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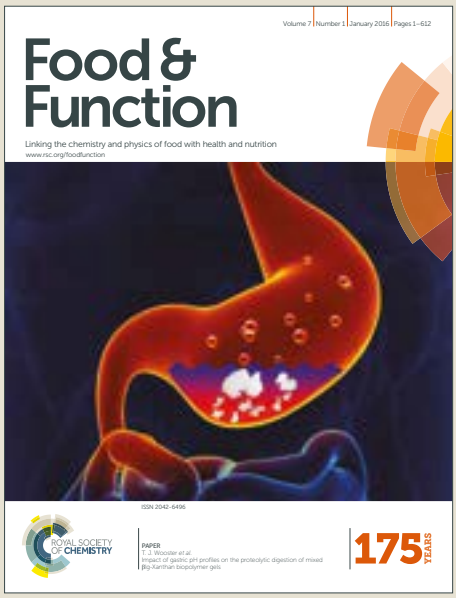

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Modulation of intestinal epithelium homeostasis by extra virgin olive oil phenolic compounds

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Abstract

Dietary habits have been strongly linked to the maintenance of intestinal epithelium homeostasis, whose alteration may contribute to the pathogenesis of inflammatory diseases and cancer. Polyphenols are among those dietary components suggested to be beneficial for gut health. Within a balanced Mediterranean type diet, a good portion of ingested polyphenols comes from olives and extra virgin olive oil (EVOO). Most of them reach the intestine, where they may be directly absorbed or metabolized under absorption. Others undergo an extensive gastrointestinal biotransformation, originating various metabolites that retain the potential beneficial effect of the parent compounds, or exert a more efficient biological action themselves. Ingested EVOO polyphenols (EVOOP) and their metabolites will be particularly concentrated in the intestinal lumen, where they might exert a significant local action. In this review we summarize the few studies that investigated the effect of EVOOP at intestinal level, focusing on the possible mechanism of action in relation to their interaction with the microbiota, and their ability to potentially modulate the oxidative status of the intestinal epithelial layer, inflammation and immune response.

Abbreviation: extra virgin olive oil, EVOO; extra virgin olive oil polyphenols, EVOOP; hydroxytyrosol, HT; tyrosol, TYR; oleuropein, OL; homovanillic acid, HVA; homovanillyl alcohol, HVAlc

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1. Introduction

The intestinal epithelium is a physical and biochemical barrier with a huge surface area, and defines the boundary between intestinal tissues and the external environment. The intestinal epithelium is specialized for nutrient and water absorption, and intestinal homeostasis depends on complex interactions among the intestinal epithelium, local and systemic immune factors, and the microbial content of the gut.

A deregulation of this equilibrium may contribute to the pathogenesis of inflammatory diseases and cancer. Dietary components strongly influence intestinal epithelium homeostasis; the "western diet" has been associated to an elevated risk of developing intestinal diseases, as it alters intestinal microbiota, increases intestinal permeability and promotes inflammation. Other dietary components, as those characteristic of the Mediterranean diet, whole-grain foods, fruits, vegetables and derived products as wine and extra virgin olive oil (EVOO), have been proved to be beneficial for gut health [1]. They are rich in bioactive compounds such as polyphenols, potentially able to exert antioxidant, anti-inflammatory and prebiotic effects at intestinal level [2]. The average intake of polyphenols is approximately 1g/day [3]. Most of them are poorly absorbed and directly or through the bile reach the colon, where they concentrate up to several hundred μM [3], in the parental form or partly metabolized. Thus, as suggested years ago by Halliwell [4], it is likely that in this site they exert a significant local action. Although the concentration of polyphenols is higher in the intestine than elsewhere, the number of studies that investigate their effect at intestinal level is quite limited.

Even more limited are studies regarding specifically EVOOP. Only few human studies have evaluated the effect of EVOOP on the intestinal homeostasis; most have been performed on intestinal cell lines and on experimental colitis animal models. Therefore, there is limited *in vivo* evidence showing a beneficial effect of EVOOP in humans at intestinal level, and we may only speculate on a protective role based on what suggested by experimental models and observational trials.

2. Extra virgin olive oil polyphenols

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EVOO is obtained solely through physical means by mechanical or direct pressing of the olives. It is not subjected to any treatment except washing, decantation, centrifugation and filtration. The oil produced from this first press is known as EVOO; it is of the highest quality and it contains also the highest levels of beneficial constituents [5-6]. The olive oil chemical composition consists of major components (triacylglycerol) that represent about 98-99% of the total oil weight, mainly oleic acid (MUFA) much higher (55-83%) than that of the other fatty acids (linoleic, palmitic, or stearic acids), which ranges between 3% and 21%. Minor components are present in small amounts (about 2% of oil weight) and include more than 230 chemical compounds such as hydrocarbons (squalene), phytosterols (β-sitosterol, stigmasterol, and campersterol), tocopherols (α-tocopherol), carotenoids (β-carotene), coloring pigments (chlorophylls), aliphalic and triterpenic alcohols, volatile compounds and phenolics, such as tyrosol (TYR) and hydroxytyrosol (HT) [7-9].

