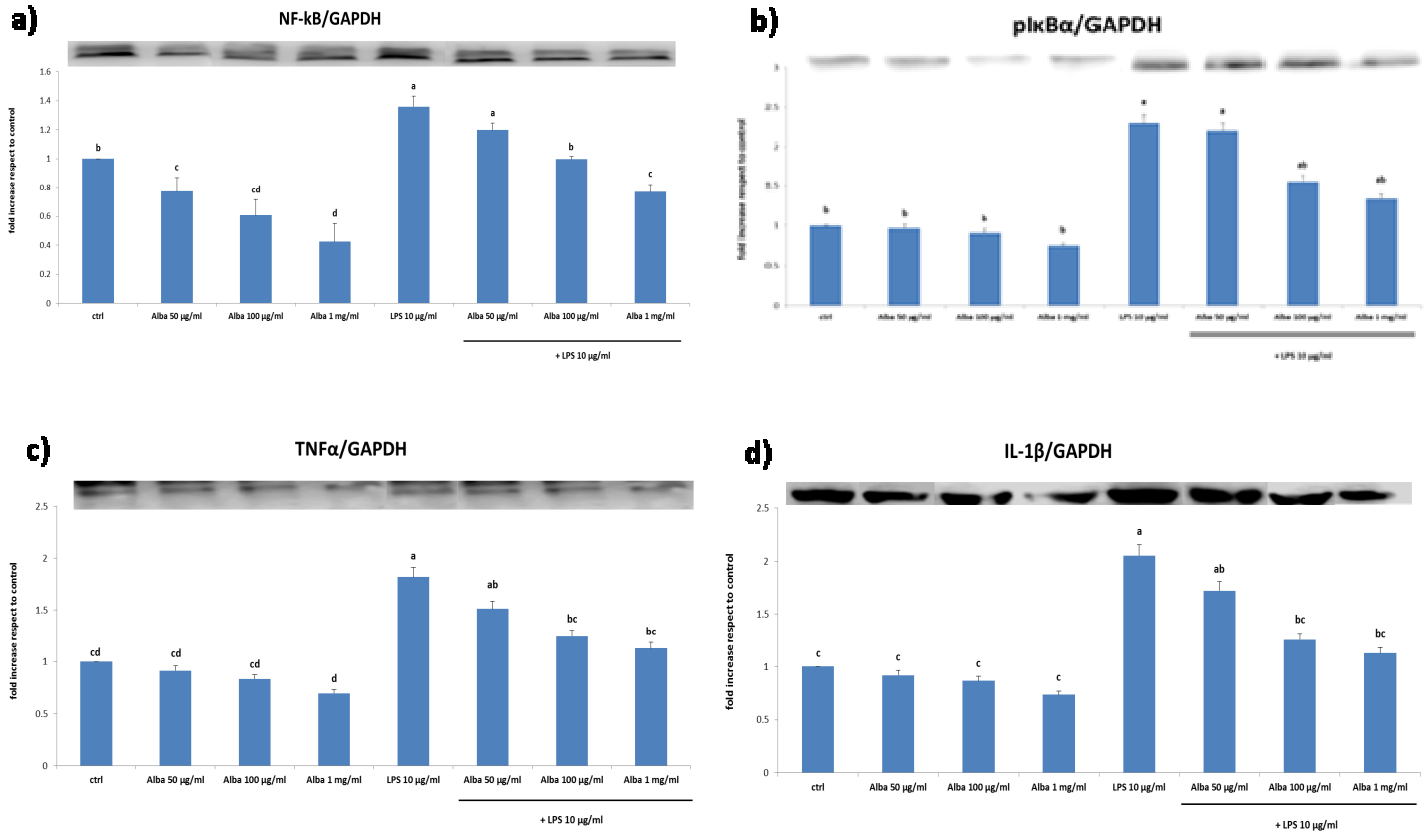


Figure 48. Effects of strawberry and LPS treatment on lipid and protein oxidation markers in HDF cells. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

Similar results were obtained with protein oxidation markers (Figure 48). Strawberry treatments improved GSH level and protein carbonyl content respect to untreated cells (25.89 and 65.54 nmol/mg prot, respectively), increasing the amount of GSH in the first case and reducing the carbonyl level in the other, significantly with Alba at 100 µg/ml in both the assays (37.63 and 41.15 nmol/mg prot, respectively). HDF cells treated with LPS showed a marked protein damage, as highlighted by the lowest value of GSH (9.72 nom/mg prot) and the highest value of carbonyl content (94.78 nmol/mg prot). The pre-treatment with strawberry extracts determined an improvement of protein damage induced by LPS in a dose-dependent manner: also in these cases, values statistically similar to the control groups were obtained with Alba at 100 µg/ml, both for GSH and carbonyl levels (22.42 and 74.56 nmol/mg prot, respectively).

4.2.3.8. Gene expression analysis

The analysis of gene expression allowed to evaluate the effect of strawberry and LPS treatment on different molecular pathway involved in HDF cells response. The different cell pellets were used to perform the analysis. First of all, the gene expressions of proteins related to inflammatory status were evaluated (Figure 49).



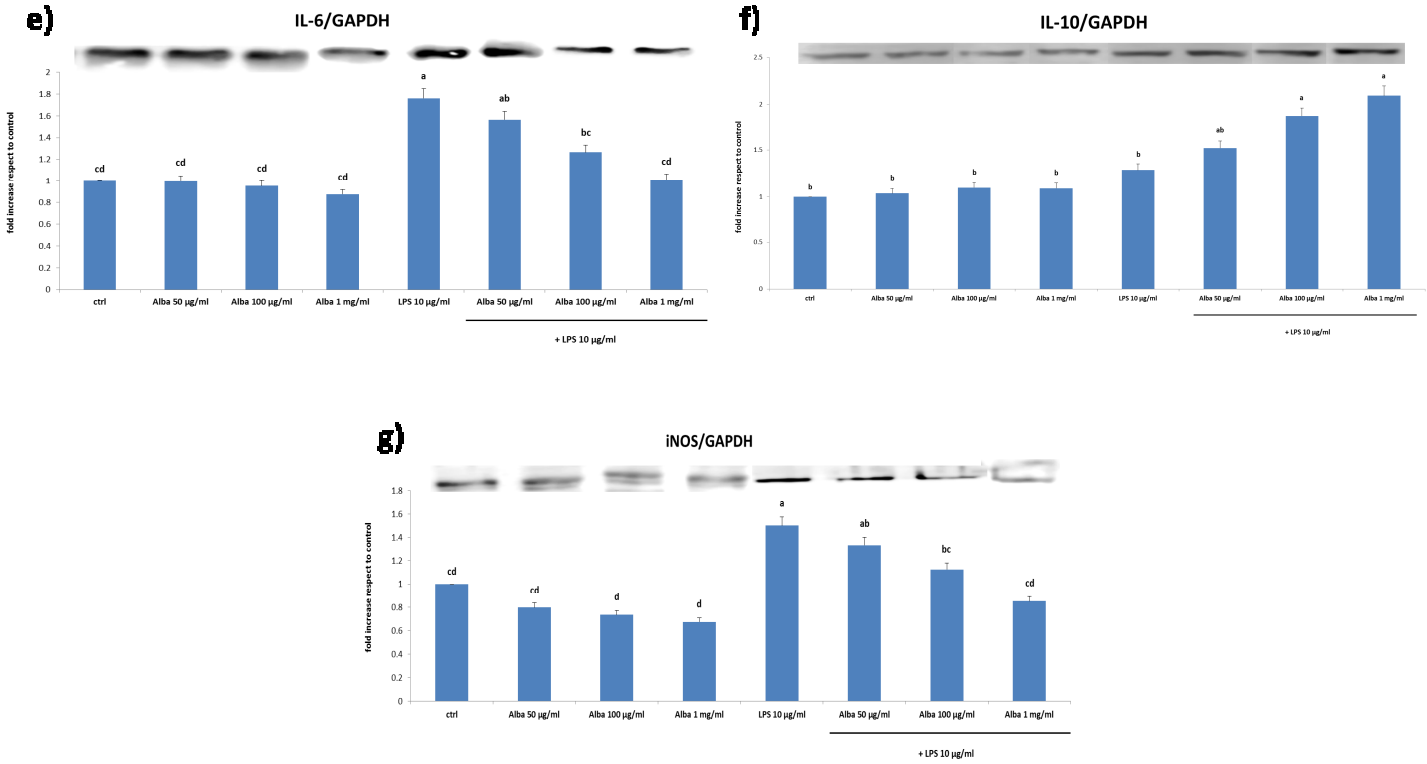


Figure 49. Effects of strawberry and LPS treatment on gene expression of inflammatory markers (NFkB, pIκBα, TNF-α, IL-1β, IL-6, IL-10 and iNOS) in HDF cells. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

Strawberry treatment lowered the level of the different inflammatory markers (NFkB, pIκBα, TNF-α, IL-1β, IL-6) at all the concentrations applied. LPS-treatment remarkably increased the expression of all the inflammatory proteins: higher values were obtained with pIκBα (fold increase of 2.29), IL-6 (fold increase of 2.02), TNF-α (fold increase of 1.82) and IL-1β (fold increase of 1.76); a lower increase was observed for NF-kB, with a fold increase of 1.36 respect to the control group. In all the cases pre-treatment with Alba extracts were able to reduce the inflammatory levels in a dose-dependent manner, restoring values similar to the control group already with 100 µg/ml (fold increase of 1.54, 1.26, 1.24, 1.25 and 1.01 for pIκBα, IL-6, TNF-α, IL-1β and NF-kB, respectively) (Figure 49a, b, c, d, e). In case of IL-10, an increase of the protein expression was observed with all the applied treatments but the raise was significative only with Alba at 100 µg/ml + LPS and Alba at 1 mg/ml + LPS, with fold increase values of 1.86 and 2.09 respectively, compared to the untreated cells (Figure 49f). Finally, taking into account iNOS expression, also in this case the LPS-treatment significantly increased the protein level (fold increase of 1.50) respect to the control group. Strawberry pre-treatment reduced the gene expression and also counteracted the LPS-

induced stress, at the different doeses applied, in particular with Alba at 100 µg/ml (fold increae of 1.12) (Figure 49g).

After that, the gene expressions of proteins related to antioxidant response were tested (Figure 50).

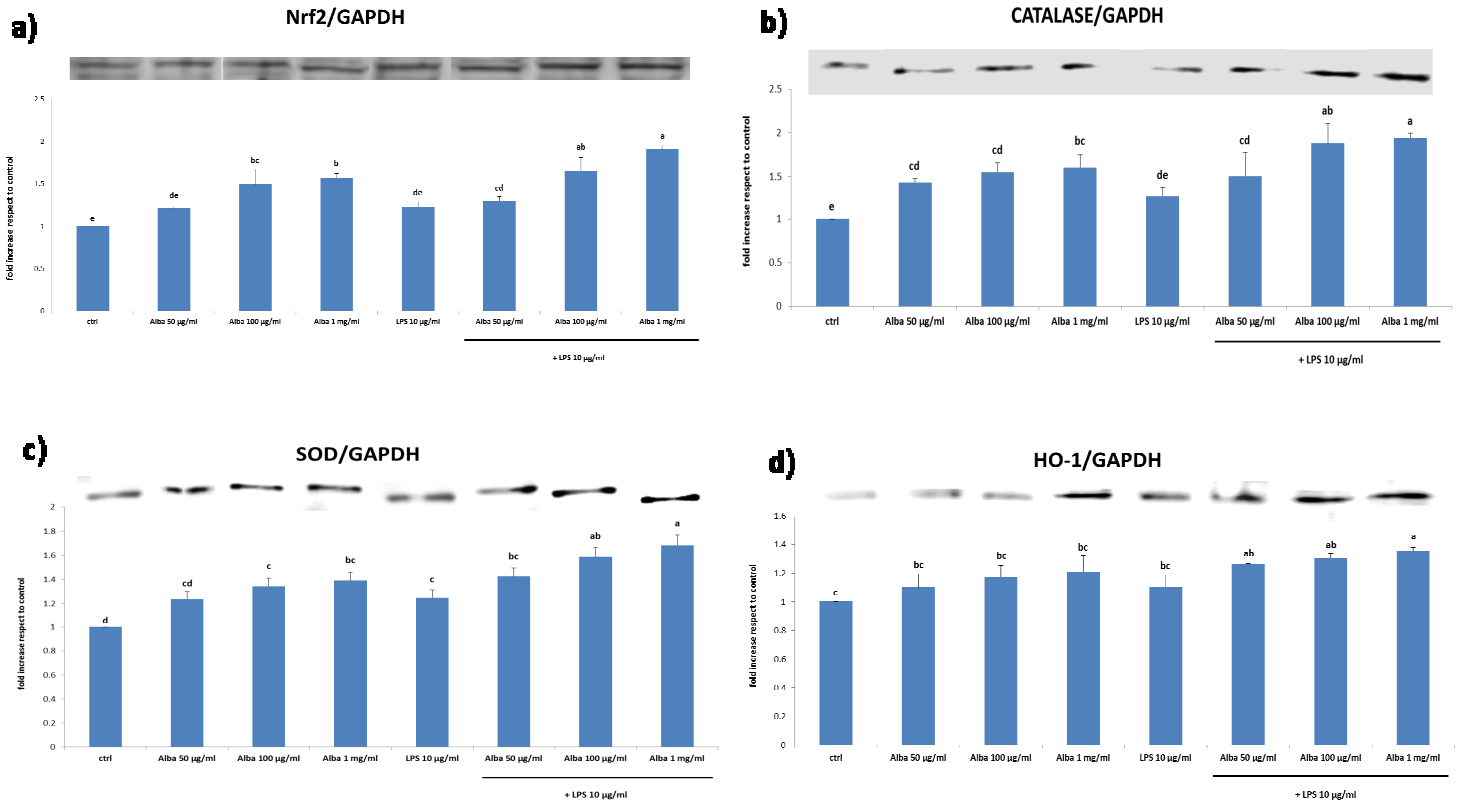


Figure 50. Effects of strawberry and LPS treatment on gene expression of protein related to antioxidant response (Nrf2, catalase, SOD, HO-1) in HDF cells. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

Alba extracts increased the level of the different proteins (Nrf2, catalase, SOD, HO-1) in a dose-dependent manner, alone or in combination with LPS. Nrf2 expression (Figure 50a) showed a significative difference respect to the control group with Alba at 100 µg/ml and 1 mg/ml (fold increase of 1.49 and 1.57, respectively) and with strawberry at 50, 100 µg/ml and 1 mg/ml + LPS (fold increase of 1.30, 1.65 and 1.90, respectively). Similar trend were obtained with catalase and SOD (Figure 50b, c): Alba improved these gene expressions at all the different concentrations applied, reaching highest values with strawberry at 1 mg/ml + LPS (fold increase of 1.94 for catalase and 1.68 for SOD). In case of HO-1 only the combination of Alba extracts and LPS

produced a significative difference respect to the untreated cells, with fold increase values of 1.26, 1.30 and 1.35 with strawberry at 50, 100 µg/ml and 1 mg/ml + LPS, respectively (Figure 50d).
The gene expressions of proteins related to the AMPK pathway were also investgated (Figure 51).