The phenolic fraction of EVOO is heterogeneous, with at least 36 structurally distinct phenolic compounds identified that can be grouped into the following classes:

- Phenolic acids. They can be divided into three subgroups, hydroxybenzoic acids, such as, gallic, protocatechuic, and 4-hydroxybenzoic acids, hydroxycinnamic acids, such as caffeic, vanillin, syringic, p- coumaric, and o-coumaric acids and other phenolic acids and derivatives. These compounds are generally present in small amounts (<10 mg per kg of oil) [10].
- Phenolic alcohols. These compounds possess a hydroxyl group attached to an aromatic hydrocarbon group, HT (3,4-dihydroxyphenyl-ethanol or 3,4-DHPEA,) and TYR (p-hydroxyphenyl-ethanol or p-HPEA). Their concentration is usually low in fresh oils but increases during oil storage due to the hydrolysis of EVOO secoiridoids (ranging from 0 to 70 mg per Kg of oil) [10-12].
- Secoiridoids. This phenolic group is characterized by the presence of either elenolic acid or elenolic acid derivatives in their molecular structure [11, 13]. The most abundant are the dialdehydic form of decarboxymethyl elenolic acid linked to HT (3,4-DHPEA) or TYR (p-HPEA) (3,4-DHPEA-EDA or p-HPEA-EDA), oleuropein (OL), its isomer, OL aglycon (HT linked to elenolic

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acid) (3,4-DHPEA-EA), and ligstroside aglycon (TYR linked to elenolic acid) (p-HPEA-EA). p-HPEA-derivates and dialdehydic forms of OL and ligstroside aglycon were also detected as minor hydrophilic phenols of EVOO [14].

- Hydroxy-isocromans. This is a class of phenolic compounds recently characterized of EVOO and the presence of 1-phenyl-6,7- dihydroxy-isochroman and 1-(39-methoxy-49-hydroxy) phenyl-6, 7-dihydroxy- isochroman has been shown in several samples [15].
- Flavonoids: These polyphenolic compounds contain two benzene rings joined by a linear three carbon chain and apigenine, luteoline, and (+)- taxifoline are the most concentrated. The amount of these compounds in EVOO is very low and generally ranges between 0 and 10 mg/kg of oil [16].
- Lignans: The exact structure of this type of phenolic is not well understood but it is based on aromatic aldehydes condensation. (+)-1-pinoresinol, (+)-1-acetoxypinoresinol and hydroxypinoresinol were characterized as the most concentrated lignans in EVOO [17]. These compounds are present in the pulp and in the woody portion of the seed [18].

TYR, HT, and their secoiridoid derivatives make up around 90 % of the total phenolic content of EVOO [19]. Not all phenolics are present in every EVOO and considerable variation has been noted in the concentration of such phenolic compounds (50 to 1000 mg/kg) [5, 20-21].

The EVOO phenolic content is determined by several factors such as olive variety (cultivar), growing area, fruit ripening, cultivation techniques, processing and storage conditions [22-24].

3. Metabolism and bioavailability

The metabolic fate of phenolic compounds after ingestion has been the subject of several studies by the scientific community to find out the mechanisms through which they exert their activity into the organism. Indeed, bioavailability of EVOOP is the key in achieving an effect in specific tissues or organs [25-26].

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Most of the studies regarding the bioavailability of these compounds have focused on the two most abundant EVOO simple phenolics: HT and TYR, amongst a few others [27]. After ingestion, EVOOP can be partially modified in the acidic environment of the stomach. The effect of such environment on aglycone secoiridoids has been examined in vitro by incubating the compounds at 37 ºC in simulated gastric pH conditions (pH 2.0) and during normal physiological time frames (up to 4 h) [28-29]. Although hydrolysis takes place releasing free phenolic alcohols, a significant amount remains intact and thus, enters the small intestine un-hydrolyzed. However, OL aglycone and its dialdehydic form, are likely not absorbed as such in the small intestine; in fact, the major metabolites detected using a perfused rat intestinal model were the glucuronide conjugates of the reduced form of both compounds [29]. In contrast, if the ingested secoiridoid is glucosylated it appears not to be subjected to gastric hydrolysis [30], meaning that phenolics such as the glucosides of OL enter the small intestine unmodified, along with high amounts of free HT and TYR and remaining secoiridoid aglycones.

Research evidence demonstrates that EVOOP are significantly absorbed (~40%–95%) in a dose-dependent manner in humans [30-37] and the major site for the absorption of these compounds is the small intestine [30, 32, 38-40].

There are different mechanisms by which absorption occurs with regards to EVOOP. The different polarities of the various phenolics has been postulated to play a role in the absorption of these compounds [30]. For instance, TYR and HT are polar compounds and their absorption has been shown to occur by a bidirectional passive diffusion mechanism across the membrane of the human enterocytes [41]. Other EVOOP, such as p-coumaric acid, pinoresinol, luteolin [25] and HT acetate [42] have shown the same mechanism of transport.

Larger compounds may be absorbed via a different mechanism to TYR and HT. It has been proposed that the polar but larger OL-glycoside may diffuse through the lipid bilayer of the epithelial cell membrane and be absorbed via a glucose transporter, but, potentially also via the paracellular route or transcellular passive diffusion [43]. Despite being well absorbed, the

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bioavailability of EVOOP is scarce due to an extensive pre-systemic first-pass metabolism in the gut and liver [27].

Once absorbed, EVOOP are, in fact, subjected to three main types of conjugation: methylation, glucuronidation and sulfation, through the respective action of catechol-O-methyl transferases (COMT), uridine-5'-diphosphate glucuronosyltransferases (UDPGT) and sulfotransferases (SULT) [44].