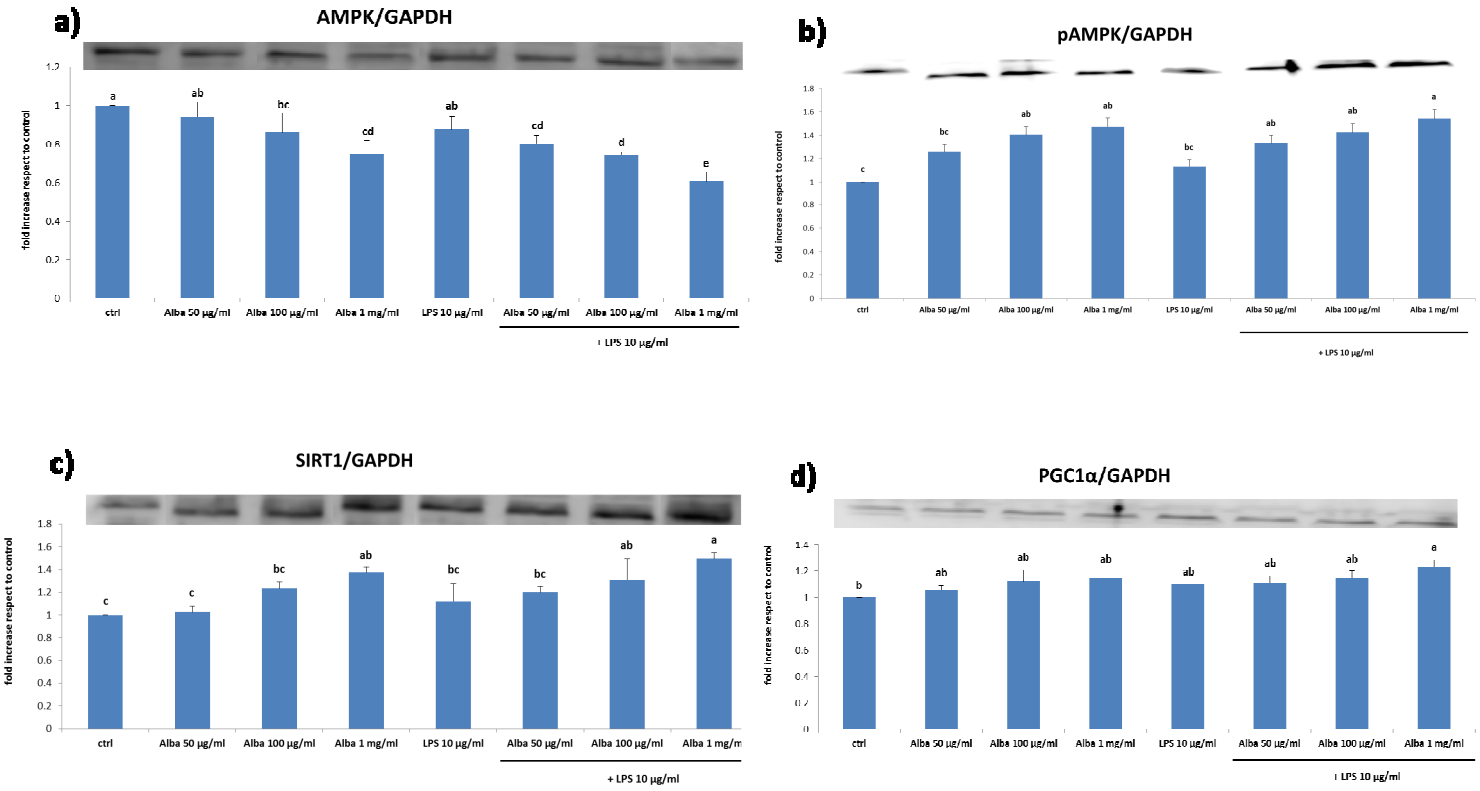


Figure 51. Effects of strawberry and LPS treatment on gene expression of protein related to AMPK pathway (AMPK, p-AMPK, SIRT1, PGC1α) in HDF cells. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

Strawberry treatments reduced AMPK level in a dose-dependent manner (Figure 51a), this effect was highlighted in combination with LPS, obtaining lower values with Alba at 100 µg/ml + LPS (fold increase of 0.74) and 1 mg/ml + LPS (fold increase of 1.60). An opposite trend was obtained with pAMPK (Figure 51b): in this case strawberry extracts significantly increased this protein expression alone (fold increase of 1.40 and 1.47 with Alba at 100 µg/ml and 1 mg/ml, respectively) or before the LPS-treatment (fold increase of 1.33, 1.42 and 1.54 with Alba at 50, 100 µg/ml and 1 mg/ml, respectively). SIRT1 expression presented a trend similar to pAMPK (Figure 51c): strawberry pre-treatment remarkably increased protein expression at 1 mg/ml (fold increase 1.37) and, in combination with LPS, at 100 µg/ml (fold increase of 1.31) and 1 mg/ml (fold increase of

1.49). Finally, taking into account PGC1α level (Figure 51d), only the pre-treatment with Alba extract at 1 mg/ml + LPS determined a significative incease of gene expression (fold increase of 1.23) respect to untreated group. The DNA damage level was also investigated, through the evaluation of OGG1 expression (Figure 52). Cell treatment with Alba extracts significantly reduced OGG1 gene expression in a dose-dependent manner (fold increase of 0.71 and 0.62 with Alba at 100 µg/ml and 1 mg/ml, respectively). On the contrary LPS-treatment increased the level of DNA damage (fold increase of 1.32), which is efficiently counteracted by pre-incubation with strawberry already at 100 µg/ml (fold increase of 0.94).

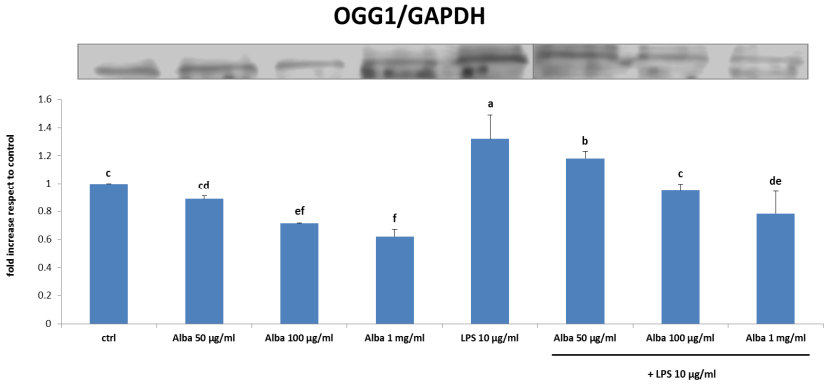


Figure 52. Effects of strawberry and LPS treatment on gene expression of protein related to DNA damage level (OGG1) in HDF cells. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

Moreover the apoptosis rate, by the measurement of caspase 3 expression, was also evaluated (Figure 53). Alba extracts reduced the caspase 3 level at the different concentrations applied (fold increase of 0.85, 0.81, 0.79 at 50, 100 µg/ml and 1 mg/ml, respectively). Also in this case, the highest value was obtained in LPS group (fold increase of 1.38), which is efficiently lowered by pre-treatment with strawberry in a dose-dependent manner, restoring values similar to the control group already with Alba extracts at 100 µg/ml (fold increase of 1.13).

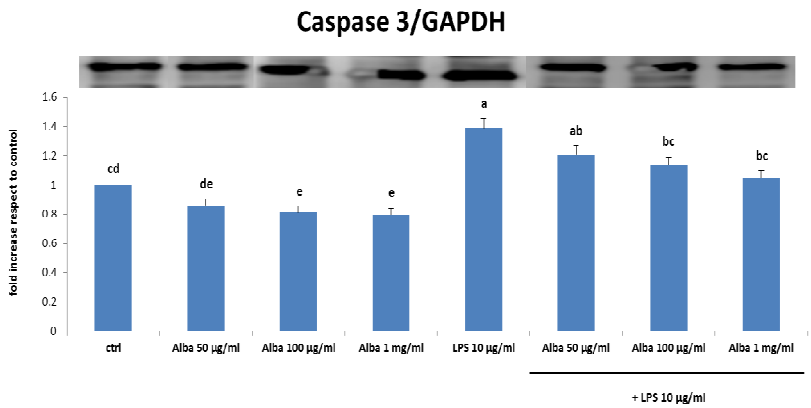


Figure 53. Effects of strawberry and LPS treatment on gene expression of protein related to apoptosis rate (caspase 3) in HDF cells. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

In conclusion, the amount of the protein analyzed in each Western blot performed was analyzed through the measurement of the expression of GAPDH, a protein with a known molecular weight that is present in each analyzed sample (Figure 54). The expression of GAPDH depends on the amount of protein analyzed so, in order to have comparable results, is necessary to find the same amount of GAPDH in each band of the Western blot.

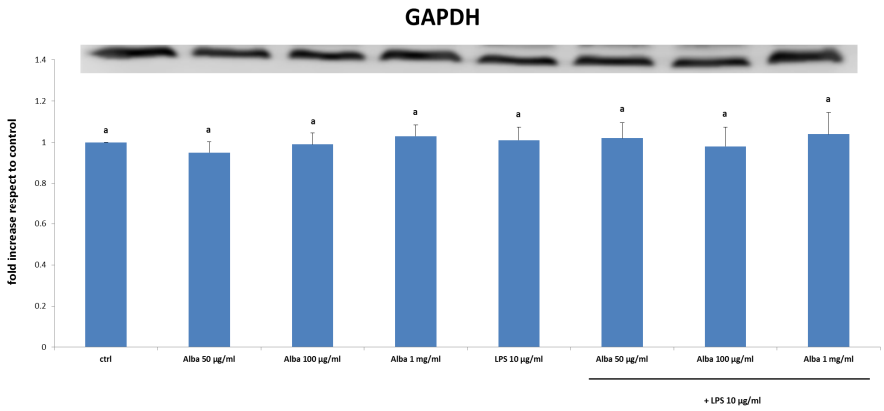


Figure 54. Representative analysis of GAPDH gene expression in HDF cells treated with strawberry and LPS. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.2.4. RAW macrophages results

4.2.4.1. MTT viability assay

RAW mcarophages were incubated for 24, 48 and 72 h with 200 µl of different concentrations of dried strawberry extracts (25, 50, 100, 250, 500 µg/ml and 1, 2.5, 5, 7.5, 10 mg/ml), in order to evaluate the possible cytotoxic effect of Alba solutions. At 24 h an increase of cell viability was observed up to 1 mg/ml of Alba (+ 24% respect to control group), after this concentration value, the viability start to decrease in a dose-dependent manner (-25% with Alba at 10 mg/ml). A similar trend was observed at 48 and 72 h, but in these cases the cell viability started to decrease from

lower values of concentration (1 mg/ml and 500 µg/ml, respectively) (Figure 55). For this reason, 24 h of treatment with a concentration range of Alba extracts from 25 µg/ml to 1 mg/ml was chosen to test the effect of strawberry in combination with LPS action.

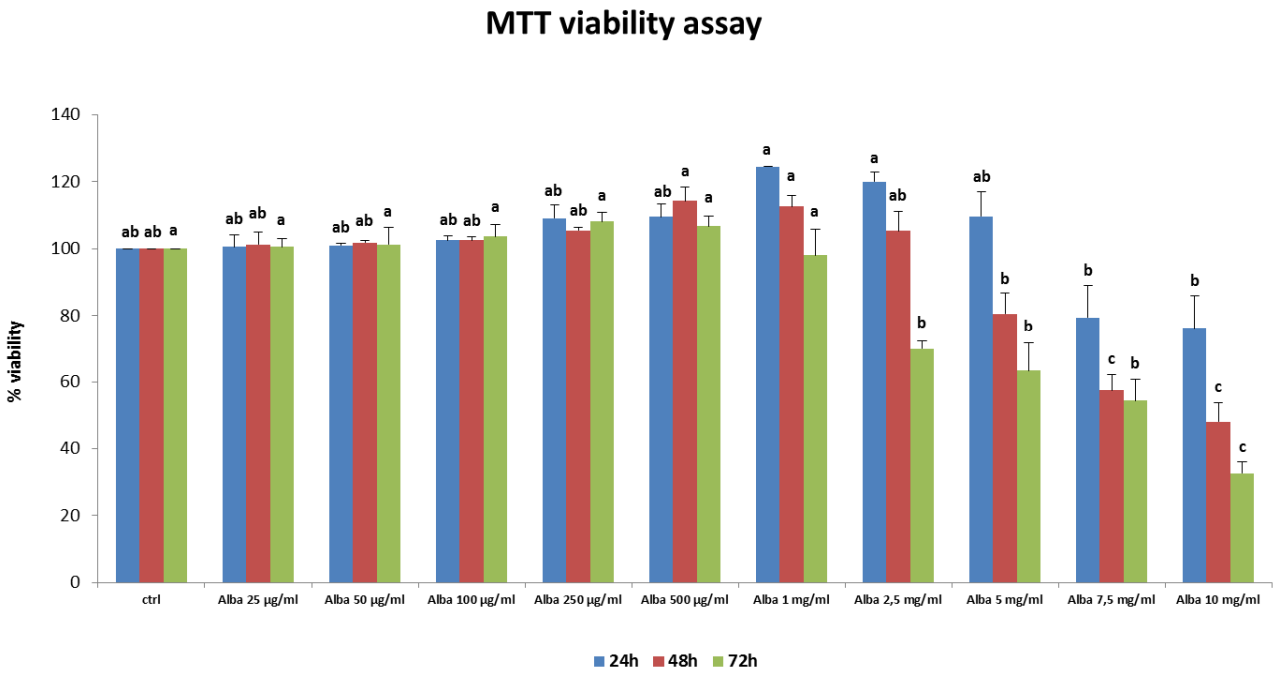


Figure 55. MTT assay for the determination of cell viability in RAW macrophages treated with different time-concentrations of strawberry extracts. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

To test the effect of LPS on cell viability, RAW macrophages were incubated with 200 µl of 20, 50, 100, 500 ng/ml and 1, 5, 10 µg/ml of LPS, for 6, 12 and 24 h. All the different concentrations applied for 6 and 12 h determined an increase on cell viability, but did not produce any statistical difference respect to untreated cells. Only at 24 h a significant improvement of cell viability respect to the control group was observed, starting from 1 µg/ml of LPS (+ 12%) (Figure 56). For this reason, 1 µg/ml of LPS, applied for 24 h, was chosen as LPS-dosage for the next steps.

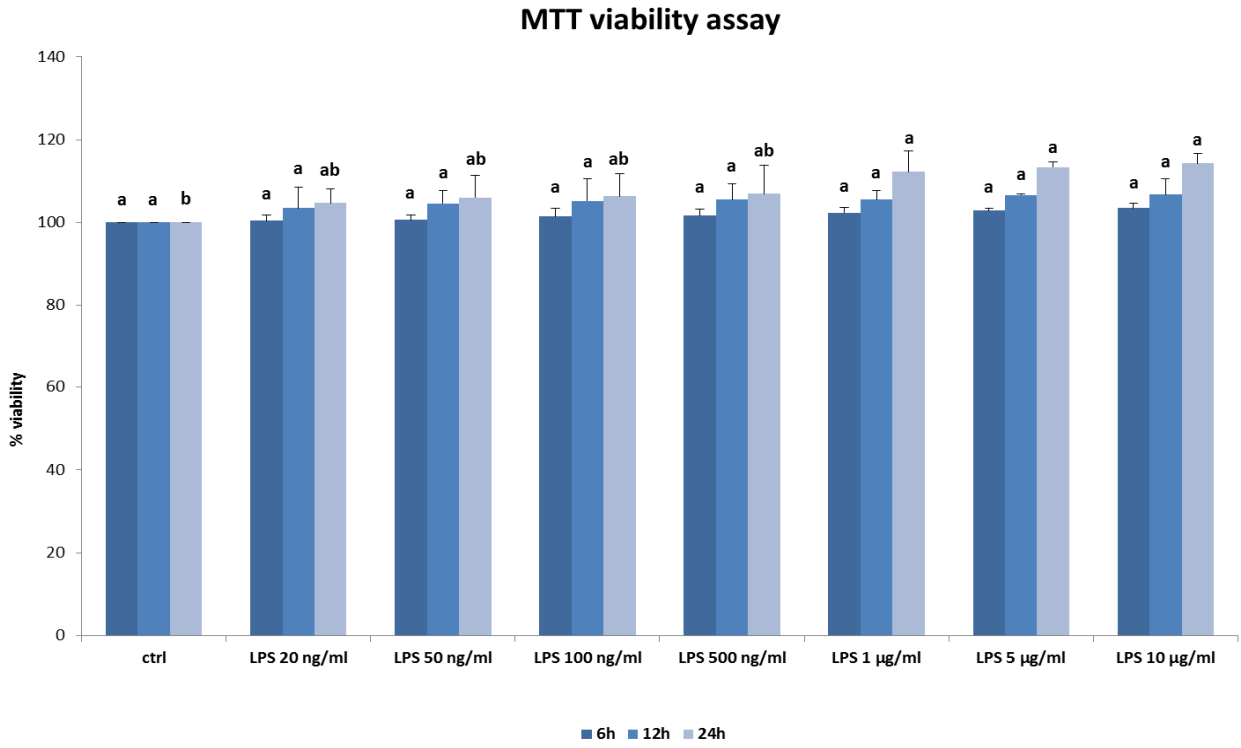


Figure 56. MTT assay for the determination of cell viability in RAW macrophages treated with different time-concentration of LPS. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

Finally, the effect of strawberry supplementation before the LPS treatment on RAW macrophages viability (strawberry at 25, 50, 100, 250, 500 µg/ml and 1 mg/ml, for 24 h + LPS at 1 µg/ml, for 24 h) was also evaluated, in order to highlight the role of strawberry pre-treatment on LPS effect. Alba extracts improved the positive effect on cell viability exerted by the LPS at all the concentration applied in a dose-dependent manner, although any statistical difference respect to LPS-treated cells was detected (Figure 57). For this reason further analysis were conducted using strawberry concentration up to 1 mg/ml, since that this value provided the maximal cell viability (+ 18%) respect to control group.

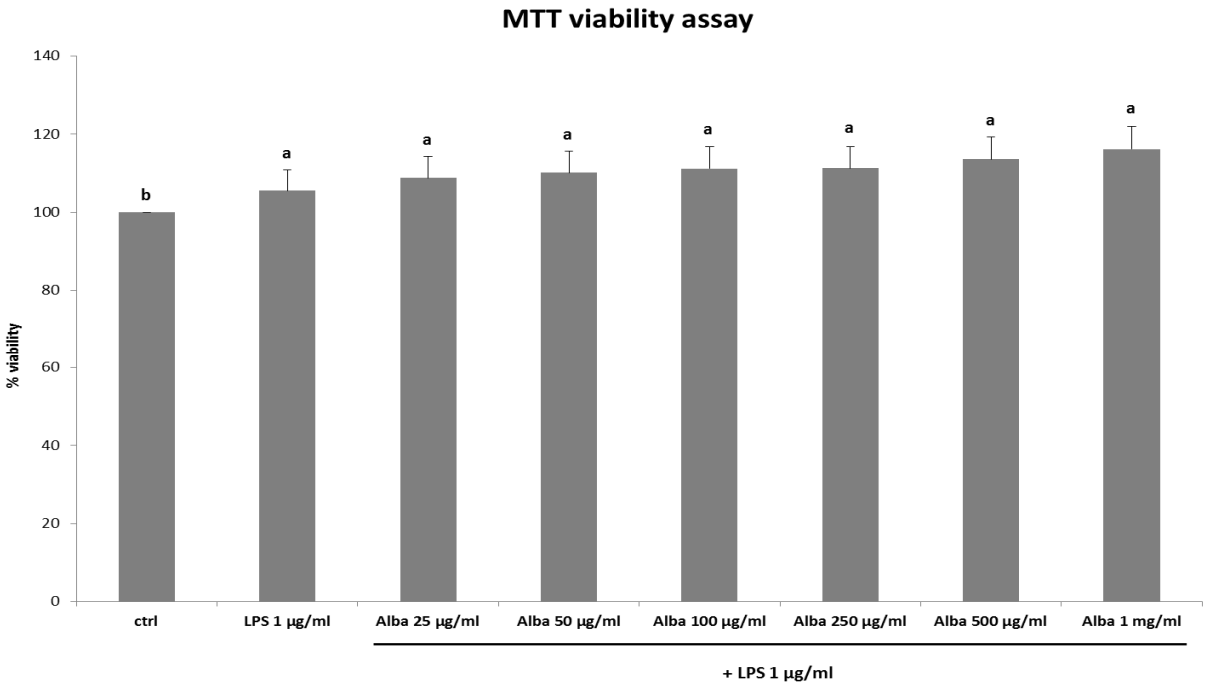


Figure 57. MTT assay for the determination of cell viability in RAW macrophages treated with different concentration of strawberry and LPS, for 24 h. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.2.4.2. TALI® ROS concentration assay

To test the effect of strawberry pre-treatment on LPS-induced ROS production, RAW macrophages were treated with Alba dried extract at different concentrations (25, 50, 100, 250, 500 µg/ml and 1mg/ml, for 24 h) before the incubation with LPS (1 µg/ml for 24 h). In figure 58 (a and b) the effect of the strawberry extract on ROS concentration is shown: Alba did not stimulate ROS production at all the different concentrations applied. In particular, already at dose of 250 µg/ml, a significant reduction of ROS level (fold increase of 0.70) was registred, respect to untreated control group. In RAW macrophages pre-treated with different strawberry extracts and stressed wtith LPS a reduction of intracellular ROS was found in a dose-dependent manner. As observd in HDF cells, a significant decrease in the amount of ROS respect to the control represented by only LPS (fold increase of 2.66) was obtained with strawberry concentrations of 50 µg/ml (fold increase of 1.88), 100 µg/ml (fold increase of 1.58) and 1 mg/ml (fold increase of 1.01). For this reason these doses of Alba extracts, in combination with LPS at 1 µg/ml, were used for all the further analysis.

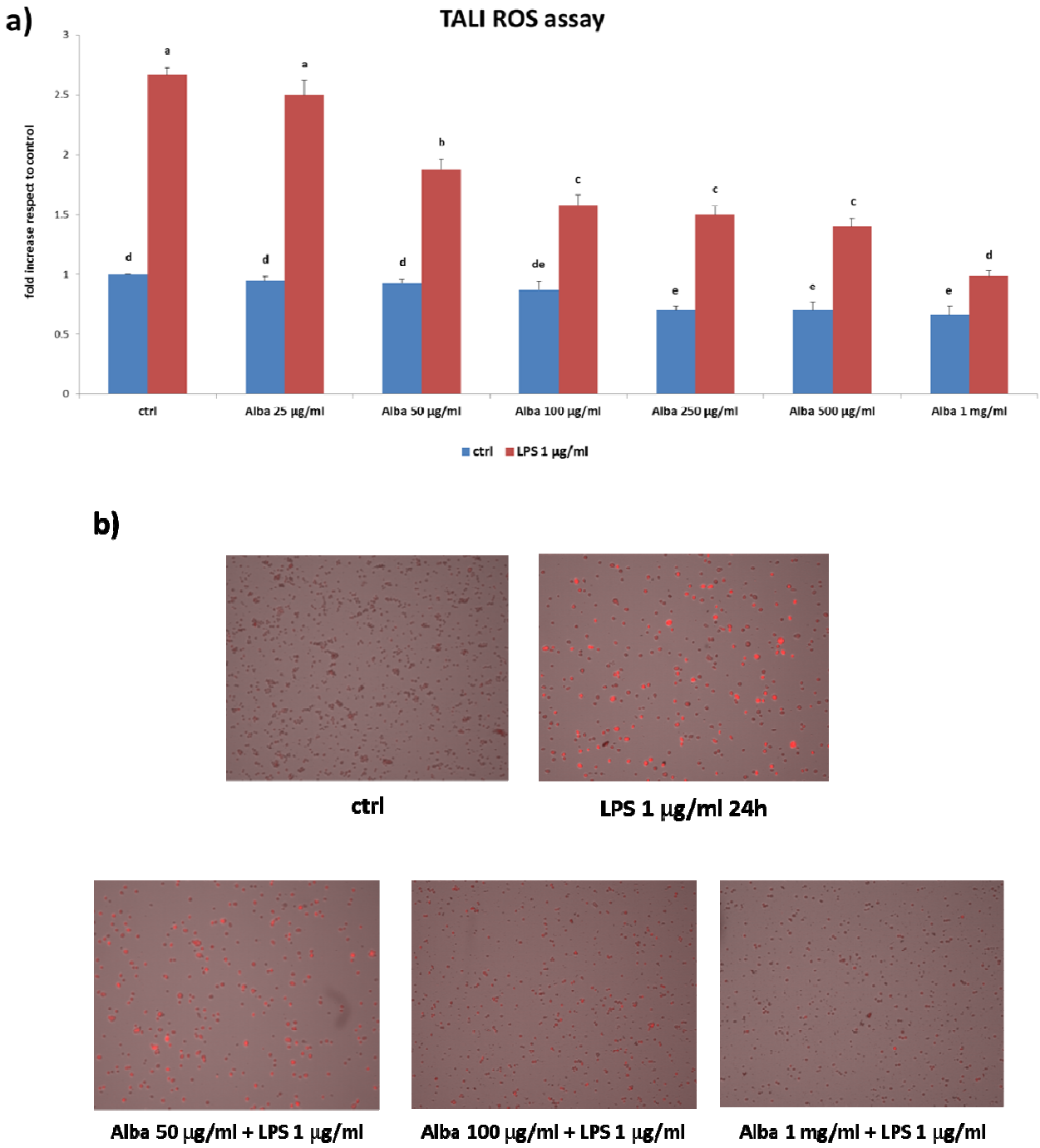


Figure 58. a) TALI ROS assay for the determination of cell ROS concentration in RAW macrophages treated with strawberry and LPS. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$). **b)** TALI ROS images of ROS concentration in RAW macrophages treated with strawberry and LPS. The amount of red point is proportional to the intracellular ROS present in the different samples.

4.2.4.3. XF24 Analyzer® respiratory capacity assay

To examine the potential role of strawberry extracts on mitochondrial function in combination with LPS stress, the respiratory capacity assay was performed on RAW macrophages treated with different strawberry concentrations (50, 100 µg/ml and 1 mg/ml, for 24 h) and subsequentally incubated with LPS (1 µg/ml, for 24 h). Figure 59 shows the trend of the different tested groups, in function of the different inhibitors applied, that were the same used for HDF assay (oligomycin, 2,4-DNP, antimycin A/rotenone). Starting from the baseline values of OCR, LPS treatment produced a considerable increasing (+73 pmol O_2/min/10^5 cells) of the oxygen consumption respect to the control group (216 pmol O_2/min/10^5 cells). Similarly, the strawberry treatment improved the mitochondrial respiration increasing the OCR level, respect to control, of 13, 35 and 47 pmol O_2/min/10^5 cells, with 50, 100 µg/ml and 1 mg/ml, respectively. Moreover, Alba extracts were also able to enhance the positive effect exerted by LPS, increasing the OCR value in a dose-dependent manner, reaching oxygen consumption values of 306 pmol O_2/min/10^5 cells with Alba at 50 µg/ml + LPS, 317 pmol O_2/min/10^5 cells with Alba at 100 µg/ml + LPS and 340 pmol O_2/min/10^5 cells with Alba at 1 mg/ml + LPS. Also for RAW macrophages, the response of the different tested groups to the inhibitors of the respiratory chain complex was the same: oligomycin produced a reduction of the respiration rate; 2,4-DNP strongly increased the OCR value and finally the antimycin A/rotenone injection determined a critical decrese of the oxygen consumption (Figure 59).

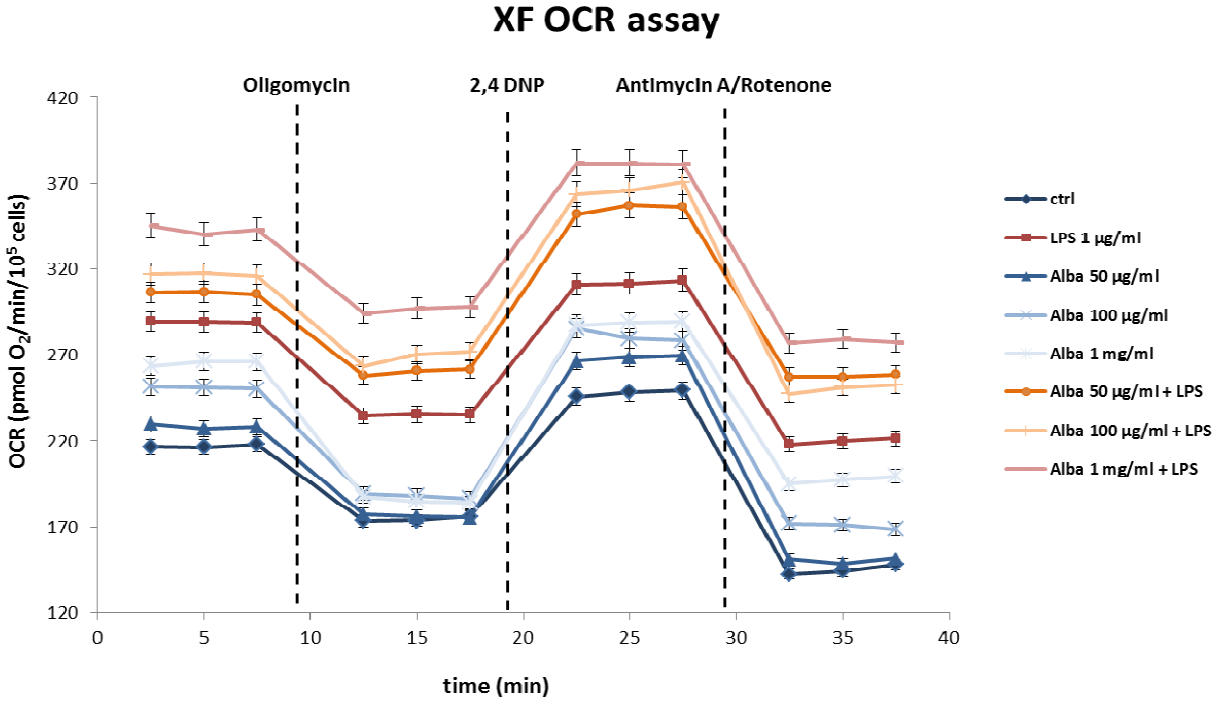


Figure 59. Effects of strawberry and LPS treatment on OCR in RAW macrophages. Mitochondria oxygen consumption was monitored with sequential injection of oligomycin, 2,4-DNP and antimycin A/rotenone at the indicated time points into each well, after baseline rate measurement. Data are expressed as mean values ± SD.