Different studies showed that HT and TYR can be metabolized to O-glucuronidated conjugates [31, 33, 40, 45-46], but HT also undergoes O-methylation, and both homovanillic acid (HVA) and homovanillyl alcohol (HVAlc) have been detected in human and animal plasma and urine after oral administration of either EVOO or pure HT and TYR [34, 40, 47-49].

The urinary excretion of HVAlc and HVA in humans was reported for the first time by Caruso et al. [45] after the intake of different EVOOs (the lowest administered dose was 7 mg of total HT/50 mL oil, and the max provided about 23 mg/50 mL oil). HVAlc contributes to 22% of the total excretion of HT and its metabolites, and HVA 56%. The excretion of both metabolites correlated with the administered dose of HT. Even at low doses, HVAlc and HVA were excreted. In a later study, Miró-Casas et al. [39] observed how urinary amounts of HT and HVAlc increased in response to EVOO ingestion, reaching the maximum peak at 0-2 h. Urinary recovery 12 h after olive oil ingestion showed that 65% of HT was in its glucuronoconjugated form and 35% in other conjugated forms.

Urinary concentrations and excretion rates of glucuronides of EVOOP were also successfully estimated in a study carried out by Khymenets et al. [46], using a dietary dose of EVOO (50 mL). About 13% of the consumed EVOOP were recovered in 24-h urine, where 75% of them were in the form of glucuronides (30- and 40-O-HT glucuronides, 40-O-glucuronides of TYR) and 25% as free compounds.

A study conducted by Corona et al. [28] about absorption, metabolism and microflora-dependent transformation of HT, TYR and their conjugated forms (e.g. OL) also showed similar results; both

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HT and TYR, transferred across human Caco-2 cell monolayers and rat segments of jejunum and ileum, were subject to classic phase I/II biotransformation. The major gastrointestinal metabolites identified were an O-methylated derivative of HT, glucuronides of HT and TYR and a novel glutathionylated conjugate of HT (HT-GSH). In contrast, there was no absorption of OL in either model [28].

On the other hand, sulfation can occur after gastrointestinal absorption, in fact in different studies conducted testing phenol-enriched virgin olive oils [37, 50-51], sulfation was the main conjugation pathway for EVOOP, whereas the glucuronidated forms were not detected. The main phenolic metabolites detected in plasma samples after ingestion of EVOO were, HT sulfate, HT acetate sulfate, HVA and HVA sulfate. HT sulfate appears to be a good biomarker for monitoring compliance of EVOO intake and a very recent study using pure HT [52] seems to reinforce this notion. In this last study, quantitatively, the total amount of HT recovered in the urine was minimal and accounted for 0.02% (only for the 25 mg dose). For the metabolites, they observed a dose-dependent increase in their excretion. And the major metabolite detected was HT 3-sulphate, which accounted for 23.1% (for the 5 mg dose) and 16.6% (for the 25 mg dose) of the administered HT, followed by HT 3-O-glucuronide with 2.78% (for the 5 mg dose) and 2.87% (for the 25 mg dose).

Suárez et al. [53] considered for the first time the absorption and disposition of flavonoids and lignans after the ingestion of EVOO. Besides the presence of those EVOOP in their conjugated forms, an important variability in the concentrations was observed between the plasma samples obtained from different volunteers. This variability may be attributed to differences in the expression of metabolizing enzymes due to genetic variability within the population [53].

Also De Bock et al. noted a large inter-individual variation in absorption and metabolism of phenolic compounds in a study with olive leaf extracts administration in humans, possibly resulting from differences in human enzymatic activity. For example, males may be more efficient at conjugating OL, which would explain their lower area under the curve (AUC) for OL but higher AUC for HT metabolites [54].

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The most comprehensive study regarding the identification of metabolites in human urine of most of the EVOOP (i.e. secoiridoids, flavanoids and phenolic alcohols) was reported by García-Villalba et al. [55]. These authors were able to achieve the tentative identification of 60 metabolites. Phenolic compounds were subjected to various phase I and phase II reactions, mainly methylation and glucuronidation. For instance, the largest number of metabolites was produced from HT, OL aglycone and oleocanthal, indicating significant post-absorption metabolism of these compounds. Conversely, the lowest number of metabolites came from TYR, luteolin, apigenin, pinoresinol and acetoxypinoresinol, suggesting that these compounds may have been excreted in faeces, destroyed in the gastrointestinal tract, excreted through another metabolic pathway or poorly absorbed [55]. A recent paper by De la Torre et al. further confirmed the presence of HT and its major methylated metabolite, 3-O-methyl-hydroxytyrosol or HVAlc, in urine following EVOO consumption in a high risk of CVD subjects, where HVAlc concentration was predictive of CVD [56].

In the case of poorly absorbed phenolic compounds, it has been suggested that these components may exert a local protective action in the large intestine, and this assumption is supported by research demonstrating, for instance, the free radical scavenging capacity of EVOOP in both the faecal matrix and intestinal epithelial cells [19].

4. Interaction with the microbiota

EVOOP can likely influence the gut microbial balance since, as reviewed in the previous paragraph, most of them are not completely absorbed into the upper parts of the gastrointestinal tract and reach the colon, where the different microbial species that inhabits the intestine reach the highest concentration [57]. The complex interaction between dietary polyphenols and the microbiota has been extensively studied, being recognized as one of the factor contributing to the beneficial effect of polyphenols consumption, although the mechanisms are still poorly understood.