With regard to the maximal respiratory capacity, untreated RAW macrophages showed the lowest values, 75.16 pmol O_2/min/10^5 cells. Strawberry treatments increased this value in a dose-dependent manner: significative differences respect to the control group were obtained with Alba at 100 µg/ml (91.71 pmol O_2/min/10^5 cells) and 1 mg/ml (98.79 pmol O_2/min/10^5 cells). Also LPS-incubation determined a remarkably improvement of maximal respiratory capacity (92.94 pmol O_2/min/10^5 cells); this result was further raised by pre-treatment with strawberry at all the different concentrations applied (102.85, 109.76 and 122.70 pmol O_2/min/10^5 cells with Alba at 50, 100 µg/ml and 1 mg/ml + LPS, respectively) (Figure 60).

	Maximal Respiratory Capacity (pmol O_2/min/10^5 cells)
ctrl	75.61 ± 3.78^{e}
Alba 50 µg/ml	83.51 ± 4.18^{de}
Alba 100 µg/ml	91.71 ± 4.59^{cd}
Alba 1 mg/ml	98.79 ± 4.94^{bc}
LPS 1 µg/ml	92.94 ± 4.65^{cd}
Alba 50 µg/ml + LPS	102.85 ± 5.14^{bc}
Alba 100 µg/ml + LPS	109.76 ± 5.49^{ab}
Alba 1 mg/ml + LPS	122.70 ± 6.14^{a}

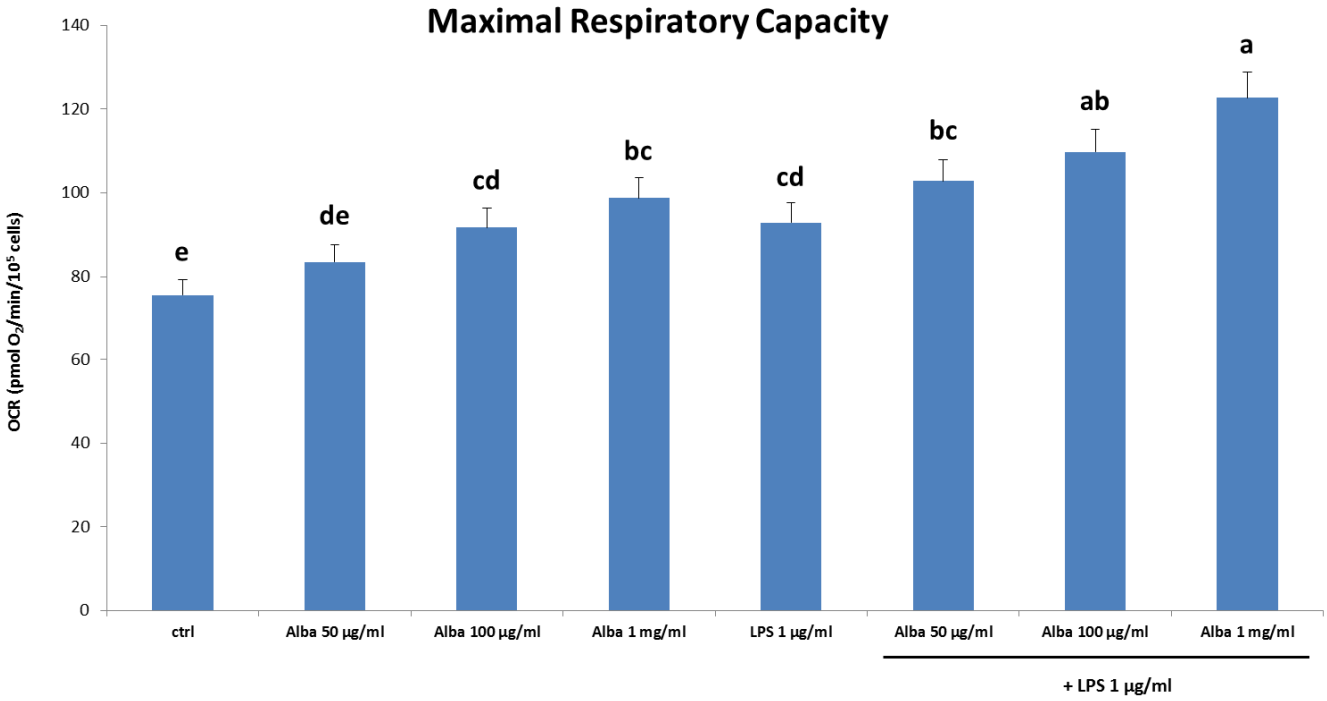


Figure 60. Effects of strawberry and LPS treatment on maximal respiratory capacity in RAW macrophages. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.2.4.4. Determination of nitrite production

RAW macrophages were pre-treated with different strawberry concentrations (50, 100 µg/ml and 1 mg/ml, for 24 h) and subsequentally incubated with LPS (1 µg/ml, for 24 h), in order to determine the nitric oxide production level of the different treatments.

As indicated in Figure 61, strawberry extracts were able to reduce the NO production in a dose-dependent manner (0.61, 0.42 and 0.31 nmol/mg prot, with 50, 100 µg/ml and 1 mg/ml of strawberry extracts, respectively), respect to the untreated RAW macrophages (0.62 nmol/mg prot). LPS-treatment significantly increased the NO level (1.60 nmol/mg prot), which was efficiently counteracted with Alba pre-treatment, and was restored to the control group value with strawberry at dose of 100 µg/ml (0.85 nmol/mg prot).

	Nitrite levels (nmol / mg prot)
ctrl	0.62 ± 0.03 ^{c}
Alba 50 µg/ml	0.61 ± 0.03 ^{c}
Alba 100 µg/ml	0.42 ± 0.02 ^{c}
Alba 1 mg/ml	0.31 ± 0.02 ^{c}
LPS 1 µg/ml	1.60 ± 0.08 ^{a}
Alba 50 µg/ml + LPS	1.23 ± 0.06 ^{ab}
Alba 100 µg/ml + LPS	0.85 ± 0.04 ^{bc}
Alba 1 mg/ml + LPS	0.69 ± 0.03 ^{bc}

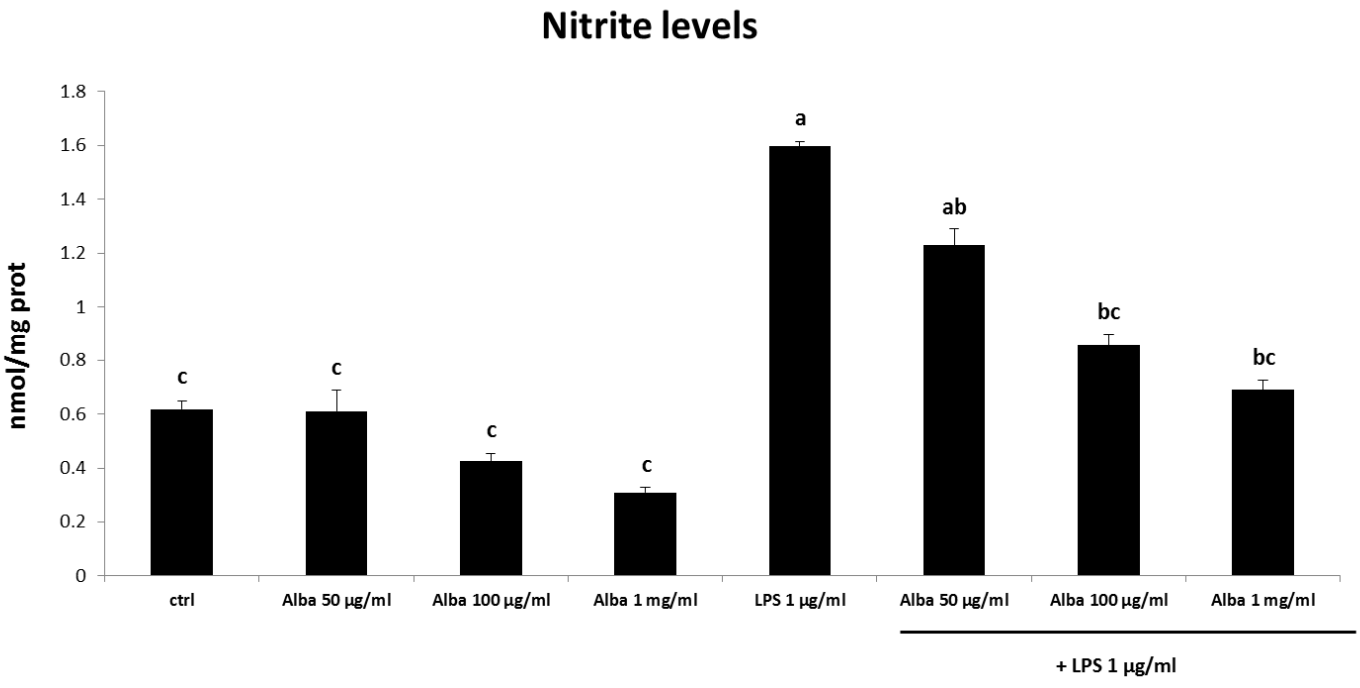


Figure 61. Effects of strawberry and LPS treatment on NO levels in RAW macrophages. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.2.4.5. Enzymatic activity assays

As for HDF cells, Cell RIPA preparation of RAW macrophages was used to evaluate the activity of different enzymes. Also in this case, a similar trend was obtained for GPx, GR and GST levels, as shown in Figure 62. Alba treatments were able to increase these enzymatic activities in a dose-dependent manner. The highest results were obtained with strawberry at concentration of 1 mg/ml with values of 509.96, 495.55 and 538.15 nmol/min/mg prot for GPx, GR and GST, respectively. With LPS treatment, a significative reduction of enzymatic activity was registred for all the tested enzymes (289.23, 201.39 and 253.09 nmol/min/mg prot, for GPx, GR and GSR respectively). These activities were improved with Alba pre-treatment at the different concentrations applied: values statistically similar to the control group (427.91, 325.32 and 387.13 nmol/min/mg prot, for GPx, GR and GST respectively) were obtained with Alba at 100 µg/ml in all the conducted assays (386.26 nmol/min/mg prot for GPx, 322.47 nmol/min/mg prot for GR and 383.44 nmol/min/mg prot for GST).

	GPx activity	GR activity	GST activity
	(nmol NADPH oxidized/min/mg prot)	(nmol NADPH oxidized/min/mg prot)	(nmol CDNB-GSH conjugate/min/mg prot)
ctrl	427.91 ± 21.39^{ab}	325.32 ± 16.27^{c}	387.13 ± 19.36^{bc}
Alba 50 µg/ml	460.23 ± 23.01^{ab}	374.64 ± 18.73^{bc}	389.26 ± 19.47^{bc}
Alba 100 µg/ml	499.32 ± 24.96^{a}	435.27 ± 21.76^{ab}	447.99 ± 22.34^{ab}
Alba 1 mg/ml	509.96 ± 25.49^{a}	495.55 ± 32.75^{a}	538.15 ± 26.91^{a}
LPS 1 µg/ml	289.23 ± 14.46^{d}	201.39 ± 10.07^{e}	253.09 ± 12.65^{d}
Alba 50 µg/ml + LPS	332.17 ± 16.61^{cd}	252.01 ± 12.60^{de}	260.88 ± 13.04^{d}
Alba 100 µg/ml + LPS	386.26 ± 19.31^{bc}	322.47 ± 16.12^{cd}	383.44 ± 19.17^{bc}
Alba 1 mg/ml + LPS	435.95 ± 21.79^{ab}	421.66 ± 21.08^{ab}	419.36 ± 20.97^{ab}

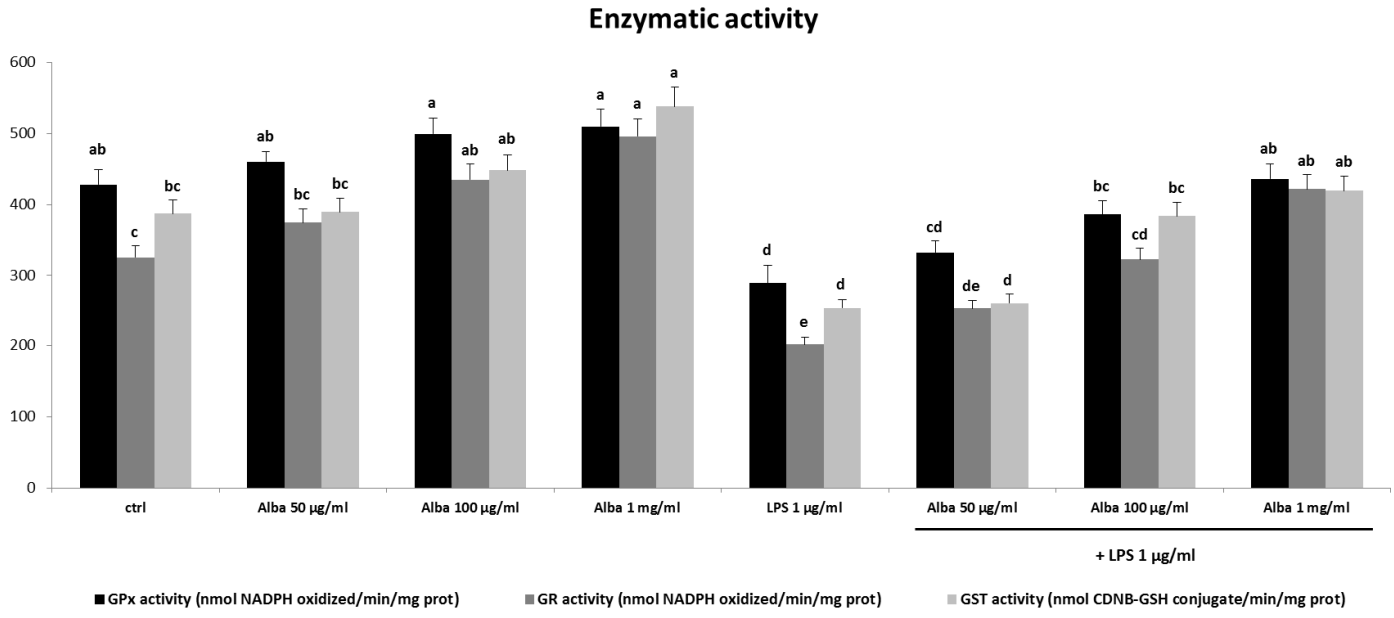


Figure 62. Effects of strawberry and LPS treatment on GPx, GR and GST activity in RAW macrophages. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

SOD and catalase activities presented similar results (Figure 63). Strawberry treaments increased the enzymatic activities in a dose-dependent manner: also in these cases the highest values were obtained with strawberry at dose of 1 mg/ml (93.82 and 26.26 U/min/mg prot, for SOD and catalase, respectively). The incubation with LPS determined a remarkably reduction of both enzymes activities (41.61 U/min/mg prot of SOD and 4.94 U/min/mg prot of catalase), that was efficiently improved by strawberry pre-treatment: values similar to those of the control groups (69.52 U/min/mg prot for SOD and 13.74 U/min/mg prot for catalase) were obtained with Alba extracts at concentration of 100 µg/ml in both the realized assays (63.59 and 10.09 U/min/mg prot, for SOD and catalase respectively),