Colon bacteria substantially contribute to the biotransformation of the polyphenols, breaking down unabsorbed compounds into a wide range of metabolites, which may be absorbed or excreted. Bacteria may also further modify enterocytes-derived metabolites [58]. On the other hand, dietary

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polyphenols and their metabolites may strongly influence microbiota composition, inhibiting the growth of harmful bacteria and exerting prebiotic-like effects towards beneficial bacteria, as nicely reviewed by Cardona et al. [58].

However, studies specifically regarding the impact of dietary intake of olives or EVOO polyphenols on the microbiota are scarce. One of the first studies on the biotransformation of ingested EVOOP by colonic microflora, was the in vitro study conducted by Corona et al.[28] cited above. The authors, using human fecal microbiota and a perfused rat intestinal model, demonstrated that these phenolic compounds undergo an extensive metabolisation in the passage through the gastrointestinal tract and are mainly absorbed as simple phenols in the small intestine. However, OL reaches the large intestine as an unmodified compounds and it is rapidly degraded in this site by the microflora to yield mainly HT. Using the same in vitro experimental model, Mosele et al. [59] reported HT as the main product of OL microbial metabolisation, together with a pool of phenolic acids resulting from further metabolisation. HT, HT acetate and TYR, tested as individual phenols, also originated phenolic acids, as phenylacetic acid, phenylpropionic acid and their hydroxylated derivatives.

A subsequent study determined in rat feces, after oral administration of OL, the presence of the parent compound together with other metabolites, identified as HT, elenolic acid and HVA [60]. In human fecal samples, obtained before and after the sustained intake of a phenol-enriched olive oil, free HT, phenylacetic acid, 2-(4'-hydroxyphenyl)acetic acid, 2-(3'-hydroxyphenyl)-acetic acid, 3-(4'-hydroxyphenyl)-propionic acid were detected; neither OL nor HVA were present in human feces, probably because of the differences in the gut metabolic responses between rat and human [59].

Microbial-derived phenolic acids have been reported to exert a significant biological activity at local and systemic level [61]; phenylacetic and phenylpropionic acids, together with their variously hydroxylated derivatives, are among the predominant structures in fecal water [62] and have shown to inhibit platelet aggregation [63] and the growth of intestinal pathogenic bacteria [64].

OL is likely to be preferentially degraded in vivo by lactic acid bacteria, as *Lactobacillus* and *Bifidobacterium* species [65], which are involved in developing the spontaneous or started lactic

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fermentation of table olives but also contribute, as probiotic bacteria, to maintain or improve microbial balance in the gut [66]. Thanks to the β-glucosidase and esterase activity [67], *L. plantarum,* that is also found as natural inhabitant of the human gastrointestinal tract, is the most effective bacteria converting OL into HT [65, 68] and it is also able to metabolize some phenolic acids as protocatechuic acid [69], ferulic, gallic and coumaric acids through inducible decarbossilase and reductase enzymes ([70] and references therein). Thus, OL possess prebiotic properties, as *Lactobacillus* and *Bifidobacterium* strains may utilize it as a carbon source, but others such as *Clostridium* and *E. coli* cannot [28]. Actually, it is assumed that EVOOP might influence the composition of the microbiota also inhibiting the growth of pathogenic bacteria. The antimicrobial activity of phenolic compounds from *Olea europaea* has been extensively studied since the early 1970s, although, depending on the experimental conditions, results have been contrasting. HT, for example, has been shown to inhibit *E. coli* growth [71], although culture media and the type of strain remarkably affected the bacterial susceptibility to HT [72]. HT exhibited also a significant antimicrobial activity against selected *Enterobacter* species [73]. Similarly, OL was effective in *E. coli* growth inhibition [74]. In general, several experimental trials showed OL and HT to be the best inhibitors of several gastrointestinal pathogens, as reported in the recent review of Thielmann et al. [75]. However, this great amount of data arises from in vitro experiments that do not mimic the in vivo conditions. To the best of our knowledge, there are only two recent reports by Martin-Pelaez et al. [76-77] and one from Conterno et al.[78] on the modulation of microbiota by EVOOP in humans. Martin-Pelaez's studies arise from the VOHF study, a randomized, controlled, double-blind, crossover clinical trial with hypercholesterolemic subjects [79]. In a subsample of 12 hypercholesterolemic adults [76], changes in faecal microbial populations were evaluated following sustained consumption of EVOOP, alone or in combination with thyme polyphenols; the study reported a slight HT modification in microbial composition following EVOOP intake, depending on the dosage, as confirmed by the parallel study in another subsample of 10 subjects [77]. A significant increase of *Bifidobacterium* group numbers was detected instead, when polyphenols from olive oil and thyme

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were ingested in combination [76]. Among the microbial phenolic metabolites, dihydroxyphenyl and hydroxyphenyl acetic acid, and a significant amount of protocatechuic acid and HT were detected in faeces after dietary interventions with polyphenols. The ingestion of a mixture of olive oil and thyme polyphenols exerted a cardio-protective effect in hypercholesterolemic subjects, mediated by the specific growth stimulation of *Bifidobacteria*, together with the increases in microbial phenolic metabolites with antioxidant activities such as protocatechuic acid and HT [76]. Conterno et al. reported small changes within the composition of the gut microbiota, showing a small increase in *Bifidobacteria*, and an up-regulation of microbial polyphenol biotransformation in the intestine, following ingestion of olive pomace extract-enriched biscuits.[78]

Although the complex interrelation between EVOOP and human microbiota is still far from being exhaustively investigated, data collected so far clearly suggest a concentration dependent impact of phenolic compounds and metabolites on bacterial growth and on the associated metabolic consequences at local and systemic level.

5. Antioxidant and anti-inflammatory effect at intestinal level

Dietary polyphenols have been claimed to exert both a protective and therapeutic effect in the management of gastro intestinal disorders, mainly those strictly linked to oxidative stress and chronic inflammation, as IBD. Being particularly concentrated in the intestinal tract, dietary polyphenols, now undoubtedly associated with scientifically validated antioxidant and anti-inflammatory properties, may act locally reducing oxidative stress and inflammatory response [2, 80].

5.1 Antioxidant effect

The gut lumen is likely to be the only site where EVOOP, together with their active metabolites, may reach a concentration high enough to enable them to act as direct antioxidants, scavenging ROS; once absorbed, they may also modulate the expression of genes linked to antioxidant cellular defenses via molecular targets. The phenolic fraction of EVOO has been shown to protect intestinal Caco-2 cells against the alteration of cellular redox status and oxidative damage to the membrane

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lipid fraction, due to the pro-oxidant action of oxidized lipids and this effect has been correlated to the activity of the most abundant phenolic compounds present in the tested fraction, HT, TYR and OL [81]. As reviewed in the first paragraph, HT, TYR and OL, together with their metabolites, are the major phenols found at intestinal level, following ingestion of EVOO, and, due to their high local concentrations, they might exert a relevant antioxidant effect. HT has been recognized as the most efficient free radical scavenger and radical chain breaker, and its catecholic structure is also able to prevent reactive species formation through metal chelation features [82-83]. It has been shown to protect Caco-2 cells against oxidative injury [84-86], because of its scavenging properties, and its major metabolites, sulfates and glucuronides, showed an efficiency in protecting Caco-2 cells [87], as well as renal cells [88] and erythrocytes [89], comparable or even better than that of the parent compound. TYR has also been shown to be effective in protecting Caco-2 cells against the cytostatic and cytotoxic effects produced by oxidized LDL [90] and to possess scavenging effects on peroxyl radicals [84, 91], O_2^- [92] and $ONOO^-$ [93]. Although there are no studies regarding specifically the intestinal compartment, trials in animal models and cell cultures demonstrated that HT is able to increase the endogenous defense system, through the modulation of related gene expression.

In human HepG2 cells HT enhanced the expression and the activity of the glutathione related enzymes, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST) [94]. The modulating activity of HT on the glutathione antioxidant network has been also demonstrated in the adipose tissue of mice fed an HT-supplemented diet [95] and in the liver of obese mice after 17 weeks supplementation [96]. HT has been shown to be a potent inducer of phase II detoxifying enzymes in retinal pigment epithelial cells [97] and to increase the expression and activity of SOD and CAT in rats fed a cholesterol-rich diet [98]. The effect of HT on the cellular antioxidant enzymes has been linked to its ability to increase the translocation of Nrf2 [94, 97] to the nucleus, thus promoting the expression of genes related to the antioxidant defense system and contributing to the protection of cells against oxidative stress. However, this hypothesis has never been proven in

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humans; indeed, a pilot study on humans demonstrated that HT administration did not significantly modify phase II enzyme expression in peripheral blood mononuclear cells [99].

Recent studies showed the ability of TYR and its sulfate metabolite to induce the GPx activity in Caco-2 cells [87] and, together with its glucuronide metabolite, to restore GSH level and related antioxidant enzymes in TNF-α treated human endothelial cells [100], as previously demonstrated in macrophages, where TYR preserved cellular antioxidant defenses against the pro-oxidant effect of oxidized LDL [101]. In a mouse model of lipopolysaccharide (LPS)-induced acute lung injury, TYR pretreatment attenuated the inflammatory response and improved expression of the antioxidant enzymes, through the activation of Nrf2 [102].

OL possesses well-documented pharmacological properties, including a potent antioxidant activity mainly due to the presence of hydroxyl groups in its chemical structure. Its free radical scavenging and metal-chelating activities enable OL to inhibit the production of a wide range of ROS and RNS in in vitro cell-free systems, as well as in cultured cells, as reported in the Hassen et al. extensive review [103]. There are also evidence for the stimulatory effect of OL on the expression of the intracellular antioxidant enzymes in free endothelial progenitor cells, via the activation of Nrf2 [104], and in normal human liver cells [105]. In vivo data confirm the ability of OL to increase the level and activities of enzymatic antioxidants in rats fed a cholesterol rich diet [106], in acute arsenic exposed rats [107], in the hypothalamus of hypertensive rats [108], in the substantia nigra of aged rats [109] and to enhance the level of non enzymatic antioxidants such as glutathione, α-tocopherol, ascorbic acid and β-carotene in alloxan-diabetic rabbits [110].

5.2 Anti-inflammatory effect

A large body of studies carried out in cell cultures, animal models and humans provides solid evidence that EVOOP are able to inhibit the inflammatory process, through the modulation of different signaling pathways regulating immune cells response, activation of pro-inflammatory enzymes and release of inflammatory mediators [111].

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There are few studies focusing on the anti-inflammatory action of EVOOP at intestinal level. In cultured Caco-2 cells stimulated with LPS or IL-1β, EVOOP are able to regulate IL-8 expression by transcriptional or posttranscriptional mechanisms, depending on the stage of inflammation [112]. We recently demonstrated that EVOOP may also counteract oxysterols-induced redox imbalance and pro-inflammatory response in Caco-2 cells, inhibiting cytokines and NO release, through the modulation of the MAPK-NF-kB pathway [113].