	SOD activity (U/min/mg prot)	Catalase activity (U/min/mg prot)
ctrl	69.52 ± 3.48^{ab}	13.74 ± 0.68^{b}
Alba 50 µg/ml	83.19 ± 4.16^{ab}	14.76 ± 0.74^{b}
Alba 100 µg/ml	86.36 ± 4.32^{ab}	23.26 ± 1.16^{a}
Alba 1 mg/ml	93.82 ± 4.69^{a}	26.25 ± 1.31^{a}
LPS 1 µg/ml	41.61 ± 2.08^{c}	4.94 ± 0.25^{c}
Alba 50 µg/ml + LPS	44.90 ± 2.25^{c}	6.59 ± 0.33^{c}
Alba 100 µg/ml + LPS	63.59 ± 3.18^{bc}	10.09 ± 0.50^{bc}
Alba 1 mg/ml + LPS	70.24 ± 3.51^{ab}	13.62 ± 0.68^{b}

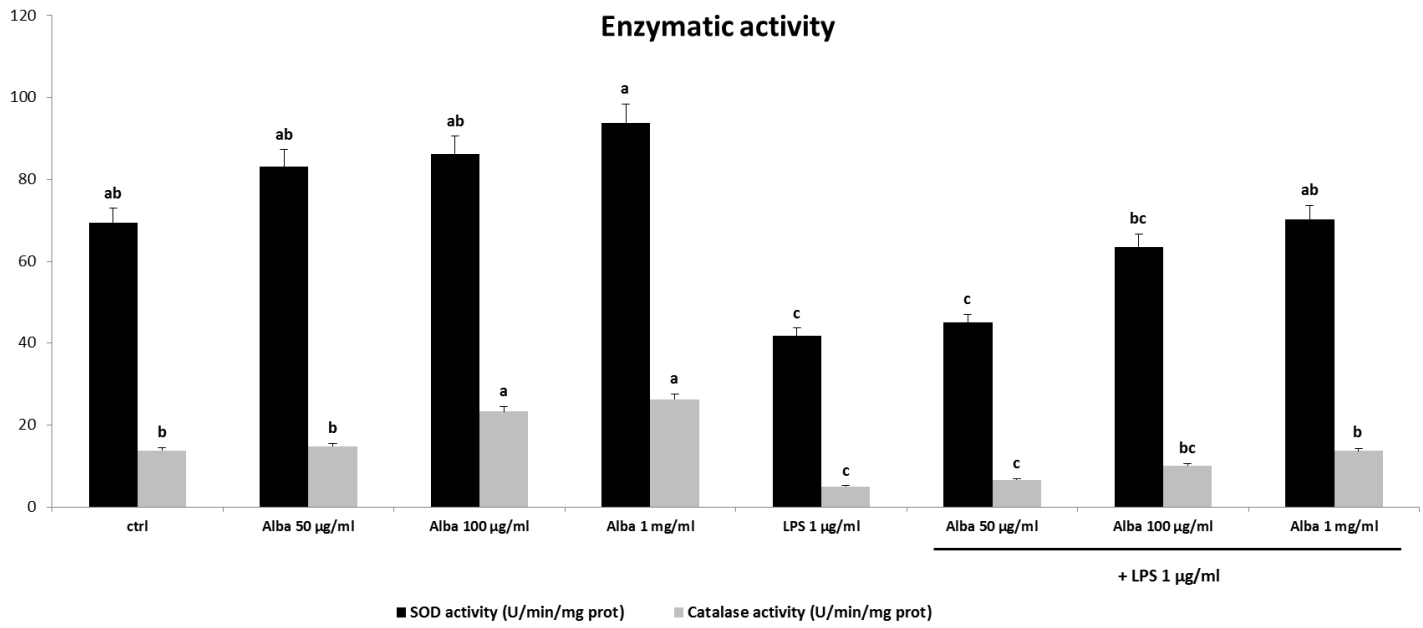


Figure 63. Effects of strawberry and LPS treatment on SOD and catalase activity in RAW macrophages. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

4.2.4.6. Lipid and protein oxidation level

Cell RIPA preparations of RAW macrophages were also used to determine the level of lipid and protein damage, through the evaluation of common markers of lipid (TBARS) and protein (GSH and protein carbonyl content) oxidation.

Alba extracts significantly reduced the TBARS level respect to the control group (66.69 nmol/100 mg prot), already at doses of 50 µg/ml (51.88 nmol/100 mg prot). On the contrary, LPS-treatment considerably raised TBARS value (95.35 nmol/100 mg prot), which was efficiently counteracted by Alba pre-treatment, restoring values similar to the control group already with 100 µg/ml (77.98 nmol/100 mg prot) (Figure 64).

	TBARS level (nmol / 100 mg prot)	GSH level (nmol / mg prot)	Protein carbonyl content (nmol / mg prot)
ctrl	66.69 ± 3.33 ^{cd}	44.79 ± 2.24 ^{cd}	44.33 ± 2.22 ^{b}
Alba 50 µg/ml	51.88 ± 2.59 ^{ef}	55.74 ± 2.78 ^{bc}	36.02 ± 1.81 ^{bc}
Alba 100 µg/ml	46.42 ± 2.32 ^{ef}	66.59 ± 3.33 ^{ab}	30.95 ± 1.55 ^{c}
Alba 1 mg/ml	40.15 ± 2.01 ^{f}	76.13 ± 3.81 ^{a}	29.52 ± 1.48 ^{c}
LPS 1 µg/ml	95.35 ± 4.77 ^{a}	16.43 ± 0.82 ^{f}	69.52 ± 3.48 ^{a}
Alba 50 µg/ml + LPS	86.97 ± 4.35 ^{ab}	27.45 ± 1.37 ^{ef}	60.78 ± 3.04 ^{a}
Alba 100 µg/ml + LPS	77.98 ± 3.89 ^{bc}	39.14 ± 1.96 ^{de}	46.04 ± 2.31 ^{b}
Alba 1 mg/ml + LPS	58.50 ± 2.93 ^{de}	50.44 ± 2.52 ^{cd}	37.81 ± 1.89 ^{bc}

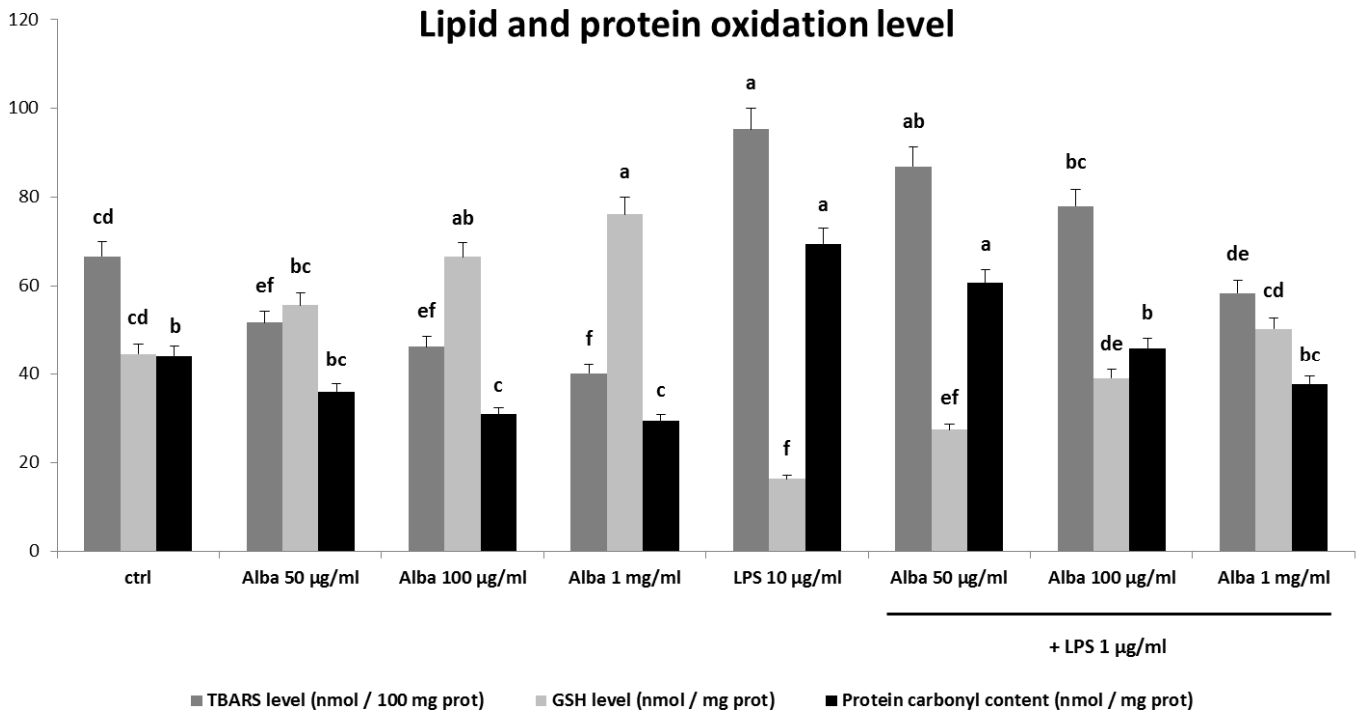
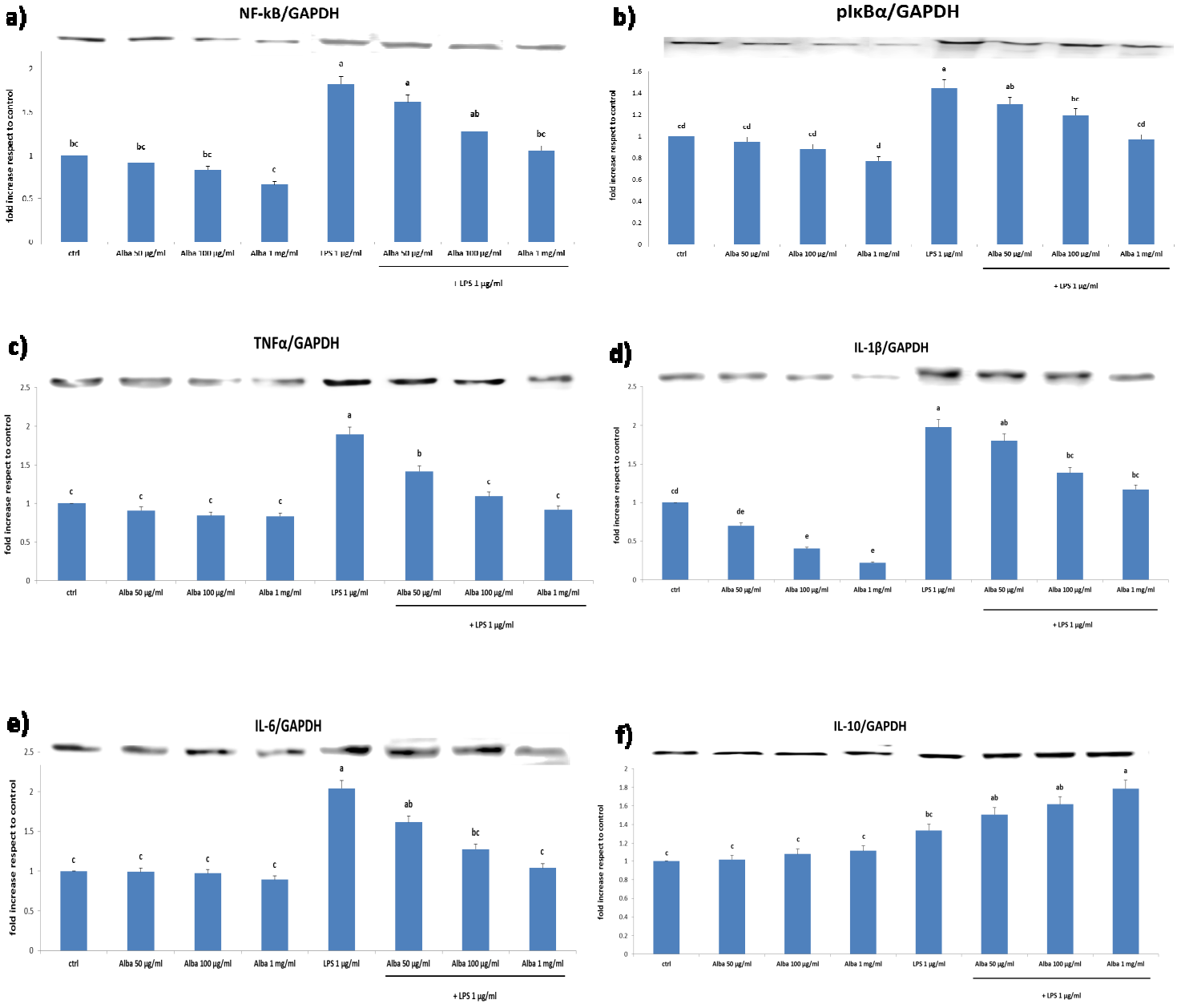


Figure 64. Effects of strawberry and LPS treatment on lipid and protein oxidation markers in RAW macrophages. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

Similar results were obtained in relation to protein oxidation markers (Figure 64). Strawberry treatments ameliorated GSH level and protein carbonyl content respect to untreated cells (44.79 and 44.33 nmol/mg prot, respectively), increasing the amount of GSH and reducing the carbonyl level, significantly with Alba at 100 µg/ml in both the assays (66.59 and 30.95 nmol/mg prot, respectively). RAW macrophages treated with LPS showed a considerable protein damage, as evidenced by the lowest value of GSH (16.43 nom/mg prot) and the highest value of carbonyl content (69.52 nmol/mg prot). Pre-treatment with strawberry extracts improved the protein damage level induced by LPS, in a dose-dependent manner: values statistically similar to the control groups were obtained with Alba at 100 µg/ml, both for GSH and carbonyl levels (39.14 and 46.04 nmol/mg prot, respectively).

4.2.4.7. Gene expression analysis

The evaluation of the effect of strawberry and LPS treatment on different molecular pathway involved in RAW macrophages response, was conducted through the gene expressions analysis. The different cell pellets were used to perform the analysis. The gene expression of proteins related to inflammatory status were firstly evaluated (Figure 65).



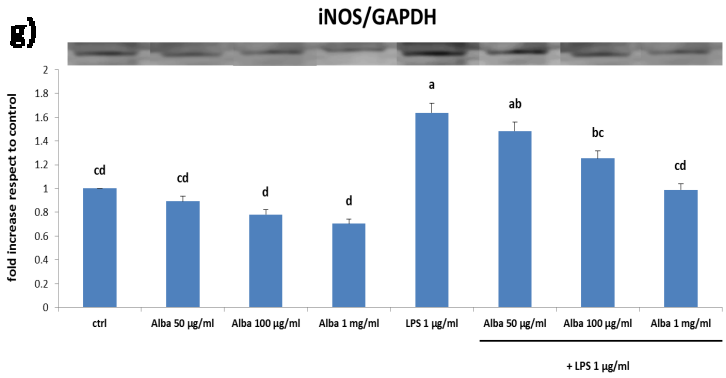


Figure 65. Effects of strawberry and LPS treatment on gene expression of inflammatory markers (NFkB, pIκBα, TNF-α, IL-1β, IL-6, IL-10 and iNOS) in RAW macrophages. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

As for HDF, strawberry treatments reduced the level of the tested inflammatory markers (NFkB, pIκBα, TNF-α, IL-1β, IL-6) at the different concentrations applied. The expression of all the inflammatory proteins was considerably increased by the LPS-treatment: fold increases of 1.81, 1.45, 1.89, 1.97 and 2.04, compared to the control group, were obtained for NF-kB, pIκBα, TNF-α, IL-1β and IL-6, respectively.