Studies in animal models show that an EVOO diet enriched with phenolic compounds mitigate the severity of DSS-induced colitis in mice, attenuating clinical and histological signs of damage of colonic segments, suppressing oxidative events and inhibiting pro-inflammatory protein expression [114-116].

The anti-inflammatory activity of the phenolic fraction is likely to be dependent on the active constituents OL, HT and oleocanthal, whose anti-inflammatory effect has been clearly demonstrated in vitro. In the same mice model of DSS-induced colitis, oral administration of OL attenuated the extent and severity of acute colitis, reducing pro-inflammatory cytokine, IL-1β, IL-6, TNF-α and NO production and enhancing anti-inflammatory cytokine levels, IL-10, in the colonic tissue. The molecular mechanism of its protective action seems at least in part linked to the down-regulation of COX-2 and iNOS proteins gene expression and to the up-regulation of annexin A1, which may mediate the suppression of p38 MAPK phosphorylation and NF-κB translocation to the nucleus [116-117]. A subsequent investigation by the same group confirmed the ability of OL to modulate intestinal immune response in DSS acute model, inhibiting Th17 response and the release of Th17-related cytokines, and, down regulating inflammatory mediators, to inhibit the development of the connected colorectal cancer [118].

A recent study conducted in colonic biopsies obtained from patients with ulcerative colitis demonstrated the ability of OL to ameliorate the inflammatory damage and reduce infiltration of CD3, CD4, and CD20 cells, while increasing CD68 numbers. In the colonic biopsies treated with

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LPS and OL the expression of COX-2 and IL-17 were significantly lower compared with those treated with LPS alone [119].

HT also demonstrated an anti-inflammatory effect in vivo, when locally applied in TNBS-induced colitic rats [120], and when administered within HT supplemented EVOO-diet to DSS-induced colitic mice. This anti-inflammatory effect has been related to the ability to modulate cytokines secretion and to reduce COX-2 and iNOS expression in colonic mucosa, by down regulating p38 MAPK pathway [121]. These observations agree with the study of Corona et al.[122] in Caco-2 cells which demonstrates that inhibition of p38 significantly reduces COX-2 expression.

A significant beneficial effect in chronic DSS-induced colitis was also exerted by HT acetate, sharing the same mechanism of action as HT [123]. There is strong evidence in vitro that also oleocanthal is an effective anti-inflammatory agent. In fact, it can efficiently inhibit COX-2 enzyme expression and activity, which is implicated in the pathogenesis of several cancers [124].

The findings of these few studies suggest that EVOOP have the potential to exert anti-inflammatory effects in the human gastrointestinal mucosa, however, no human studies, up to now, have specifically dealt with this issue.

6. Anti-carcinogenic effect at intestinal level

Over the past decades, epidemiological studies have indicated an inverse correlation between EVOO consumption and the incidence of different type of cancers, although the scientific evidence in support of this correlation is still limited [125]. It has been shown that the Mediterranean diet, and EVOO seem to be protective against colon cancer [126-127]. A systematic review and meta-analysis analyzed 19 case-control studies (13800 cancer patients and 23340 controls) and found that high olive oil consumption was associated with lower odds of having any type of cancer [128]. Moreover, high olive oil consumption was associated with lower odds of developing breast cancer (logOR = -0,45 95% CI -0.78 to -0.12), and a cancer of the digestive system (logOR = -0,36 95% CI -0.50 to -0.21), compared with the lowest intake [128]. In addition, another systematic review and meta-analysis

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included 25 studies, and concluded that high olive oil consumption decreased the risk of upper digestive and respiratory tract neoplasms, breast and, possibly, colorectal and other cancer sites [125]. More recently, a systematic review reported the association between EVOOP and other Mediterranean diet components with a reduction of colorectal cancer initiation, promotion and progression [129]. Several nutrients play a significant role in colorectal cancer development, and the importance of monounsaturated fatty acids has been highlighted [130].

In addition, EVOOP, including phenolic alcohols, lignans and secoiridoids, are thought to be, in part, responsible for EVOO reported anti-carcinogenic effects [131]. EVOOP have been shown to influence carcinogenesis and tumor development at various levels [132-134]: by exerting antioxidant activities [135-136], by modulating detoxification enzyme systems [137], and the immune system [138], by reacting with activated carcinogens and mutagens [139-140], and by exerting actions on proteins controlling cell cycle progression [122, 141-142], and gene expression [143-144].

The ability of EVOO to inhibit colon cancer development has been demonstrated in large intestinal cancer cell models [122, 144-145], in animals [140, 146] and in humans [131, 147]. In experimental models, olive oil consumption has been shown to prevent benzo(a)pyrene [B(a)P]-induced colon carcinogenesis in Apc(Min) mice [140], reduce the incidence of aberrant crypt foci in azoxymethane-treated rats [146] and dimethyl-benz(a)antracene-induced mammary carcinogenesis [148], and has been shown to induce significant levels of apoptosis in large intestinal cancer cells [136, 145]. In animal models, n9 fatty acids present in olive oil have been able to prevent the development of aberrant crypt foci and colon carcinomas [146]. Thus, EVOOP have also been shown to play an important role, due to their ability to inhibit the initiation, promotion and metastasis of the carcinogenetic process in human colon adenocarcinoma cells [149-150]. Furthermore, EVOO has been shown to down-regulate the expression of COX-2 and Bcl-2 proteins that have a crucial role in colorectal carcinogenesis [145].

A study conducted using different colon cancer cell lines (p53 proficient, mutant and knocked out), demonstrated that a pinoresinol-rich olive oil extract was capable of reducing cancer cell viability (particularly in p53-proficient cells), inducing apoptosis, inducing a G2/M cell cycle block and

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causing the up-regulating of ATM and a parallel decrease of cyclin B/cdc2 [151]. Similar experiments conducted with purified pinoresinol resulted in similar effects, although higher concentrations were required, indicating a possible synergistic effect between pinoresinol and other polyphenols in EVOO [151].

The cellular mechanism by which EVOOP exert anticancer effects can also be linked to the modulation of MAPK kinases and COX-2 [122]. COX-2 is over-expressed in colorectal cancer, and its over-expression has a strong association with colorectal neoplasia, by promoting cell survival, cell growth, migration, invasion and angiogenesis [152]. An efficient inhibitor of COX-2, oleocanthal, repressed cell viability and induced apoptosis in human colon carcinoma HT-29 cells, via AMPK activation and COX-2 suppression [153], and it has also been proven to reduce proliferation and migration in different cancer cells, deactivating the activity of various mediators in addition to COX-2, which result in tumorogenesis [124].

The MAPK signaling pathway has long been viewed as an attractive pathway for anticancer therapies, based on its central role in regulating the growth and survival of cells from a broad spectrum of human cancers [154], and it also modulates the transcriptional and post-transcriptional activation of COX-2 [155].

An EVOO phenolic extract has been shown to exert a strong inhibitory effect on the growth of colon adenocarcinoma cells through the inhibition of p38/CREB signaling, a decrease in COX-2 expression and the stimulation of a G2/M phase cell cycle block [122]. In contrast, HT exerts its anti-proliferative effects via its ability to strongly inhibit ERK1/2 phosphorylation and downstream cyclin D1 expression [142]. These findings are of particular relevance due to the high colonic bioavailability of HT compared to the other EVOOP and may help explain the inverse link between colon cancer and EVOO consumption.

Furthermore, HT inhibits colon cancer cell proliferation [156] and induces cancer cell apoptosis [157] through a mechanism of action linked to a prolonged stress of the endoplasmic reticulum (activation of unfolded proteins) and over-expression of pro-apoptotic factors, such as Ser/thr phosphatase 2A,

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a key protein involved in the induction of apoptosis in colon cancer cells [157]. TYR on the other hand, has been found to reverse a number of effects induced by oxidized lipids, including ROS overproduction, GSH depletion, the impairment in antioxidant enzyme activity and the increase in the expression of p66Shc protein [101, 158-159]. All of these findings suggest that the ability of EVOOP as intestinal anti-cancer agents should be reappraised, as it is clear that their actions on the process of carcinogenesis are many-fold and involve more than simple antioxidant effect.

7. Conclusions

A large body of evidence suggests the potential for EVOOP to promote beneficial health effects in the prevention and amelioration of several chronic diseases, mainly cardiovascular diseases, neurodegenerative disorders and cancer, as recently outlined by Visioli et al., who critically summarized the main reported findings on the effects of EVOO consumption on human health, discussed in the last International Olive Council Conference [160]. Studies on the absorption and metabolization of EVOOP show that some complex polyphenols reach the intestine, where they may be directly absorbed or metabolized during absorption, while others undergo an extensive gastrointestinal biotransformation. Therefore, a significant amount of bioactive compounds, mainly simple phenols and metabolites, will be present in the small and large intestine, concentrating at this site.

Considering that dietary intake of EVOOP in the Mediterranean area has been estimated to be around 9 mg, based on 25 - 50 ml of EVOO daily consumption [19], EVOOP may significantly contribute to preserve intestinal epithelium homeostasis. As suggested by the few studies summarized in this review (Table 1), EVOOP may help to counteract oxidative stress and can modulate intestinal inflammation, gut microbiota and immune response, thus helping to prevent the onset or delay the progression of inflammatory/degenerative diseases. Although more studies are necessary to validate the important role of EVOOP in the maintenance of intestinal homeostasis, the

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457 regular consumption of EVOO should be highly promoted also in view of their possible role in
458 preventing intestinal diseases.
459

460 **Conflicts of interest**

461 No conflicts of interest.

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Table 1. Overview of EVOOP actions at intestinal level