Pre-treatment with Alba extracts was able to reduce the inflammatory marker levels in a dose-dependent manner, restoring values similar to the control group already with 100 µg/ml (fold increase of 1.27, 1.19, 1.09, 1.39 and 1.27 for NFkB, pIκBα, TNF-α, IL-1β and IL-6, respectively) (Figure 65a, b, c, d, e). A different trend was registred for IL-10: in this case an increase of the protein expression was observed with strawberry, LPS and strawberry + LPS treatments, with a significative raise, compared to the untreated cells, obtained already with Alba at 50 µg/ml + LPS (fold increase of 1.50) (Figure 65f). Finally, LPS-treatment significantly increased the level of iNOS expression (fold increase of 1.63) respect to the control group. Strawberry pre-treatment reduced the gene expression and also counteracted the LPS-induced stimulation, at all the different concentrations applied, significantly with Alba at 100 µg/ml (fold increae of 1.25) (Figure 65g).

The gene expressions of proteins related to antioxidant response were also tested (Figure 66).

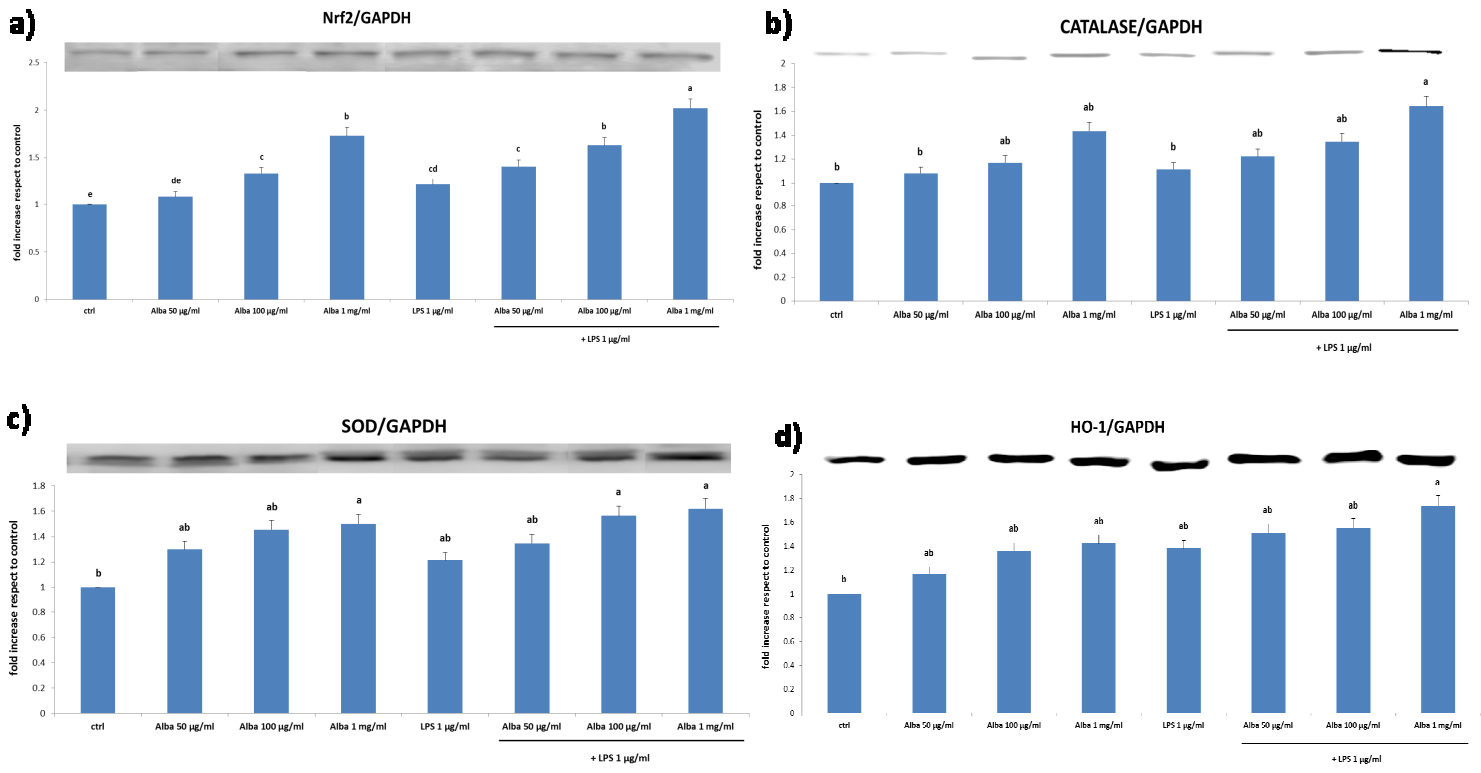


Figure 66. Effects of strawberry and LPS treatment on gene expression of protein related to antioxidant response (Nrf2, catalase, SOD, HO-1) in RAW macrophages. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

Strawberry extracts increased the level of the different proteins (Nrf2, catalase, SOD, HO-1) in a dose-dependent manner, a similar effect was obtained with LPS and with strawberry + LPS treatments. Nrf2 expression (Figure 66a) presented a significative difference respect to the control group with Alba at 100 µg/ml and 1 mg/ml (fold increase of 1.33 and 1.73, respectively), with LPS-treatment (fold increase of 1.21) and with strawberry at 50, 100 µg/ml and 1 mg/ml + LPS (fold increase of 1.40, 1.63 and 2.01, respectively). A similar trend was obtained with the other genes investigated. In case of SOD (Figure 66c) a significative increase compared to the untreated cells was obtained with Alba treatment at dose of 1 mg/ml (fold increase of 1.49) and with strawberry pre-treatment at 100 µg/ml and 1 mg/ml before LPS incubation, with fold increase values of 1.56 and 1.62, respectively. Similar results were obtained for catalase and HO-1 (Figura 66b, d): in both gene expressions, values statistically different from to the control group were obtained with strawberry at 1 mg/ml + LPS-treatment, with fold increase values of 1.64 and 1.73, respectively.

Moreover, the gene expressions of proteins related to the AMPK pathway were also studied (Figure 67).

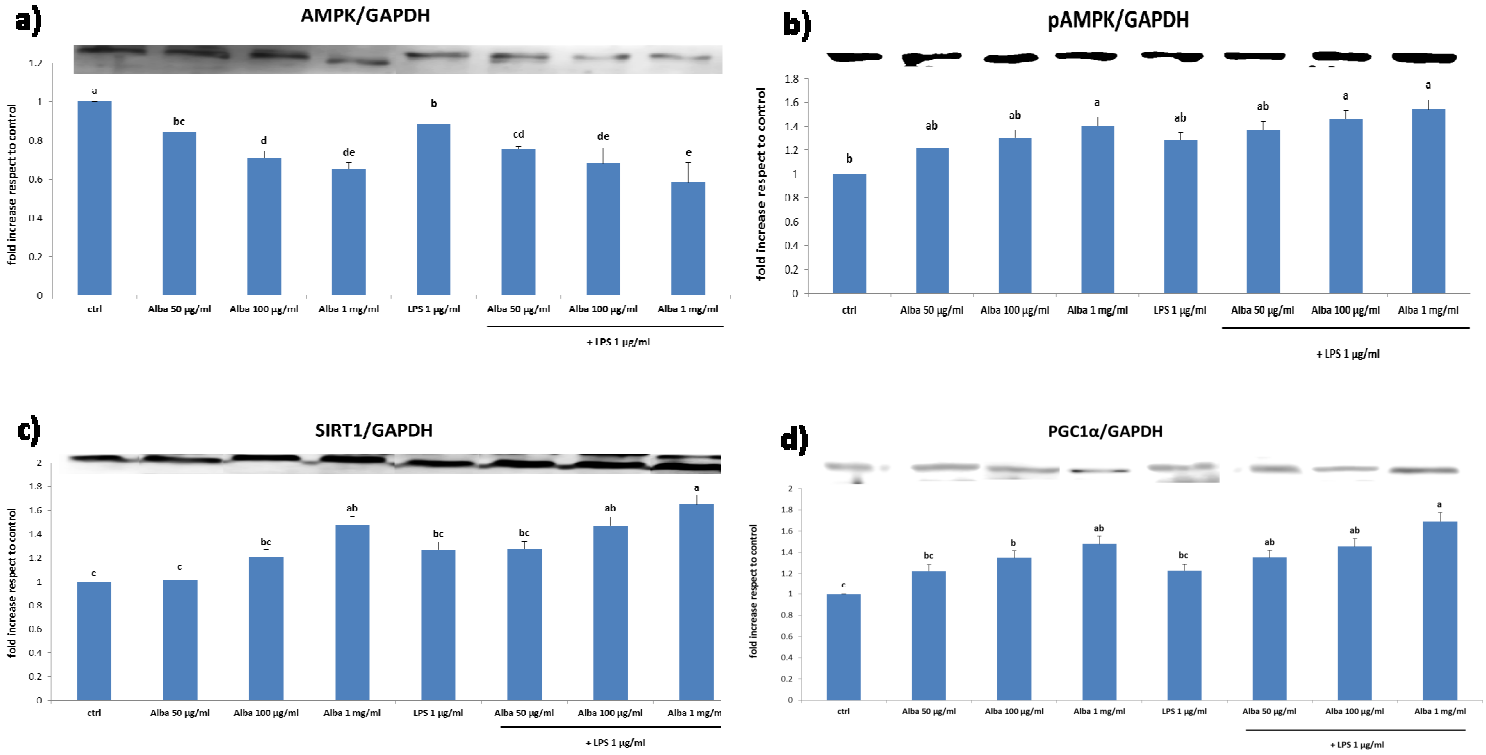


Figure 67. Effects of strawberry and LPS treatment on gene expression of protein related to AMPK pathway (AMPK, p-AMPK, SIRT1, PGC1α) in RAW macrophages. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

LPS and strawberry treatments (at the different concentrations applied) significantly reduced AMPK level (Figure 67a). This effect was further marked by the Alba pre-treatment before LPS-incubation, obtaining fold increase values of 0.75, 0.68 and 0.58 with Alba at 50, 100 µg/ml and 1 mg/ml + LPS, respectively. As for HDF, an opposite trend was obtained with pAMPK (Figure 67b): in this case strawberry extracts significantly increased this protein expression alone, at 1 mg/ml (fold increase of 1.40) or before the LPS-treatment, at 100 µg/ml and 1 mg/ml (fold increase of 1.46 and 1.54, respectively). Similar to pAMPK, the results obtained with SIRT1 (Figure 67c): strawberry pre-treatment remarkably increased protein expression at 1 mg/ml (fold increase 1.47) and, in combination with LPS, (fold increase of 1.64). Taking into account PGC1α level (Figure 67d), a significative increase of gene expression respect to untreated group was obtained with strawberry extracts at 100 µg/ml (fold increase of 1.35) and 1 mg/ml (fold increase of 1.47), and

before LPS-treatment, with Alba at all the concentration used (fold increase of 1.35, 1.45, 1.69 with strawberry at 50, 100 µg/ml and 1 mg/ml + LPS, respectively). The evaluation of OGG1 expression allowed to estimate the DNA damage level in RAW macrophages (Figure 68). Alba extracts reduced OGG1 gene expression in a dose-dependent manner. On the contrary LPS-treatment increased the level of DNA damage (fold increase of 1.69), which was efficiently counteracted by pre-incubation with strawberry already at 100 µg/ml (fold increase of 1.12).

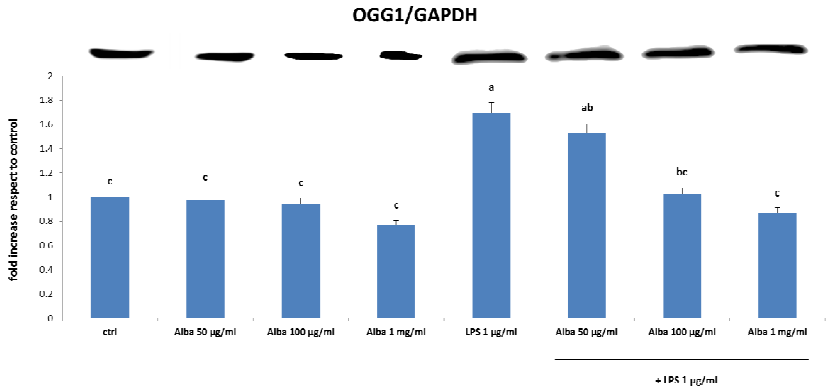


Figure 68. Effects of strawberry and LPS treatment on gene expression of protein related to DNA damage level (OGG1) in RAW macrophages. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

Finally, the expression of GAPDH (Figure 69), a protein with a known molecular weight that is present in each analyzed sample, was tested in order to determine the amount of the protein analyzed in each Western blot performed.

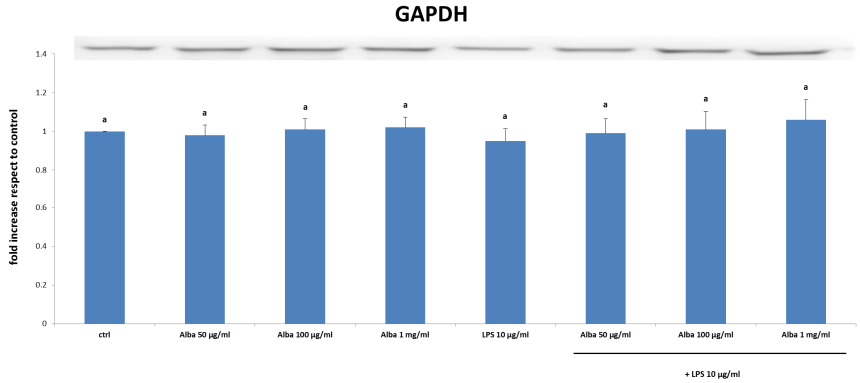


Figure 69. Representative analysis of GAPDH gene expression in RAW macrophages treated with strawberry and LPS. Data are expressed as mean values ± SD. Columns belonging to the same set of data with different superscript letters are significantly different ($p < 0.05$).

In the present study, we found that the anti-inflammatory role of strawberry is primarily involved in the activation of Nrf2 pathway, which is markedly AMPK-dependent in LPS-stimulated inflammatory responses.

4.2.5. Discussion

The second part of the PhD thesis evaluated the effects of methanolic purified extracts from Alba cultivar on inflammatory status induced by *E.Coli* LPS on two different cell lines, HDF and RAW 264.7 macrophages.

HDF represent a standard *in vitro* cell model for different kind of toxicity determinations (Agyare et al., 2011; Awang et al., 2014; Galandakova et al., 2015); actually, it is known that they recruit immune cells via soluble mediators and contribute to the progression of inflammatory processes (Tardif et al., 2004; Wheater et al., 2012). For this reason it is very important to understand better how fibroblasts produce and secrete different inflammatory mediators, related in this case to LPS-induced stress. Simultaneously, macrophages play an important role in immune reactions, allergy and are considered the cells predominantly involved in the inflammatory response (Suzuki et al., 2009; Choi et al., 2015). Once activated (by different exogenous or endogenous stimuli, see chapter 3.3 Oxidative stress and inflammation), macrophages induce the expression of pro-inflammatory cytokines and other factors directly involved in the progression of inflammatory condition and of its related diseases (Suzuki et al., 2009; Choi et al., 2015). As highlighted by the available literature, RAW 264.7 macrophages are often used as model for cell culture studies, expecially in LPS-induced inflammation researches (Hill et al., 2005; Zong et al., 2012; Guimaraes et al., 2013; He et al., 2013).

Firstly, to evaluate the effects of strawberry extracts, LPS and strawberry + LPS treatments on cellulr viability, MTT assays were performed. Alba dried extracts positively affected both HDF cells and RAW macrophages viability after 24 h of treatment, at all the different concentrations applied in case of HDF, and up to 1 mg/ml in case of macrophages, confirming the anti-cytotoxic effect of strawberry treatment on cellular viability (Giampieri et al., 2012b; 20014a; 2014b). After 48 and 72 h of treatments with Alba, the cellular viability decreased in both the studied lines: for this reason 24 h of treatment with different concentrations of strawberry were chosen for further analysis. LPS exerted a significative negative effect on cellular viability in HDF at 10 μg/ml after 12 and 24 h of treatment, while in RAW macrophages the increase of viability percentage became already relevant at 1 μg/ml after 24 h of treatment. These different trends could be ascribed to the fact that RAW macrophages are stimulated by LPS-treatment, as previously shown (Park et al., 2009; Nan et al., 2012; Zhang et al., 2012; Oliveira et al., 2014). Finally, the effects of the

strawberry pre-treatment before LPS incubation were evaluated. In both cellular models, Alba extracts improved the effect on cell viability exerted by LPS, in a dose-dependent manner. The preliminary MTT test permitted to obtain a first view of the range of concentrations of strawberry extracts and LPS, useful for the following analysis.

As previously indicated (see chapter 3.3 Oxidative stress and inflammation), the interaction of the cellular immune system with endogenous or exogenous inflammatory stimuli determines the generation of ROS, that can result in hyperactivation of inflammatory responses leading to tissue damage and oxidative stress phenomena (Khansari et al., 2009; Schieber and Chandel, 2014). In this context the measure of ROS intracellular production could represent a very useful tool to quantify the oxidative damage induced by LPS (Hsu et al., 2002; Ramana et al., 2006; de Souza et al., 2007), and to evaluate the possible protective effect exerted by strawberry.

In both studied models, LPS induced a significative raise of ROS production at different dosis, after 24 h of treatment: in case of HDF cells 10 μg/ml were necessary to obtain a fold increase value of 2.5, while for RAW macrophages a similar result was obtained with 1 μg/ml of LPS. This is probably connected to the high sensibility of macrophages to LPS supplementation (Park et al., 2009; Nan et al., 2012; Zhang et al., 2012; Oliveira et al., 2014). Alba extracts were able to efficiently counteract the LPS-induced oxidative stress, at 50, 100 μg/ml and 1 mg/ml, in both cellular lines: with these concentrations, statistically differences were obtained with respect to LPS-treated cells, and for this reason these dosis were chosen for further analysis. Moreover, the results obtained with strawberry are in line with ones previously showed by several authors, which tested the efficacy of different bioactive compounds against LPS-induced damage (de Souza et al., 2007; Park et al., 2013b; Mo et al., 2014; Sagar et al., 2014; Choi et al., 2015).

Cell death can be induced by three different mechanisms: autophagy (programmed cell death II), oncosis (programmed cell death III or necrosis) and apoptosis (programmed cell death I). Apoptosis can be defined as a mechanism characterized by a series of different biochemical and morphological changes, including increase in ROS level, activation of caspases, cell shrinkage, chromatin condensation and nucleosomal degradation (Forbes-Hernandez et al., 2014). Various phytochemicals are involved in the apoptotic process, through the regulation of intrinsic pathway related to a variety of stimuli from inside the cells like DNA damage and ROS generation (Somasagar et al., 2012). For this reason, in the present study, the apoptosis rate of HDF cells treated with strawberry extracts and LPS was investigated in order to evaluate the influence of these treatments in the apoptotic process and its link to the reduction of cell viability (as highlighted by the MTT assay, chapter 4.2.3.1.).

The apoptotic rate was tested through the TALI apoptosis assay. The results demonstrated that LPS-treatment slightly increased the number of apoptotic cells, while Alba extracts reduced this number in a dose-dependent manner. However, both the treatments did not produce significative differences with respect to untreated cells. The apoptosis test was conducted only in HDF cells, since LPS treatment in RAW macrophages exerted a stimulatory action on cell viability and did not produce any cytotoxic effect. The Western Blot analysis confirmed the above results, through the evaluation of caspase 3 expression, that is one of the main enzymes linked to the apoptosis progression (Forbes-Hernandez et al., 2014). Caspase 3 level significantly increased after LPS treatment and it was restored to control level in association with strawberry treatment at 100 μg/ml. These results confirmed the concomitant results found in previously works in which LPS-treatment significantly reduced cell viability and increased apoptosis rate (Schneider et al., 2003; Ben et al., 2011; Bullon et al., 2012). In our work, it is showed for the first time the protective role of strawberry methanolic extracts in LPS-induced apoptosis rate and in caspase 3-related expression.

The most important site of ROS production is represented by the ETC in the mitochondria (Forbes-Hernandez et al., 2014). In this process, in fact, a small percentage of electrons directly reacts with oxygen, determining the formation of ROS as secondary ETC products (Forbes-Hernandez et al., 2014). In this context and in agreement with the result obtained by ROS analysis, we investigated the implication of mitochondria dysfunction in HDF cells and RAW macrophages, after strawberry and LPS-treatment, through the OCR measurement (see chapter material and methods, 4.2.2.6. Mitochondrial functionality with Seahorse XF24 Analyzer®: respiratory capacity assay). Basal respiration is predominantly controlled by the parallel re-entry pathways through the ATP synthase and proton leak. Addition of oligomycin blocks the ATP synthase and the residual respiration is due to the proton leak. The decrease compared to basal respiration provides the coupling efficiency. The addition of a carefully calibrated concentration of the protonophore 2,4 DNP introduces a high artificial proton conductance into the membrane. This maximal respiration is now controlled by electron transport chain activity and/or substrate delivery. The increased respiratory capacity above basal respiration provides the maximal respiratory capacity. Finally, ETC inhibitors are added: antimycin A/rotenone that block complex III and I respectively; in this way any residual respiration is non-mitochondrial and needs to be subtracted from the other rates. In HDF cells LPS treatment produced a depressive action on oxygen consumption, which was efficiently counteracted by strawberry pre-treatment in a dose dependent manner. Also for the maximal respiratory capacity a similar situation was observed: the LPS group presents the lowest result and the pre-treatment with Alba extract at 100 μg/ml restores a value statistically similar to the control group. These results support and expand previously published evidence regarding the presence of mitochondrial

dysfunction and oxidative stress in LPS-treated cells (Apostolova et al., 2011; Bullon et al., 2011; Choumar et al., 2011). Different results were obtained for RAW macrophages. These cells presented an opposite trend compared to that obtained in HDF: in this case the LPS-treatment determined an increase of oxygen consumption which was further enhanced by Alba extracts in a dose-dependent manner. This appears to be contradictory with the ROS values, that were high in LPS group. Obviously, also for maximal respiratory capacity the same trend was respected. To our knowledge, there are no published studies that underline the effect of LPS-treatment on mitochondrial functionality of RAW macophages, with particular attention to the OCR results. One possible explanation could be related to the stimulatory effect exerted by this endotoxin on RAW macrophages (Park et al., 2009; Nan et al., 2012; Zhang et al., 2012; Oliveira et al., 2014), that could produce a raise of oxyen consumption, despite the ROS obtained results. For this reason further studies are required to clarify this apparently contradictory aspect.

Different studies have suggested that exposure of cells to LPS can lead to the release of proinflammatory cytokines and in turn activate a second level of inflammatory cascades including cytokines, lipid mediators and adhesion molecules such as NO (Zong et al., 2012).

NO is an important regulatory and effector molecule with different biological functions and is considered a fundamental component involved in many physiological and pathophysiological processes (Chakravortty et al., 2001).

Its production is catalysed by at least three different types of NOSs: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Pekarova et al., 2011). Once iNOS is induced, it can produce a large amounts of NO that profoundly influences cell and tissue function, creating oxidative stress and damage. In our work, both studied cell lines showed a significant increase in NO level after LPS-treatment, which was efficiently counteracted with Alba at dose of 100 μg/ml. These results confirmed what previously obtained by other authors on RAW macrophages, which provide the best-studied example of the regulation of NO production (Park et al., 2005; Park et al., 2011; Zhang et al., 2012 Mo et al., 2014; Choi et al., 2015), and represent one of the first studies that investigate the involvement of NO production in HDF cells.

These results were further confirmed observing the iNOS gene expression. In both HDF cells and RAW macrophages, LPS-treatment significantly induced iNOS expression, while strawberry extracts, in particular at 100 μg/ml, reduced the gene level restoring values similar to those of untreated cells. The strong association between NO production and iNOS expression was demonstrated in our study, as previously shown by different authors (Victor et al., 2004; Ben et al., 2011; Pekarova et al., 2011; Xu et al., 2012; Lee et al., 2014; Mo et al., 2014; Choi et al., 2015).

If the strawberry really acted as anti-oxidant agent against the LPS-induced oxidative stress, the immediate consequence could be that the antioxidant reservoir of the studied cell models would be consumed for counteracting the damage caused by the endotoxin. Polyphenols may offer an indirect antioxidant protection by activating endogenous defense system, mainly by modulating the expression of some antioxidant enzymes. Earlier studies indicate that different bioactive compounds are able to modulate the activity of diverse antioxidant enzymes (GPx GR, GST, SOD, catalase), reducing the damage induced by LPS and other different stress, and restoring condition similar to control level, both in HDF cells (Camera et al., 2009; Ramachandran et al., 2010; Feng et al., 2013; Sagar et al., 2014) and in RAW macrophages (Ferret et al., 2002: Park et al., 2005; Park et al., 2011; Choi et al., 2015). GPx ubiquitously exists both in cytosol and mitochondria of the hepatocytes; it removes H_2O_2 by coupling its reduction to H_2O with reduction of GSH into GSSG. Moreover, it can act on peroxides other than H_2O_2, catalyzing GSH-dependent reduction of fatty acid hydroperoxides, cholesterol 7β-hydroperoxide and various synthetic hydroperoxides such as cumene. GST locates in hepatocytes cytosol and plays an important role in detoxification and excretion of xenobiotics (Bansal et al., 2005). GST catalyzes the conjugation of the thiol functional groups of GSH to electrophilic xenobiotics (chloroform, DDT, aflatoxin, paracetamol, etc), leading to elimination or conversion of xenobiotic-GSH conjugate (Rao et al., 2006). In such reaction, the GSH is oxidized into GSSG, which can be reduced to GSH by GR with the consumption of NADPH (Fang et al., 2002). In addition to GSH-related antioxidant enzymes, also other enzymes including SOD and catalase play important role in the antioxidant defense system. SOD is widespread in tissue animals and is located in cytoplasm (CuZnSOD) and mitochondria (MnSOD); it catalyzes the dismutation of two superoxide radicals to H_2O2 and O_2. Catalase acts as supporting antioxidant enzymes by transforming H_2O_2 to H_2O e O_2, thereby providing protection against ROS (Vasquez-Garzon et al., 2009). In our work, the enzymatic assays of all these enzymes were performed. The expected results were that if LPS really elicited an oxidative damage, the activity of all the tested enzymes should decrease in treated cells, with respect to control cells, because the reservoir was used to counteract the damage. On the contrary, strawberry could reduce the LPS-effect, improving the enzyme activities and decreasing the induced damage. The obtained results confirmed such hypothesis: LPS significantly decreased all the enzymatic activities, due to ROS and NO production that it provoked (Park et al., 2005), while Alba counteracted these effects, restoring values similar to those of control groups at concentration of 100 µg/ml, both in HDF cells and RAW macrophages, suggesting that the increase of endogenous antioxidant enzyme activities might be one of the important mechanisms for strawberry against oxidative stress damage. Moreover, the tendency of strawberry to increase and subsequently recover the basal level of these

enzymes might be interpreted as the consequence of the initial alteration of cellular steady-state function followed by stress-induction phenomena tending to reestablish the primitive situation (Huertas et al., 1992; Battino et al., 1995; Battino et al., 2000).

In our work, the antioxidant capacity provided by strawberry resulted also in enhanced protection against lipid, protein and DNA damage induced by LPS-oxidative stress. Lipid peroxidation is a free radical-mediated propagation of oxidative insult to PUFA involving several types of free radicals, and termination occurs through enzymatic means or by free radical scavenging by antioxidants (Babujanarthanam et al., 2011). Protein oxidation, measured as an increase in carbonyl groups, was shown to be an early event in oxidative stress *in vitro* (Pacifici and Davies, 1990) and was used to highlight accumulation of oxidative damage to proteins over the longer term, as in aging studies (Ciolino and Levine, 1997). Free radical induced oxidation appears to play an important role in the generation of protein carbonyls *in vitro* (Fucci et al., 1983) and also *in vivo* model (Oliver et al., 1990). GSH level is another marker of protein oxidation. It plays an important role in maintaining the normal reduced state of cells, counteracting the harmful effects of oxidative stress and detoxifying xenobiotics (DeLeve and Kaplowitz, 1990). GSH is involved in many metabolic processes, including vitamin C metabolism, maintaining communication between cells (Barhoumi et al., 1993), and in general preventing protein SH groups from oxidizing and cross-linking. Moreover, it is also involved in intracellular copper transport (Pederson et al., 1996). Furthermore, it was demonstrated that in free radical-mediated cell injury, consumption of hepatocellular GSH is associated with the initiation of cell damage with lipid peroxides formation (Nishida et al., 1997).

Our results demonstrated that both in HDF cells and RAW macrophages LPS treatment determined a significant increase in TBARS and protein carbonyl content with a concomitant reduction of GSH level, as previously shown by different autohrs (Park et al., 2005; Park et al., 2011; Zhai et al., 2012; Sagar et al., 2014). Strawberry treatment efficiently counteracted lipid and protein damage in a dose-dependent manner: in both studied cell lines a normal condition was restored with Alba extract at 100 µg/ml, in all the performed assays. This could be related to the ability of strawberry antioxidant compounds in scavenging free radicals and in the activation of antioxidant enzymes.

In cellular compartments, mitochondrion and nucleus, two major targets of oxidative stress, contain a variety of enzymes to repair oxidant-induced DNA modifications (Hacker et al., 2006). Basically, DNA damage most likely occurs when the endogenous antioxidant network and DNA repair systems are flooded (Yamamoto et al., 2003). The expression level of 8-Oxo-7,8-dihydro-2-deoxyguanosine (8-OHdG) in DNA reflects its rate of generation and of repair. In an earlier study, Smart et al. (Smart et al., 2006) showed that 8-OHdG in DNA is repaired primarily via the DNA

base excision repair pathway that includes 8-oxoG DNA glycosylase (OGG1) (Baumeister et al., 1998). Therefore, OGG1 could represent a sensitive marker of LPS-induced DNA damage. In our work, OGG1 expression was measured in both studied models. LPS-treatment remarkably increased OGG1 level, as previously shown by Sagar et al. (Sagar et al., 2014), while Alba extract at 100 µg/ml, efficiently lowered this gene expression, counteracting the DNA damage induced by LPS. The collected results highlighted the marked protective effect of Alba extract on the different biological macromolecules against the oxidative stress induced by LPS.