Compound	Experimental system	Mechanism	Ref.
Interaction with microbiota			
OL, HT, TYR	in vitro batch colonic fermentation/ perfused rat intestinal model	increase of bioactive phenolic metabolites	28
TYR, HT, HT acetate and OL/phenols-enriched OO	in vitro batch colonic fermentation/ human intervention study	increase of bioactive phenolic metabolites in faeces	59
OL	oral administration in rats	increase of bioactive phenolic metabolites in faeces	60
HT,TYR	broth dilution	growth inhibition of *E. coli* (ATCC no. 25922)	71
HT	broth dilution	growth inhibition of *E. coli* (CECT 533, 4972, and 679 grown in LB and E. coli 4972 grown in ISO)	72
HT	agar plates	growth inhibition of *E. coli*, *Enterobacter* and *Enterococcus* species	73
OL, HT/ phenolic extract	broth dilution	growth inhibition of *E. coli (C7085L)*	74
phenols-enriched OO/ phenols and thyme phenols-enriched OO	double-blind, cros-sover human trial	increase of *Bifidobacteria* increase of bioactive phenolic metabolites in faeces	76 77
olive pomace enriched biscuits	double-blind, parallel dietary intervention in human subjects	increase of *Bifidobacteria*	78
Antioxidant effect			
HT, TYR, homovanillic alcohol	TBH treated human colon adenocarcinoma cells (Caco-2)	inhibition of oxidative modification of membrane lipid fraction	84
HT	H_2O_2 or xanthine oxidase/xanthine treated Caco-2 cells	inhibition of lipid peroxidation and monolayer permeability changes	85
HT	acrylamide treated Caco-2 cells	prevention of ROS overproduction	86
HT, TYR and sulfate metabolites	oxydized cholesterol treated Caco-2 cells	inhibition of ROS and MDA production and GSH depletion	87
TYR	oxidized LDL treated Caco-2 cells	inhibition of morphological and functional alterations	90
phenolic extract	oxysterols treated Caco-2 cells	reduction of ROS production and GSH depletion	113
Anti-inflammatory effect			
phenolic extract	LPS or IL-1β treated Caco-2 cells	prevention of IL-8 expression and secretion, regulation of IL-8 mRNA transcription and stability	112
phenolic extract	oxysterols treated Caco-2 cells	inhibition of IL-6, IL-8 and NO release, modulation of MAPK-NF-kB pathway	113

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phenols-enriched EVOO	DSS-induced chronic colitis in mice	attenuated damage of colonic segments, PPARγ up-regulation, NF-kB, MAPK and downstream inflammatory cascade inhibition	114
EVOO unsaponifiable fraction	DSS-induced acute colitis in mice	attenuated damage of colonic segments, decreased MCP-1 and TNF-α levels, iNOS and COX-2 overexpression and p38 MAPK activation	115
OL	DSS-induced acute colitis in mice	reduction of neutrophil infiltration, NO, IL-1β, IL-6, and TNF-α production, iNOS, COX-2, and MMP-9 expression	116
OL	DSS-induced chronic colitis in mice	attenuated colon damage, reduction of COX-2 and iNOS expression and IL-1β and IL-6 release; increase of IL-10	117
OL	DSS-induced acute colitis in mice	inhibition of Th17 response and Th17-related cytokines release	118
OL	LPS treated colonic biopsies from UC patients	reduced expression of COX-2, IL-17 and infiltration of CD3,CD4 and CD20 cells	119
HT	TNBS- induced colitis in rats	reduced inflammatory infiltration	120
HT-enriched EVOO	DSS-induced chronic colitis in mice	attenuated colon damage, reduced TNF-α, COX-2 and iNOS expression, downregulation of p38 MAPK; increase of IL10	121
HT acetate	DSS-induced acute colitis in mice	improved histological damage, reduction of COX-2 and iNOS expression, inhibition of JNK MAPK and NF-kB	123
Anti-carcinogenic effect			
phenolic extract	CaCo-2 cells	inhibition of cell proliferation, induction of G2/M phase cell cycle block, inhibition of p38 and CREB activation, reduction in COX-2 expression.	122
HT	adenocarcinoma cells (DLD1 cells)	ROS generation, apoptotic cell death, mitochondrial dysfunction, phosphoinositide 3-kinase/Akt pathway activation, FOXO3a phosphorylation, FOXO3a's target genes downregulation.	136
HT	CaCo-2 cells	inhibition of cell proliferation, induction of G2/M phase cell cycle block, inhibition of ERK1/2 activation, reduction of cyclin D1 expression.	142

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EVOO, phenolic extract, HT	CaCo-2 cells, rat colon	up-regulation of CNR1 gene in CaCo-2 cells, reduced DNA methylation at CNR1 promoter in CaCo-2 cells, reduced cell proliferation; increase in CB(1) expression in rat colon, reduction of CpG methylation of rat Cnr1 promoter, miR23a and miR-301a	144
phenolic extract	colon cancer cells (HT-29), intestinal barrier function (CaCo-2 cell monolayers), matrigel invasion assay (HT115 cells)	reduction of DNA damage (HT-29), improved barrier function (CaCo-2), inhibition of HT115 invasion, inhibition of HT115 cell attachment	149
phenolic extract, HT, TYR, pinoresinol, caffeic acid	Matrigel invasion assay (HT115 cells)	anti-invasive effects, no citotocixity observed, no effects on cell attachment	150
pinoresinol-rich phenolic extract, oleocanthal-ric phenolic extract	p53 proficient (RKO and HCT116), and p53 knocked out (SW480 and HCT116 p53-/-) cell lines	reduction of cell viability,increased apoptosis, cell cycle arrest at G(2)/M, up-regulation of ATM signalling pathway, decrease of cyclin B/cdc2	151
HT	HT-29	inhibition of cell proliferation	156
HT	HT-29	induction of cell growth arrest, induction of apoptosis, prolonged stress of the endoplasmic reticulum (ER), activation of UPR, overexpression of CHOP/GADD153, activation of JNK, modulation of Akt/PKB, inhibition of TNFα-induced NF-kB	157

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GRAPHICAL ABSTRACT

Extravirgin olive oil polyphenols concentrate at intestinal level and, modulating microbiota, oxidative status and inflammation, contribute to prevent the onset or delay the progression of inflammatory/degenerative diseases.