All the previous findings give a quite comprehensive picture of the protective effect of strawberry pre-treatment on LPS-mediated viability, ROS/NO generation, apoptosis induction, mitochondrial functionality, antioxidant status and macromolecules damage in HDF cells and RAW macrophages. To complete the present study, Western blot analysis were performed to evaluate the gene expressions of several proteins related to the most probable molecular pathways involved in strawberry and LPS mechanisms of action.

First of all, the gene expressions of proteins related to inflammatory status were evaluated. NF-κB is a key transcriptional regulator of the inflammatory response, and plays an important role in the regulation of inflammation and in the development of cellular injuries (Ou et al., 2013). It is activated in response to various extracellular stimuli, including cytokines, LPS, and oxidative stress (Park et al., 2005). NF-κB exists ubiquitously in the cytoplasm as a heterodimer consisting of p50 and p65 as an inactive form by creating a complex with the inhibitor protein IκBα. In response to inflammatory stimuli (ROS, LPS) IκBα is phosphorylated (pIκBα) and released from NF-κB that, once activated, migrates to the nucleus and up-regulates inflammation-related genes such as iNOS and pro and anti-inflammatory cytokines (Park et al., 2011). Earlier studies investigated the role of different bioactive compounds on LPS-mediated NF-κB response, both in HDF cells (Perfetto et al., 2003) and in RAW macrophages (Park et al., 2005; Park et al., 2011; Ji et al., 2012; Choi et al., 2015). Our results confirmed what previously shown: LPS-treatment significantly increased NF-κB expression in both the cellular models. Alba pre-treatment on the contrary efficiently decreased these inflammatory responses in a dose-dependent manner, restoring values similar to control group already at concentration of 100 µg/ml. Strictly related to NF-κB, is pIκBα expression. As we expected, and as also found by other autohrs (Ji et al., 2012; Perfetto et al., 2013), pIκBα expression followed the same trend of NF-κB in both tested cell models. LPS treatment determined the activation of IκBα, which became phosphorylated and consequently relased NF-κB into the nucleus. Strawberry pre-treatment efficiently counteracted this process at all the different concentrations applied, but most significantly at 100 µg/ml. Accordingly to the obtained results, the gene expressions of pro- and anti-inflammatory cytokines were conducted. TNF-α, IL-1β and IL-6 level

markedly increased after LPS treatment; these genes' expressions were efficiently reduced by Alba extract, restoring values similar to those of untreated cells at 100 µg/ml. In case of IL-10, that is an anti-inflammatory cytokine, its expression was stimulated by LPS, in response to the stress, and in a more pronounced manner by Alba pre-treatment before LPS supplementation, highlighting the significant contribute of strawberry in the modulation of the inflammatory response. These results confirm the data obtained in other studies conducted in both HDF cells (Perfetto et al., 2003; Tardif et al., 2004; Wang et al., 2011; Rizzo et al., 2012) and RAW macrophages (Ci et al., 2010; Yao et al., 2012; Zong et al., 2012; Zhang et al., 2012; Ji et al., 2012; Lee et al., 2014; Mo et al., 2014; Choi et al., 2015), in which the expression of pro and anti-inflammatory cytokines induced by LPS was improved by different bioactive compounds.

Through the enzymatic assays previously performed, we demonstrated that the LPS-mediated ROS production consumed the antioxidant reservoir of the tested cells, lowering the activity of SOD and catalase in both the cell lines. But what happens to the gene expression of these antioxidant enzymes (SOD, catalase and HO-1) and their related pathway (Nrf2)?

Nuclear factor (erythroid-derived 2)-like 2 or Nrf2 is a basic leucine zipper transcription factor that binds to the promoter sequence "antioxidant responsive element" (ARE) leading to coordinated up-regulation of ARE driven detoxification and antioxidant genes. Since the expression of a wide array of antioxidant and detoxification genes are positively regulated by the ARE sequence, Nrf2 may serve as a master regulator of the ARE-driven cellular defense system against oxidative stress (Mo et al., 2014). The anti-oxidant properties of ARE enzymes are related to the inhibition of formation of adhesion molecules, such as NO, and reduction of oxidative stress (Lee et al., 2014b).

In our work, contrarily to what happened to the enzymes activities, the expression of SOD, catalase and HO-1 were up-regulated in both cell lines not only by strawberry treatment, but also after LPS supplementation, as indicated by different authors (Ferret et al., 2002; Rushworth et al., 2005; Lee et al., 2014b; Mo et al., 2014). These anti-oxidant enzyme expressions reached the highest value with the concomitant action of strawberry and LPS treatment. This situation, apparently controversial, could be explained if we hypothesize that the oxidative stress induced by LPS treatment stimulated both HDF cells and RAW macrophages to protect from this oxidative insult, augmenting the synthesis of their antioxidant defenses (SOD, catalase and HO-1). For this reason the studied cell lines upregulated the expression of genes related to the expression of the different enzymes. On the basis of the obtained results, if SOD, catalase and HO-1 expressions were up-regulated by strawberry and LPS treatment, a similar trend should be observed for Nrf2 expression. Also in this case, in fact, strawberry, LPS and strawberry + LPS treatments significantly increased the Nrf2 expression, in both HDF cells and RAW macrophages, confirming the link between Nrf2

and ARE genes in LPS-stimulated cells, as indicated in recent works (Lee et al., 2014b; Mo et al., 2014).

Finally, the gene expressions of proteins related to the 5' AMP-activated protein kinase or AMPK pathway were also investgated. The involvement of cellular energy metabolism in inflammation suppression has recently become an area of wide interest, although the exact mechanisms are still poorly understood (Mo et al., 2014). AMPK is a sensor of intracellular energy status and represents an attractive target for inflammation control. Emerging evidence show that AMPK activation can decrease the oxidative stress and inhibit inflammation, serving as a potential target to treat inflammation-related disorders (Zong et al., 2012; Mo et al., 2014). Mechanistic connections between AMPK and inflammation have been limited to links with NF-κB pathway (Mo et al., 2014). It has been shown in fact that chemical activators of AMPK decrease NF-κB-mediated transcription, and that constitutively activate AMPK suppressed NF-κB signaling and fatty acid-induced inflammation in macrophages, although NF-κB subunits are not the direct targets of AMPK (Mo et al., 2014). Consistent with the unequivocal action of Nrf2-pathway on ROS clearance and the suppression of inflammation, the potential for the crosstalk between Nrf2 and AMPK pathways has been also noted (Mo et al., 2014). For this reason we decided to investigate the potential role of a functional interaction between Nrf2 and AMPK pathways in the inflammatory system induced by LPS in both HDF cells and RAW macrophages. The analysis on Western blot conducted on the AMPK and in particular on its activated form, pAMPK, showed that both strawberry and LPS treatments positively affected pAMPK expressions, increasing its level in both cellular models. These results are of great importance and innovation because demonstrate a direct effect of strawberry and expecially LPS treatments on AMPK/pAMPK expression, and are also in agreement with the few previous findings deriving from literature (Zong et al., 2012; Mo et al., 2014). AMPK, protein kinase B and p38 mitogen-activated protein kinase phosphorylate and thus activate PGC1α, which is considered the master regulator of mitochondria biogenesis (Wenz, 2013; Forbes-Hernandez et al., 2014). The activity of PGC1α is also dependent on the activity of sirtuin 1 or SIRT1, a NAD^+-dependent deacetylase located in the cell nucleus, which possesses a bidirectional interaction with pAMPK (Cantó and Auwerx, 2009). The activity of SIRT1 in fact depends on the NAD^+/NADH ratio, which in turn is increased by pAMPK. Following this theory, the results of our study should present in strawberry and LPS treated HDF cells and RAW macrophages high levels of pAMPK, of SIRT1 and, as a consequence, of PGC1α. The obtained results confirmed this hypothesis: SIRT1 and PGC1α gene expressions presented an increased level after both strawberry and LPS treatment, highlighting a similar trend to those obtained with pAMPK, and confirming previously results obtained by other authors on different cellular models (Hickson-Bick et al., 2008;

Labuzek et al., 2010; Han et al., 2012; Rayamajhi et al., 2013). All the collected evidence underline that strawberry extracts were able to counterarct the LPS-mediated inflammatory response, acting on the AMPK related pathways. The increment of pAMPK expression improved the Nrf2 cascade signaling, also stimulated by LPS-mediated ROS production, which finally lead to the incresase of ARE-antioxidant enzymes expression. The augment of antioxidant defense efficiently reduced the inflammatory damage created by endotoxin LPS, through the modulation of NF-κB pathwyas and the reduction of NO and inflammatory cytokines production.

5. CONCLUSIONS

The strong association between a diet rich in fruits and vegetables and a lower incidence of different pathologies (i.e., obesity, infections, inflammation, cardiovascular and neurodegenerative diseases, cancer) is wide documented through a growing number of epidemiological studies (Siegel et al., 2010; Urpi-Sarda et al., 2012; Wallace et al., 2013; Elwood et al., 2013; Charlton et al., 2014; Kruk, 2014). For this reason fruit and vegetables consumption is assuming more importance from the nutritional point of view and the consumers are aware that a consumption of fruit rich in health-promoting compounds is an appropriate strategy to enjoy their benefits. Recently, individual subgroups of fruits have been taken into account, to facilitate the observation and promote their specific health benefits. Among these, strawberry represents one of the most consumed berry in Italy and also an important sources of bioactive compounds with antioxidant activity. In this context the increasing attention of consumers on the health benefits of fruits, and strawberry in particular, is driving the fruit breeders to create new strawberry varieties that could be rich of bioactive compounds, such as vitamin C, folates and polyphenols, which strawberries high antioxidant and health benefit potentials. This is a great new for the breeding program, considering that, until a few years ago, the majority of researchers were focused on the creation of varieties which contain great commercial qualities, such as commercial production, fruit size and plant yield.

The first aim of this study was to assess and compare the nutritional and phytochemical quality of strawberry fruit extracts of different commercially available varieties, obtained through specific breeding programs (Adria, Alba, Cristina, Romina and Sveva), with the purpose to evaluate the influence of genetic background on these parameters. All genotypes were evaluated for their nutritional value, by measuring the TAC, TPC, TFC, ACY of the different strawberry extracts. In addition to these common general measurements, vitamin C, anthocyanins and folates amounts were also quantified. According to these results, the strawberry cultivar chosen for the second part of the study was Alba. It possesed the highest TAC value and very high concentrations of vit C,

ACY, TFC and folates. Moreover, Alba cultivar was recently used in our research group studies, obtaining very interesting results both *in vitro* (Giampieri et al., 2014a; Giampieri et al., 2014b) and *in vivo* models (Romandini et al., 2013; Alvarez-Suarez et al., 2014).

The second aim of this PhD project was to evaluate the effects of methanolic purified extracts from Alba cultivar on inflammatory status induced by *E.Coli* LPS on two different cell lines, HDF and RAW 264.7 macrophages. HDF represent a standard *in vitro* cell model for different kind of toxicity, while RAW 264.7 macrophages are often used as model for cell culture studies, expecially in LPS-induced inflammation researches. The preliminary MTT viability test permitted to obtain a first view of the range of the concentration of strawberry extracts and LPS, useful for the following analysis. Moreover, in order to evaluate the influence of strawberry and LPS treatments in the apoptotic process and its link to the reduction of the cellular viability in HDF cells, the apoptotic rate was tested through the TALI apoptosis assay and caspase 3 expression. The obtained results showed, for the first time, the protective role of strawberry methanolic extracts in LPS-induced apoptosis rate and in caspase 3-related expression. ROS intracellular production could represent a very useful tool to quantify the oxidative damage induced by LPS and to evaluate the possible protective effect exerted by strawberry. In both studied models strawberry pre-treatment efficiently counteracted the LPS-induced oxidative damage remarkably reducing the ROS amount, in a dose-dependent manner. Since the most important site of ROS production is represented by the ETC in the mitochondria, the implication of mitochondria dysfunction in HDF cells and RAW macrophages, after strawberry and LPS-treatment, through the OCR measurement, was investigated. In HDF cells, LPS-treatment produced a depressive action on oxygen consumption, which was efficiently counteracted by strawberry pre-treatment in a dose dependent manner. On the contrary, in RAW macrophages LPS-treatment determined an increase of oxygen consumption wihich was further enanched by Alba extracts in a dose-dependent manner. This apparently contradictory aspect could be related to the stimulatory effect exerted by this endotoxin on RAW macrophages (Park et al., 2009; Nan et al., 2012; Zhang et al., 2012; Oliveira et al., 2014), that could produce a raise of oxyen consumption, despite the ROS obtained results. The exposure of cells to LPS can lead to the release of proinflammatory cytokines and in turn activate a second level of inflammatory cascades, including lipid mediators and adhesion molecules such as NO, that is an important regulatory and effector molecule with different biological functions, involved in many physiological and pathophysiological processes. In our work, both studied cell lines showed a significant increase in NO level after LPS-treatment, which was efficiently counteracted with Alba pre-incubation. These results confirmed that RAW macrophages provide one of the best-studied example of the regulation of NO production and represent one of the first studies that investigate the

involvement of NO production in HDF cells. Moreover, the strong association between NO production and iNOS expression was demonstrated in our study: in both HDF cells and RAW macrophages, LPS-treatment significantly induced iNOS expression, while strawberry extracts reduced the gene level restoring values similar to those of untreated cells. If the strawberry really acted as anti-oxidant agent against the LPS-induced oxidative stress, the immediate consequence could be that the antioxidant reservoir of the studied cell models would be consumed for counteracting the damage caused by the endotoxin. The obtained results confirmed these hypothesis: LPS significantly decreased all the enzymatic activities, due to ROS and NO production that it provoked, while Alba extracts counteracted these effects increasing the antioxidant defences, suggesting that the improvement of endogenous antioxidant enzyme activities might be one of the important mechanisms for strawberry against oxidative stress damage. Furthermore, the antioxidant capacity provided by strawberry resulted also in enhanced protection against lipid, protein and DNA damage induced by LPS-oxidative damage. The results of TBARS level, GSH amount, protein carbonyl content and OGG1 expression highlighted the marked protective effect of Alba extracts on the different biological macromolecules against the oxidative stress produced by LPS. Finally, Western blot analysis were performed to evaluate the gene expressions of several proteins related to the most probable molecular pathways involved in strawberry and LPS mechanisms of action. In the present study, we found that the anti-inflammatory role of strawberry is primarily involved in the activation of Nrf2 pathway, which is markedly AMPK-dependent in LPS-stimulated inflammatory responses.

The preliminary results obtained in this work provided an interesting starting point. Additional *in vitro* and *in vivo* studies are necessary to characterize the bioactive compounds which play a fundamental role against oxidative stress and to completely understand the molecular pathwyas involved in the strawberry-mediated anti-inflammatory response.

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